

Lipid Metabolism in Pisum sativum infected by
Erysiphe pisi

by

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ROBERT INGLIS SLOSS: Lipid metabolism in Pisum sativum infected by
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ABSTRACT

The project was set up to investigate the host-parasite interface in powdery mildew infected pea plants. The interface consists of a double membrane system containing both host and parasite membranes through which all the solutes required by the pathogen must pass. As membranes contain large amounts of phospholipid it was decided to look for changes in the phospholipid components which may become apparent during the development of the disease.

Initially a quantitative extraction and analytical procedure was developed for plant lipids. Once this system was developed a study using lipid extracts from whole leaves was undertaken which revealed no changes in lipid composition in leaves up to 21 days after infection. Epidermal strips were then used and it was seen that infection caused a decrease in the chloroplast lipids PG, DGDG and MGDG and an increase in the unsaturation of polar lipid-bound fatty acids. Later studies showed that these changes were not caused by the presence of fungal lipids within the epidermal cells but were a result of premature senescence of the tissue.

When individual membranes were isolated and analyzed it was shown that these changes were consistent for all membranes in the epidermal cells.

These results demonstrated the efficiency of the biotrophic interaction between the parasite and its host. The results were discussed in relation to membrane permeability phenomena and possible mechanisms for early changes in membrane structure resulting in nutrient transfer from host to parasite were speculated upon.

LIST OF ABBREVIATIONS

Eu	-	Arrhenius activation energy
BHT	-	Butylated hydroxy toluene
cm	-	centimeter(s)
°C	-	degrees centigrade
DGDG	-	digalactosyl diglyceride
esr	-	electron spin resonance
FAMES	-	fatty acid methyl esters
fig	-	figure
glc	-	gas liquid chromatography
g	-	gram(s)
Ltd	-	limited
l	-	litre
m	-	metre(s)
µg	-	microgram(s)
µmoles	-	micromoles
ml	-	millilitre(s)
min	-	minute(s)
M	-	molar
MGDG	-	monogalactosyl diglyceride
nm	-	nanometer(s)
P _l	-	permeability coefficient
PA	-	phosphatidic acid
PC	-	phosphatidyl choline
PE	-	phosphatidyl ethanolamine
PG	-	phosphatidyl glycerol
PI	-	phosphatidyl inositol
PS	-	phosphatidyl serine
R _t	-	retention time
sec	-	second
tlc	-	thin layer chromatography
UV	-	ultraviolet (light)
V/V	-	volume per volume
W/V	-	weight per volume
wt	-	weight

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1. Introduction

The aim of this project was to examine the host parasite interface in a plant disease caused by a biotrophic fungal parasite. The experimental system comprised of powdery mildew of peas Pisum sativum caused by Erysiphe pisi. The reason for choosing this system was that the powdery mildew host parasite interface is restricted to the epidermal cells. This means that any method for analysis can be confined to a single tissue, the epidermal layer enabling a closer examination of the effects of the disease on the host. Another important consideration in the choice of the disease system was the fact that a considerable amount of work has been carried out on the microscopic characteristics of this host parasite interface and it was also possible to separate the haustorial complexes from the epidermal cells.

The most important characteristic of any fungal disease is the fact that the fungus is able to direct host metabolites from the host to the fungus. In order to fully understand a disease situation it is important to uncover how this occurs. The main objective of this study was to observe, chemically, the changes that take place in the plant membrane composition throughout the course of the disease and in this way to come to an understanding of how the pathogen alters the permeability of the membranes in order to obtain nutrients from the host. In particular the changes in membrane lipids were studied as the barrier properties of biological membranes depend to a considerable extent on the nature of the hydrocarbon chains of the membrane lipids. Studies using monomolecular films and liposomes have shown that alterations in the fine structure of phospholipids has a profound effect upon the permeability of biological membranes and so it was thought that an investigation into the changes in phospholipid structure may reveal some information about the nature of the membrane permeability changes in the disease state.

2 Literature Review

2.1 The Fungus

Powdery mildew of peas caused by Erysiphe pisi has a world wide occurrence. It is found on members of the Papilionaceae, mainly on the

genera Pisum, Dorcyinium, Medicago and Vicia and less frequently on Lupinus sp, Lens sp and Trifolium dubium (Kapoor, 1967). The mycelium is variable, thin effused and arachnoid, the conidia, usually formed singly or occasionally in very short chains are elipsoid whilst the cleistothecia are globose.

Three biologic forms of Erysiphe pisi have been described (Hammerlund, 1925); f. sp. pisi on Pisum sativum and Pisum arvense, f. sp. medicagnis sativae on Medicago sativa and Medicago fulcata and f. sp. vicioe sativae on Vicia sativa, Vicia sepium and Vicia sylvatica.

2.2 The Disease

The fungus grows on both leaf surfaces and stems as powdery greyish white colonies which eventually coalesce (Dixon, 1978). Mildew infection adversely affects the total plant weight, the number of pods per plant, the plant height, the number of nodes per plant, the weight of shelled peas and the numbers of peas per pod. Infection also raises the "tenderometer" reading and thus depresses quality (Gritton and Ebert, 1975).

Oku et al (1975) demonstrated phytoalexin production in peas infected by Erysiphe pisi. The phytoalexin so produced was pisatin.

Development on the host has not been studied to the same extent as has Erysiphe graminis but it does follow the same pattern of morphologically identifiable stages, spore germination, formation of appressorial initials, maturation of appressorial initials, maturation of appressoria, formation of a penetration peg and formation of haustoria in epidermal cells.

2.3 Haustorial structure and function

The haustorium is defined as a specialised outgrowth of fungal cells which functions as a feeding cell (Bushnell, 1976). It is the only structure of the fungus which makes intimate contact with host cells.

The haustoria in Erysiphe pisi have an elipsoid central body with short finger like projections extending over the surface of the body (Gil and

Gay, 1977). The haustorium has a single nucleus and is separated from the remaining hyphal network by a septum in the neck region normally perforated to provide hyphal continuity throughout the colony (Bushnell, 1976). The haustorial cytoplasm lacks a large vacuole but commonly has several small ones, the cytoplasm is usually rich with ribosomes and sometimes glycogen granules are present (Perrera and Gay, 1976). There are many mitochondria in the lobes and the peripheral cytoplasm.

The haustorium lies within an extrahaustorial membrane (Ehrlich and Ehrlich, 1971) which can be seen using an electron microscope. The membrane is thought to be an invagination of the host cell plasma membrane.

Dekhuizen (1966) was the first to attempt to isolate haustoria for studies on host-parasite physiology. The structures which he isolated from peas comprised the haustorium proper together with a surrounding intact extrahaustorial membrane. When haustoria are mounted in water the matrix region between the membranes undergoes osmotic swelling revealing that the two membranes are tightly bound in the neck region (Fig. 1). Although isolated haustoria are detached from the main hyphal network they retain their cytoplasm due to the pore at the junction with the neck sealing when the mycelium is disrupted. Apart from the removal of the neck cytoplasm isolation does not alter haustorial structure (Bushnell and Gay, 1978).

The extrahaustorial membrane differs structurally from the plasma membrane lining the cell wall of the infected cell despite being ultimately derived from and continuous with it. The extrahaustorial membrane in barley is thicker than the normal plasma membrane (Bracker, 1968) and this has also been reported for peas (Gil and Gay, 1977). The extrahaustorial membrane resists treatment with chemicals which solubilize "normal" cell membranes e.g. Triton x 100 and Teepol (Gil and Gay 1977).

Freeze fracture studies of isolated and infected epidermal cells have shown that the surface of the extrahaustorial membrane which faces away from the host cytoplasm, the A surface, shows crater like structures which

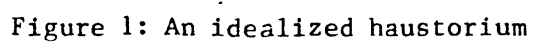
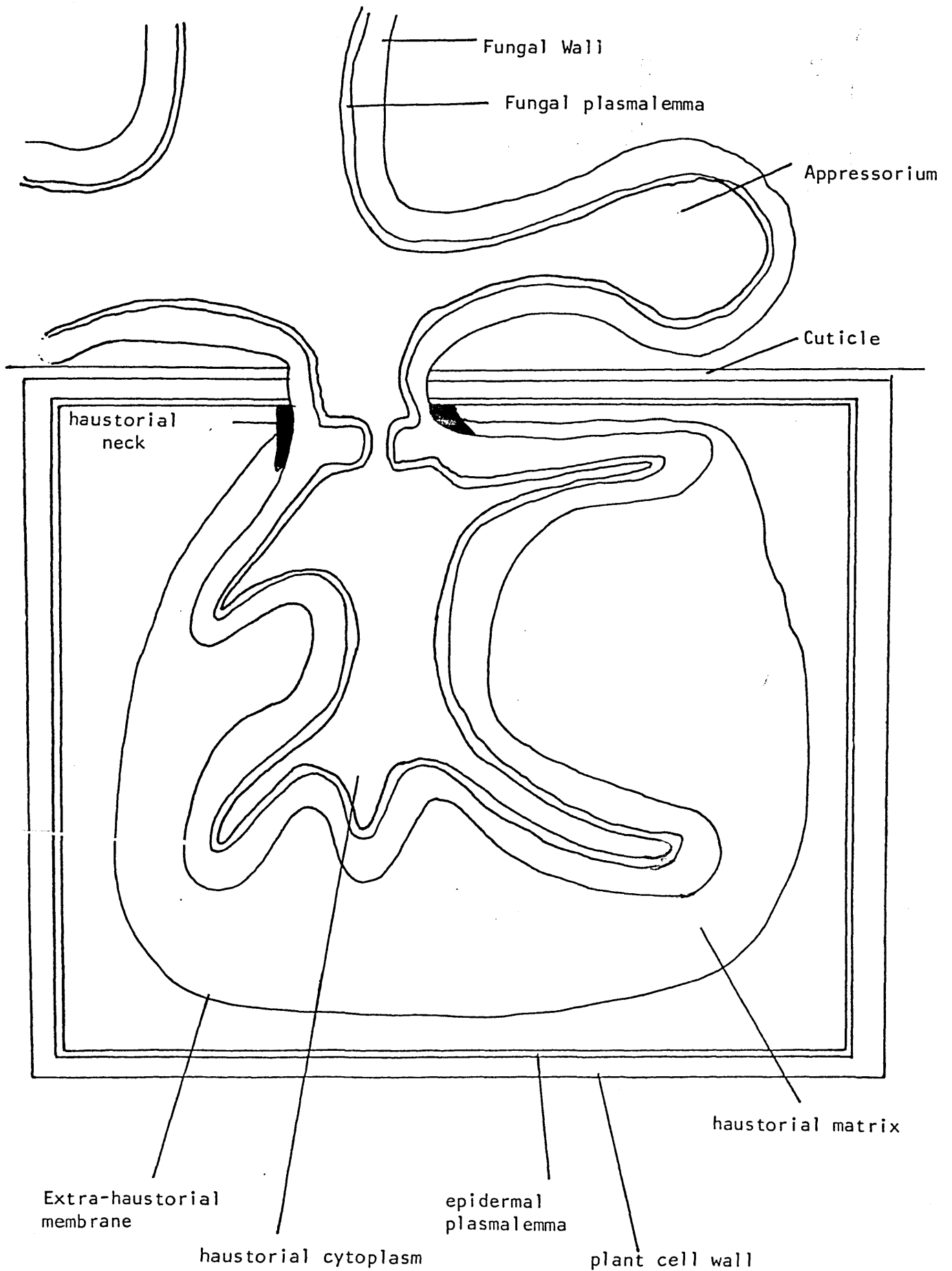


Figure 1: An idealized haustorium

Figure 1



represent the edges of broken microvilli projecting into the extrahaustorial matrix (Gil and Gay, 1977). This surface did not contain the small particles found on the A surface of the plasma membrane of infected cells which are thought to be responsible for microfibril synthesis during cell wall deposition (Preston, 1974).

Gil and Gay (1977) have suggested that in infected peas the extrahaustorial matrix probably contains pectic and hemicellulosic substances. They found signs of intensive Golgi activity in adjacent host cytoplasm, along with sites on the extrahaustorial membrane which suggested fusion with Golgi vesicles. They suggested that haustoria cause a derangement of cell wall metabolism with an inhibition of cellulose synthesis and an increase of Golgi mediated synthesis of pectic and hemicellulosic substances which accumulate to form the extrahaustorial matrix.

The swelling of the extrahaustorial matrix of isolated haustorial complexes in water occurs without any sign of disruption of the matrix leading Gil and Gay (1977) to conclude that the extrahaustorial matrix was fluid or at least a gel

The extrahaustorial matrix is separated from the apoplast of the rest of the leaf by two annular structures in the neck region, the A and B bands. The A band adjoins the inner face of the host cell wall of infected cells, this usually being the point where the neck breaks when the complexes are isolated (Gil and Gay, 1977). The B band is separate and nearer to the haustorial body. It seals the extrahaustorial membrane to the haustorial neck, thus creating the isolated apoplast referred to as the matrix. As a consequence of this isolation all solutes entering the extra haustorial matrix must do so by traversing one of the two bounding membranes.

Studies using isolated haustorial complexes have provided evidence that haustoria have a role in the nutrition of obligate parasites. Radiolabelled ^{14}C sucrose was detected in haustorial complexes isolated immediately after exposure of infected leaves to $^{14}\text{CO}_2$, thus demonstrating that products of photosynthesis were transported to the

fungus. Little label was found in sucrose after a 20 hours chase with unlabelled CO_2 which indicated that further metabolism or translocation had occurred.

Spencer-Phillips and Gay, 1980, found that the radioactive isotope count in the lobes was twice that found in the body and suggested that this was due either to a higher rate of uptake associated with the large surface area and abundant mitochondria or, more likely, it is correlated with growth as expressed as an increase in the number and length of haustorial lobes which occurs as haustoria age. The extrahaustorial membrane was heavily labelled as was the matrix suggesting a host origin for both.

Spencer-Phillips and Gay, 1980, recognised three distinct types of haustorial complex; 1) healthy, 2) pre necrotic and 3) necrotic. The first symptom of pre necrosis seen under the electron microscope was an alteration of the extrahaustorial membrane manifested as a reduction in extrahaustorial membrane folding. Osmiophilic sites appeared on the extrahaustorial and host cell membranes which indicated a change in the membrane rather than a deposition of material. The sites are similar to those found on plant leaf membranes damaged by freezing or osmotic shock (Pierce and McDonald, 1977; Singh, 1979), where dehydration of the membrane is thought to be the direct cause; the postulated mechanism being a transformation from a bilayer to a micellar conformation (Simon, 1974).

This reduction in convolution of the extrahaustorial membrane may result from a rearrangement of membrane components and the loss of phospholipid which occurs in senescent cells (Ferguson and Simon, 1973). The consequent decrease in membrane area reduces the passage of translocates and the disorganisation of host cytoplasm observed may contribute to the decreased isotope incorporation by pre necrotic complexes. It is likely that a precise molecular arrangement in the extrahaustorial membrane is essential for metabolite passage and Manners (1979) has suggested that transport is an active facultative process.

Analysis of haustorial complexes isolated from infected leaves following pulse-chase feeding of $^{14}\text{CO}_2$ suggests that sucrose is the major

compound which traverses the extrahaustorial membrane (Manners, 1979). Evidence from other biotrophic associations suggests that sucrose is probably hydrolysed prior to uptake by the fungi (Whipps and Lewis, 1981). However Edwards and Allen (1966) showed, after exposing barley leaves infected by Erysiphe graminis to $^{14}\text{CO}_2$ for 15 minutes, that 57% of the isotope in the mycelium was sucrose. Sucrose is transported intact across many plant cell membranes (Giaquinta, 1977) and for powdery mildew at least the consensus view favours the uptake of the intact disaccharide (Gay and Manners, 1981).

In mycelium of E. pisi the primary sink for photosynthates is mannitol (Manners, 1979). It comprises almost half of the ^{14}C extracted from the mycelium after the pulse exposure, but within the 24 hour chase the proportion of radioactivity in the polysols declines and glycogen becomes the major sink. Glycogen is a major constituent of the conidia of E. pisi (Martin and Gay, 1981).

Analysis of $^{14}\text{CO}_2$ labelled haustorial complexes hydrolysed in 2M trifluoroacetic acid showed that they contained mainly glucose (88%) with small proportions of galactose (7%) and mannose (5%) and thus differed significantly from the host fraction (glucose, 54% and galactose, 45%). The cell walls of mycelium contain glucose, galactose and mannose in a 3:1:1 ratio. Thus qualitatively the polysaccharides of the complexes resembled those of the parasite while quantitatively an excessive proportion of glucose is present which suggests that polysaccharides are present in the complexes.

Lipid analysis of haustorial complexes indicated approximately 50% triglycerides and 40% polar lipids. The triglycerides are thought to correspond to the osmiophilic spherical structures observed in electron micrographs of haustoria (Manners, 1979).

The polar lipid found in haustorial complexes is probably derived from membrane constituents. The haustorial plasmalemma is extensive, lining the many lobes extending from the body of the haustorium and the extrahaustorial membrane, is a highly convoluted structure (Manners, 1979).

Solutes entering the haustoria are directed through the epidermal cells which, since they contain few chloroplasts are essentially parasitic on the mesophyll. Plasmodesmata are infrequently found between mesophyll and epidermal cells, therefore it is most likely that the rate of entry into epidermal cells is influenced by the concentrations of photosynthates in the apoplast (Gay and Manners, 1981). Mildew infection increases the loss of electrolytes from leaf cells (Ayres, 1977) but the concentration of photosynthates is slightly decreased by infection (Manners, 1979).

The cytoplasm of epidermal cells may play an essential role in transporting assimilates into powdery mildew haustoria. Unfortunately the evidence for this is equivocal since the experiments that support it have relied upon isolated haustorial complexes and are open to criticism for two main reasons. Firstly isolated haustorial complexes lack the major sink normally provided by the mycelium. Secondly the process involved in removing haustorial complexes, cold osmotic shock and glycerol treatments, remove certain constituents of membranes (Rubenstein, Mahar and Tottar, 1977). Nevertheless Manners (1979) calculated the flux of photosynthates across the extrahaustorial membrane to be much greater than sucrose effluxes calculated from plant cells down large concentration gradients (Edelman, Schoolar and Bornar, 1971) but was similar to the ATP coupled sucrose influx for maize scutellum (Humphreys, 1978). Manners proposed that transport across the haustorial interface was limited by an active or facultative process. Using an alternative approach Spencer-Phillips and Gay (1981) examined the distribution of ATPase and glycerophosphatase activities at pH 5.5 and 7.2 in infected cells. They found that the plasmalemma lining the epidermal wall showed the high level of ATPase activity characteristic of these cells which the portion of the membrane invaginated around haustoria was completely devoid of any phosphatase activity. The transition in activity occurred where the invagination began and coincided with the edge of the A neckband.

Gay and Manners, 1981, proposed a model for the mechanism of assimilate transport at the interface as follows. Entry into the epidermal cells is presumed to be almost exclusively by transmembrane import and the high ATP

ase activity of the plasmalemma suggests that the epidermal cells scavenge solutes from the apoplast. The ATP ase activity of the haustorial plasmalemma is assumed to deplete the solutes in the extrahaustorial matrix simultaneously and thus a high concentration gradient is maintained across the extrahaustorial membrane thus constituting a transport system.

2.4 Lipids

2.4.1 Structure of lipids

Lipids are defined as substances which are insoluble in water, soluble in non-polar organic solvents, contain long chain hydrocarbon groups in their molecules and are present in or derived from living organisms (Kates, 1972). This definition covers a wide range of compounds and includes long chain hydrocarbons, alcohols, aldehydes, fatty acids and their derivatives e.g. glycerides, wax esters, phospho-lipids, glycolipids and sulpholipids. This definition also includes substances which belong to other groups of substances e.g. fat soluble vitamins A, D, E and K and their derivatives as well as carotenoids, sterols and their fatty acid esters.

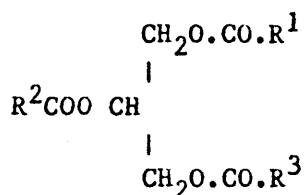
Within plant cells they are bound in two distinct ways, by weak Van der Waals forces by which non polar neutral lipids are bound by relatively weak non covalent forces and through hydrocarbon chains to other lipids and to hydrophobic regions of proteins.

The largest group of plant acyl lipids are those based on glycerol. Of these there are three main groups, the neutral glycerides, the phosphoglycerides and the glycosyl diglycerides. The neutral glycerides are represented mainly by fully esterified triglycerides (Fig. 2a), the properties of which depend upon the fatty acids present, their relative amounts and positions in the molecule.

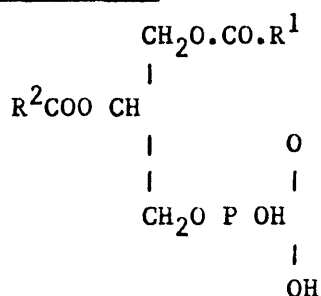
Lipids based on phosphatidic acid (PA) (Fig. 2b) are more polar than the neutral glycerides and tend to be associated with cell membranes. Phosphatidyl choline (PC), the choline ester of PA together with phosphatidyl ethanolamine (PE) the ethanolamine ester of PA are the main phospholipid components of non-photosynthetic tissues.

Figure 2. Generalised Structures of Lipids

a). Triglycerides



b). Phosphatidic Acid



Key

R: Fatty acid chain

A major phospholipid of the chloroplasts is phosphatidyl glycerol (PG) the glycerol ester of PA. Other less abundant phospholipids in plants include phosphatidyl inositol (PI) and phosphatidyl serine (PS). Lysophospholipids, lipids with only one acyl chain, may be minor natural plant phospholipids but this is difficult to assess as they may also be art facts of lipid breakdown during the extraction process.

The third major group of glycerides, associated mainly with chloroplasts (Roughan and Batt, 1969) are the glycosyldiglycerides, the most important of which are the mono- and di galactosyl diglycerides (MGDG and DGDG respectively) which occur in a wide variety of plant tissues. Small amounts of tri- and tetra- galactosyldiglycerides have occasionally been found (Hitchcock, 1971).

The major fatty acids found in plants are long chain saturated or unsaturated monocarboxylic acids with an even-numbered aliphatic chain (Hitchcock and Nichols, 1971), the more common fatty acids are oleic, linoleic, linolenic, myristic, palmitic and stearic acids. Some plants also contain small but significant amounts of unusual fatty acids e.g. non conjugated ethylenic acids found in conifer leaves (Jamieson and Reid,

1972).

Higher plant leaves have a relatively low content of total lipid between 7% and 10% of dry weight (Galliard, 1973) whereas oily seeds have a lipid content of up to 70% by weight (Nichols, 1964). Neutral lipids are the major lipids of seeds (Nichols, 1964) whereas polar lipids predominate in leaves. Since leaf lipids are the topic of the present study their structural characteristics will be described in detail.

Wintermans (1960) demonstrated that the major leaf lipid classes are the galactosyl diglycerides, sulpholipid, PG, PI, PC and PE. However the relative concentrations of different lipid classes varies greatly between different species (Roughan and Batt, 1969). In maize leaves the composition is as follows (units μ moles lipid/g fresh weight) MGDG 3.1, DGDG 2.3, sulpholipid 0.35, PC 0.45, PE 0.48 and PI 0.12 whereas in tomato leaves it is MGDG 5.1, DGDG 2.5, sulpholipid 0.3, PC 1.1, PG 0.4 and PI 0.1. In a later section it will be shown that relative composition can vary greatly depending on the growth conditions and age of the plant even within a single species.

Dark-grown etiolated leaves contain lower levels of glycolipid and polar lipids than light-grown leaves (Winterman, 1960). In general polar lipids tend to be associated with membraneous structures in plant cells whereas triglycerides and waxes are not strongly membrane bound.

Chloroplasts have a high lipid content (about 50%) most of which is located in the lamellae and osmiophillic grana. The major chloroplast lipids are MGDG, DGDG, sulpholipid and PG (Galliard, 1973). Shibuya et al (1965) found sulpholipid localized in lamellae. Mckender and Leech (1974) found that galactolipids were the major components of both lamellar and envelope membranes of chloroplasts from Vicia faba, however, the galactolipid: phospholipid ratio is higher in the lamellar membrane than in the envelope. PC is the major phospholipid in the envelope whereas PG was in the lamellar membrane.

Schvertner and Biale (1973) examining mitochondria from various plants found that they all contained PC, PE, PI, PG, DGDG and MGDG and that phospholipids composed 50-60% of the total lipid in the mitochondria.

Musgrove et al. (1976) found that the microsomal fraction from pea stems contained the following lipids PC, PE, PI, PS, PG, PA, MGDG and DGDG. Phospholipids comprised 64.4% of the total pea lipids, glycolipids 3.7%, the rest being composed of sterol lipids. The major lipids were PC and PE. All the lipids had a lower than usual linolenic acid content. In higher plant leaves the most abundant fatty acids are α linolenic, linoleic and palmitic acids. Trans 3 hexadecanoic acid is ubiquitous in green leaves though it is not found in non-green tissue: etiolated leaves contain no trans-3-hexadecanoic acid and a smaller proportion of polyunsaturated fatty acids than light-grown tissue.

Fatty acids are rarely distributed at random between different lipid classes and frequently exhibit tendencies to associate with specific lipids. Absolute specificity is rare with the notable exception of trans-3-hexadecanoic acid which is restricted to PG (Allen et al., 1964).

Most highly unsaturated fatty acids have a marked tendency to accumulate in the galactosyl diglycerides fractions. In higher plant leaves, linolenic acid often comprises more than 90% of the component fatty acids of MGDG and more than 80% in DGDG. Phospholipids from higher plant leaves contain less linolenic but more linoleic acid. In PC and PE saturated fatty acids tend to accumulate at the 1 position and unsaturated fatty acids at the 2 position; this is the same in mammalian phospholipids.

2.4.2 Biosynthesis of Glycerolipids

Several review articles have covered this subject (Kates & Marshall, 1975; Mudd and Garcia, 1975).

Figure 3 shows the major biosynthetic sequences. The key intermediate in this series of reactions is PA. The general scheme is that PG, PI and PS are derived from CDP diglyceride which is itself synthesized from PA and CTP. PC and PE are mainly synthesized by the nucleotide pathway involving 1,2 diglyceride and CDP choline or CDP ethanolamine. In addition PC is synthesized by stepwise methylation of PE by S-adenosyl methionine. PE is also synthesized by a minor route involving decarboxylation of PS. MGDG is formed by galactosylation (using UDP galactose) of 1,2 diglyceride and

DGDG is derived from a second galactosylation. Sulpholipid is synthesized by a transfer of a sulphoquinovose group to a 1,2 diglyceride.

Galactolipid synthesis occurs in chloroplasts while most phospholipids are synthesized in microsomes. Exchange of phospholipids between microsomal and other membranes has been demonstrated (Mazliak, 1975).

2.4.3 Fatty Acid Biosynthesis

Fatty acid biosynthesis has been reviewed by Hitchcock and Nicholls (1971), Harwood (1975) and Stumpf (1975).

Fatty acid biosynthesis in plants is identical to that established for bacteria and other organisms. The ultimate source of fatty acids is the condensation of acetate units. The components of the system leading to de novo formation of saturated fatty acid, palmitate and stearate from acetate (Acetyl CoS carboxylase, fatty acid synthetase, acyl carrier protein and cofactor requirement) were studied in detail by Stumpf (1975).

Formation of unsaturated fatty acids by plants was shown to be an aerobic process by James (1963) who fed ^{14}C labelled fatty acids to castor bean leaves and obtained evidence that the formation of the major unsaturated fatty acids of plants, oleic, linoleic and linolenic acids was by sequential desaturation stearic acid $\rightarrow$ oleic acid $\rightarrow$ linoleic acid $\rightarrow$ linolenic acid. Nichols et al. (1967) who fed ^{14}C acetate to Chlorella vulgaris found that PG, MGDG, PC and neutral glyceride had a higher turnover rate for certain fatty acids than was the case for DGDG, sulpholipid, PE and PI. They suggested that lipids with a rapid fatty acid turnover were involved in desaturation.

Tremolieres and Mazliak (1974) who studied in vivo and in vitro incorporation of ^{14}C labelled acetate, oleate and linoleate into lipids of developing pea leaves showed that oleate is desaturated to linoleate in microsomes where it resided mainly in PC, PG and PE.

Nichols (1968) suggested that oleate is transferred directly from lipid to a desaturase enzyme and the linoleate produced was transferred back to the lipid. This mechanism would permit simultaneous chain

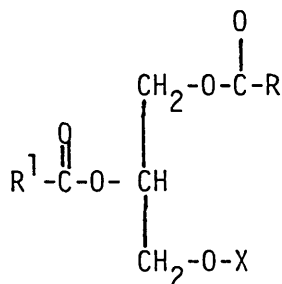
elongation and desaturation of fatty acids. However, there is strong evidence that desaturation occurs while the fatty acid is still esterified to lipid (Willemot & Verret, 1973).

2.4.4 Plant Lipid Degradation

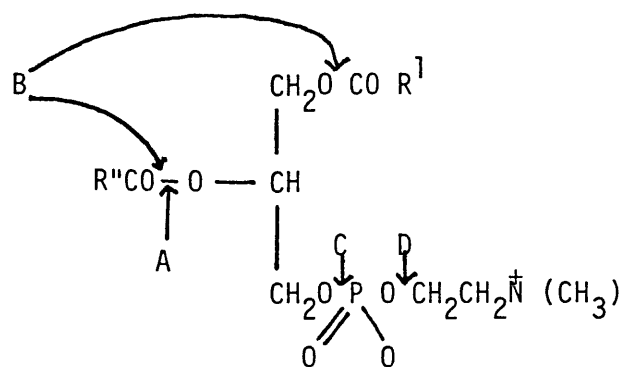
The enzymic breakdown of plant lipids can be divided into hydrolytic and oxidative processes.

Lipase has been used loosely as a term for an enzyme which liberates free fatty acids from acyl lipids. A true lipase is an enzyme which hydrolyses emulsified triacylglycerides at an oil water interface.

Numerous classes of phospholipid hydrolysing enzymes, lipolytic acyl hydrolases have been characterized. These enzymes deacylate glycerolipids with the following general formula:-



Although various acyl hydrolases have been described in no case has an absolute specificity been established e.g. the galactolipid-hydrolysing enzyme found in Phaseolus vulgaris (Sastry & Kates, 1964) shows a higher specific activity with lysophospholipids and mono-glycerides as substrates than with diglycerides and triglycerides (Galliard and Dennis, 1974). With this proviso 4 phospholipases have been described. The sites of attack for these enzymes are shown below:-



A range of acyl hydrolases with various polar lipid substrates is listed in table 1.

Table 1

Table 1: Polar lipid acyl hydrolases from plants

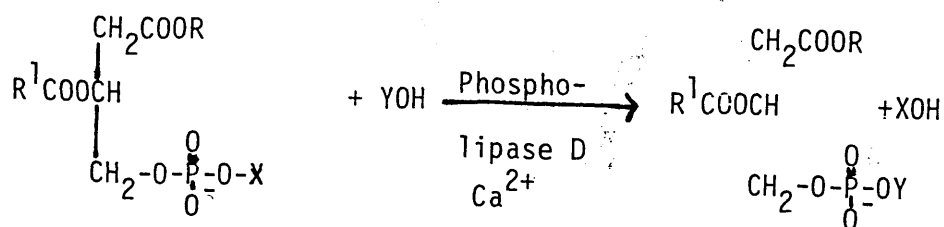
Substrates	Source	Description of enzyme	Products formed in addition of fatty acids	Other lipids hydrolysed	Reference
MGDG and DGDG	Bean leaves	Galactolipase	Mono and digalactosyl glycerol	PC diglyceride	Sastry & Kates (1964)
MGDG + DGDG	Potato tubers + leaves	Lipolytic acyl hydrolase	Mono and digalactosyl glycerol	Diacyl lipids	Galliard (1971)
Sulpholipid	Alfalfa and corn roots Alfalfa leaves	Sulpholipase	Sulphoquinovosylglycerol	Diacyl phospholipids	Yagi & Benson (1962)
PC	Germinating barley	Phospholipase B	Glycerylphosphoryl choline	—	Rebam & Acker (1973)
Mono acyl and diacyl phospholipids	Potato tuber leaves	Lipolytic acyl hydrolase	Glycerylphosphoryl esters	Monoglycerides	Galliard & Dennis (1974)

Surprisingly little attention has been paid to phospholipid acyl hydrolase enzymes in plants compared with animals and micro-organisms. Using germinating barley seeds Acker and Bucking (1956) found an enzyme which removed fatty acids from the 1- and 2- positions of PC and this they characterized as a phospholipase B.

A lipolytic acyl hydrolase which was partially purified from potato tubers catalysed the hydrolysis of both phospholipids and galactolipids. Additionally the same enzyme preparation also deacylated monoglycerides and diglycerides and some artificial esters of long chain fatty acids (Galliard, 1971). The specific activity of the partially purified enzyme towards galactolipids was seventy times greater than that of a purified homogeneous galactolipase enzyme from Phaseolus multiflorus leaves (Helmsing, 1969). Since the Phaseolus galactolipase also showed some activity towards PC (Galliard, 1973) and had properties very similar to the potato enzyme and since crude extracts of barley deacylate both phospholipids and galactolipids, it is possible that the same low specificity lipolytic acyl hydrolase enzyme is concerned in all these tissues and that a specific description such as galactolipase or phospholipase B is misleading (Galliard, 1973).

Phospholipase A is the enzyme which removes an acyl residue on the 2 position of a phosphoglyceride, there is little evidence for its presence in plants as lysophospholipids are only rarely found in plant extracts. This is not, however totally convincing especially in view of the ability of plant cells to degrade mono acyl complex lipids rapidly (Nichols, 1971).

Phospholipase D is the only fully characterized enzyme from plant tissues which attacks the polar groups of acyl lipids. The reaction catalysed by the enzyme can be generalised as follows:-



The enzyme was first described from carrot root and cabbage leaves (Hanahan and Chaikoff, 1947). It was originally thought to be localised in plastids (Kates, 1954) but there is no evidence that the enzyme is present mainly in non particulate fractions of extracts (Quarles and Dawson, 1969). It is completely absent from chloroplasts of maize and spinach leaves when these organelles are prepared under conditions which eliminate contamination by other subcellular fractions (Clermont and Douce, 1970). Similar observations were made with mitochondria from cauliflower inflorescence and hypocotyls from Phaseolus aureus.

Figure 3: Pathways of Glycerolipid Biosynthesis

The specificity of the enzyme has not been unequivocally determined, its activity in vitro on a given substrate is affected by the presence or absence of organic solvents and detergents e.g. diethyl ether stimulates the hydrolysis of PC by phospholipase D from most plant tissues.

In addition to hydrolysis the enzyme also catalyses transphosphatidization reactions which are Ca^{2+} dependent (Galliard, 1975) e.g. $\text{PC} + \text{glycerol} \rightarrow \text{PG} + \text{choline}$.

The other physiologically significant enzymes involved in lipid degradations are oxidative in nature. Enzymic oxidation usually involves the prior release of fatty acids from lipids. These are 6 different types of reaction involved in the oxidation and oxygenation of fatty acids by plants; α oxidation, β oxidation, ω oxidation, β hydroxylation, in-chain hydroxylation and peroxidation (Galliard, 1975).

There are two different processes of α oxidation in plants. In both cases a free fatty acid is oxidised at the terminal end losing 1 carbon unit to become the aldehyde of the corresponding C^{n-1} acid and further oxidation of the aldehyde yields a C^{n-1} fatty acid. Initially it was thought that there were two separate systems. One system was a peroxidase process where the hydrogen peroxide generating system was required (Markonetz et al., 1972) in peanut cotyledons. The other system found in peanut leaves did not require hydrogen peroxide but used molecular oxygen (Hitchcock and Nichols, 1971). More recently Shine and Stumpf (1974) have shown that a common system exists in both (figure 4).

Reviews on β oxidation in plants include those by Hitchcock and Nichols (1971) and Galliard (1975). Two different roles have been suggested for β oxidation, one in seeds which contain storage lipids where β oxidation is involved in the conversion of seed oil via acetyl Co A to the products of the glyoxylate cycle giving a net conversion of lipids to carbohydrate. The other role is that of energy production which is associated with mitochondria.

ω oxidation concerns fatty acids with an oxygen function (Hydroxyl, keto and carboxyl groups) at the ω position such as occur in some seed oils and cutin and suberin. These fatty acids are hydroxylated by in-chain

reactions which involve a mixed function oxygenase and coenzyme A thiolester (Galliard, 1975).

Lipoxygenase catalyses peroxidation of fatty acids. Lipoxygenases are distributed widely throughout higher plants (Grosch, 1972). The enzymes from different sources differ in several aspects including substrate specificity, pH optima, the effects of inhibition and the isomeric form of the products (Galliard, 1975).

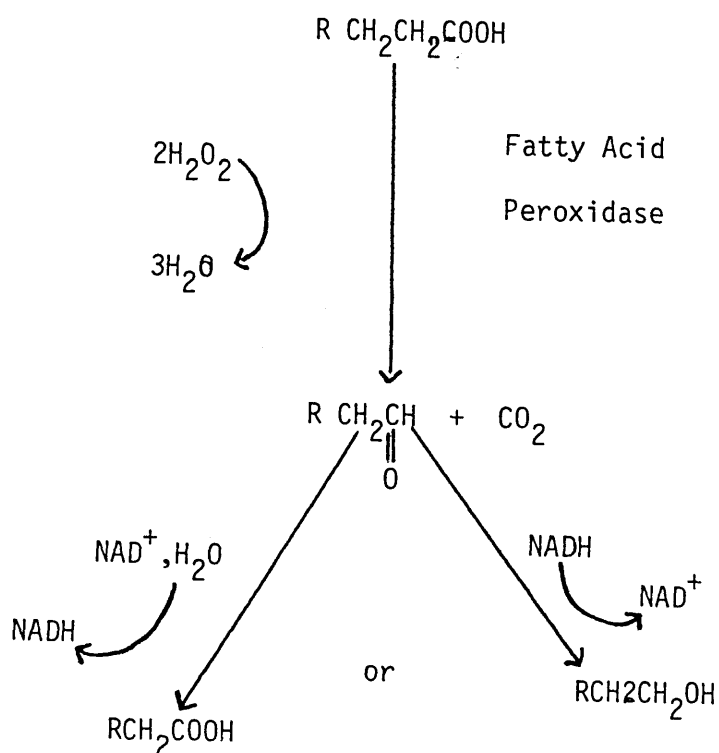
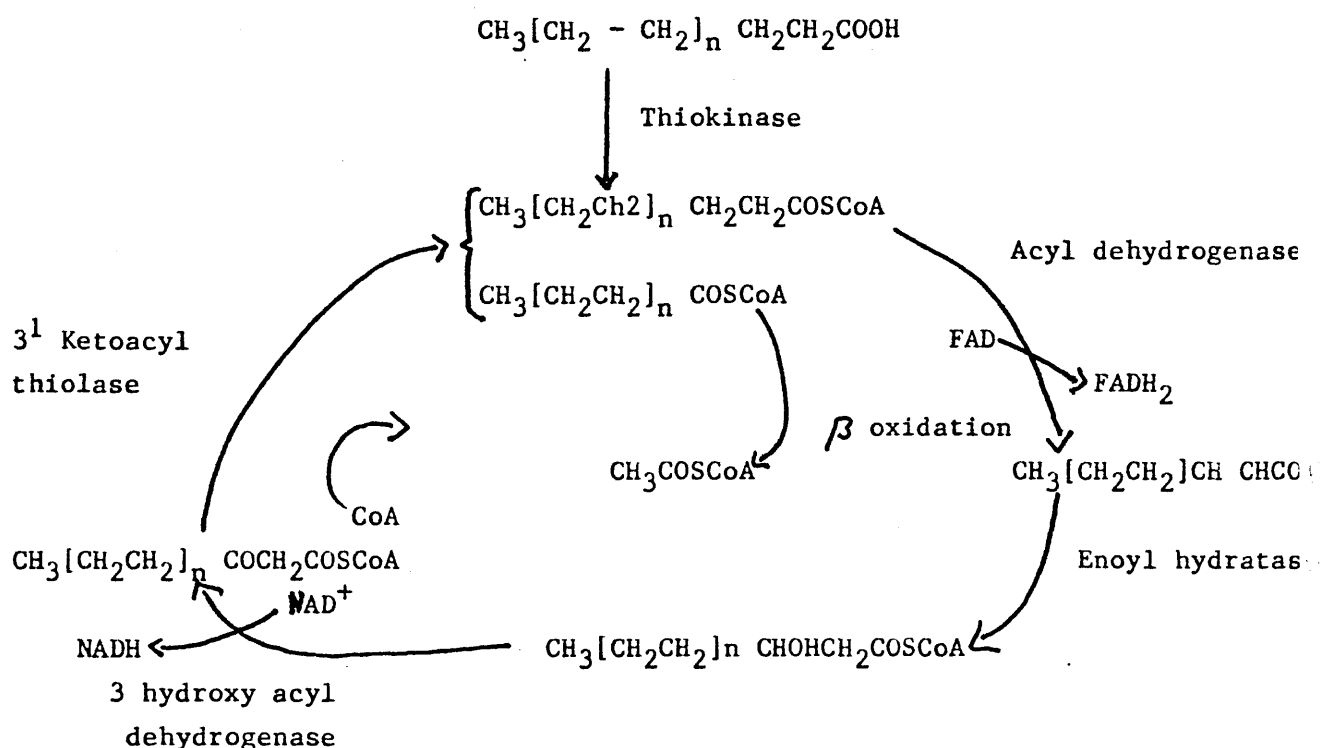


Figure 5: Mechanisms of β Oxidation



All lipoxygenase enzymes specifically attack fatty acids with *cis, cis* -1,4 pentadiene structures and convert them to *cis trans* diene hydroperoxides. The detailed mechanism of action of lipoxygenase has not been elucidated. Recent developments suggests that the lipoxygenase reaction is a free radical process involving a novel type of interaction between both the substrate and oxygen and the enzyme.

The role of lipoxygenase in plants is unclear. Levels of the enzyme are known to fall during the early stages of germination and increase later on (Anstey & Friend, 1974). It may function as a defence mechanism as its activity increases in damaged tissue (Lipetz, 1970). Its activity also increases during the ripening of climacteric fruits such as apples, at the same stage as the evolution of volatile flavour compounds and ethylene (Woolerton *et al.*, 1965) and the loss of endogenous galactolipids, rich in linoleic and linolenic acids (Galliard, 1968). Volatile aldehydes with characteristic flavours are formed from lipoxygenase substrates in damaged plant tissue.

Fatty acid hydroperoxides formed by the action of lipoxygenase are toxic to living tissues. Plants are known to contain systems which metabolise these hydroperoxides to the corresponding hydroxydiene derivatives e.g. Soyabean (Grosch, 1972) and potato tubers (Galliard et al., 1975). It is thought that reduction may be non enzymic as fatty acid hydroperoxides are reduced by Fe^{2+} ions or by Fe^{3+} ions in the presense of thiols, though the yield of hydroxydiene is low compared with other products.

Liperoxidase has been described in plants. Hieman et al. (1973) described a hyperoxidase from oats which reduced fatty acid hydroxyperoxides to corresponding hydroxyacids in the presenCe of phenyldiamine but they were unable to separate the peroxidase and lipoxygenase functions and therefore proposed that a multienzyme complex was involved.

2.4.5 Fungal Lipids

The lipid content of vegetative hyphae varies between 1 and 50% of the dry weight depending on the species, development stage of growth and cultural conditions. Although many fungi have a high capacity for lipid production, most mycelial species contain between 6% and 9% lipid when grown under favorable conditions (Weete, 1980). Fungal spore lipid abundancy also vary considerably depending on the species but range between 1% and 35% of the spore dry weight. The ability of spores to germinate without an exogenous carbon source appears to be related to the abundance of reserve lipid.

The fungal cell wall consists of 80-90% polysaccharide with the remainder consisting of protein and lipids. Fungal cell wall preparations consist of both freely extractable and bound lipids (Kessler and Nickerson, 1959). Care, should, however, be taken when looking at the analysis of fungal cell wall lipids since it has been shown that carefully isolated cell envelopes of yeast contain plasma membrane fragments (Suomalainen and Nurminen, 1970). Suomalainen (1969) reported that yeast cell wall lipids are composed of neutral lipids, phospholipids and that the predominant fatty acids are palmitoleic acid and oleic acid.

A major portion of the intracellular lipids of fungi are in the membranes. However at various growth stages, some lipid accumulates as droplets or globules in the cytoplasm. These globules are thought to consist of mainly triglycerides.

Protoplast membranes from Saccharomyces cerevisiae account for 13-20% of the dry cell weight and contain 39% lipid, 49% protein, 6% sterol and traces of RNA and carbohydrates. The principal structural lipid components were phospholipids varying between 15% and 25% of the membrane. The principal fatty acids were Palmitic, Palmitoleic and Oleic acid. The membranes also contained mono-, di- and triglycerides, free sterols, sterol esters, PC, PE, PI, PS and LPC (Longley et al., 1968).

Anderson et al. (1964) found lipid composition of Claviceps purpurea mitochondria was the same as typical mammalian and plant mitochondria.

Phospholipid distributions in various organisms have been reviewed by Weete (1980). PC is the most abundant phospholipid in both yeast and mycelial forms of fungi. Total PC concentrations range from 35-50% of the total phospholipid fraction. The next most abundant phospholipid in many fungi is PE which ranges from 14-35% of the total fraction. PI and PS have been found in most fungi studied and generally represent less than 25% of the phospholipid fraction, PS from 4%-20% and PI from 8-23%.

In general fatty acid distribution in fungi are very similar to those of other biological systems. The major components are palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids. Other fatty acids are found and they vary from species to species.

The metabolism of lipids and fatty acids in fungi is similar to that in plants with a few species-specific differences e.g. in yeast the fatty acid synthetase enzyme while performing the same function as the same enzyme in plant and bacterial cells is structurally very different. However in general the metabolic pathways are the same.

The presense of phospholipase enzymes in phytopathogenic fungi is discussed in Section 2.7.5.

2.5 Function of Lipids

There are two major functions of lipids, firstly as structural components of all membranes and secondly for energy storage particularly in seeds.

For the purposes of this study only the role of lipids as structural components of cell membranes will be examined in depth. Membranes consist 95% of lipids and proteins (Kotyk, 1977). The most widely accepted model of membrane structure is that of Singer and Nicholson (1972) in which proteins are more or less embedded in the lipid bilayer. Some proteins extend through the membrane, integral proteins, whilst others are confined to one side of the membrane only, peripheral proteins. Membrane proteins, like the phospholipids are amphipathic and it is this property that defines their location in the membrane; their polar and ionic groups project into the aqueous interior, while their hydrophobic regions remain embedded within the membrane in association with the non polar hydrocarbon chains of the phospholipid.

Since the leakage of substances through membranes is implicit in the nutrition of obligate plant pathogens, the details of membrane construction are important in so far as they can show the ways by which permeability could become altered. According to the model of Singer and Nicholson (1972) phospholipid and protein both contribute to the structure of the membrane so that a cytoplasmic solute might be prevented from diffusing through a membrane by phospholipids in one region or by protein molecules in another. It follows that the modification of either component might enhance membrane permeability, effectively there being two channels in parallel.

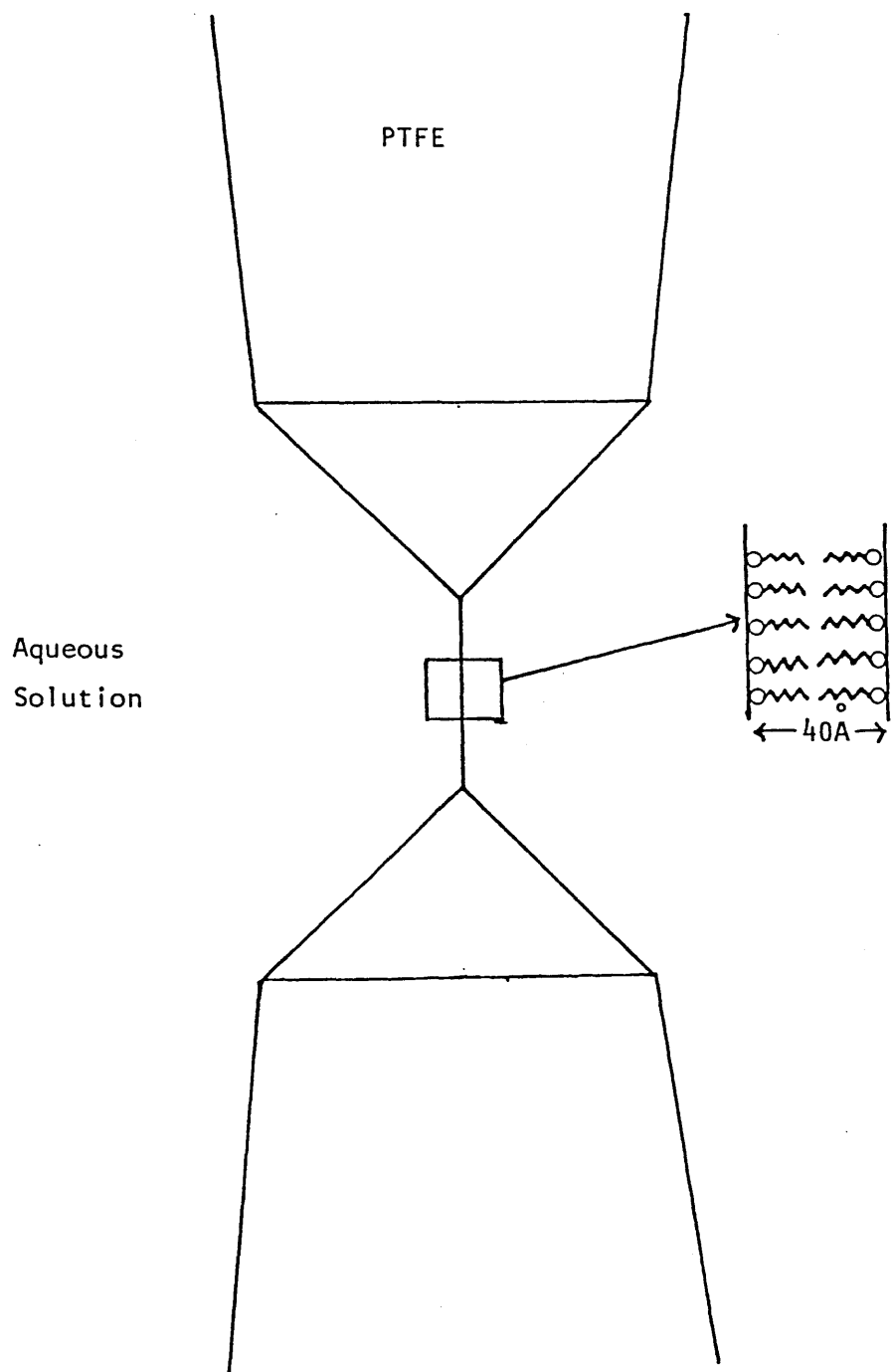
Many permeability studies of membranes have been conducted on one of two artificial model systems, either optically black films or liposomes. These studies have been the subject of a review by Fettiplace and Haydon (1980). Two methods have been used to measure the water permeability of membranes. In one the diffusional permeability coefficient (P_d) has been determined by measuring the exchange of radioactively labelled water between the cell and its surroundings. Alternatively a permeability

coefficient (Pl) has been obtained from the hydraulic conductivity of the membrane, the latter being inferred from the net water flux generated by a given gradient of an osmotic or a hydrostatic pressure. This usually involves measuring the rate of change of volume of a cell when it is immersed in a non isotonic solution. There have been difficulties in interpreting results using these techniques, based upon doubts as to whether the cell membrane really constitutes the rate limiting step in the transport of water and solutes. It has been argued that the diffusion of water both within the cytoplasm (Dick, 1964) and in unstirred layers outside the cell (Dainty, 1963) may pose extra limitations to transport.

A black film is formed by extending a solution of a lipid in a non polar solvent across a hole in a suitable support, under an aqueous solution (figure 6). This black film is never quite comparable to the lipid leaflet found in biological membranes as there is always some residual non-polar solvent in the membrane (Fettiplace and Haydon, 1980). It has been possible to study the permeability of lipid bilayers using this method as it allows the production of artificial membrane systems of different compositions. If the barrier to the movement of water across lipid bilayers is thought to be non-polar, interior variations in the lipid that would alter the fluidity and thickness of the hydrocarbon core might be expected to produce permeability changes. Increasing the degree of unsaturation of the acyl chains of the lipid increases the water permeability (Fettiplace, 1978). Conversely hydrogenating the lipid or using analogues with saturated chains can reduce the permeability (Fettiplace, 1978).

Figure 6: Cross Section through a black lipid film in aqueous solution

Figure 6



The maximum variation observed by altering the unsaturation is of the order of $\pm$ 50%. Increasing the length of the chains also decreases permeability (Fettiplace, 1978).

Membrane fluidity and water permeability properties can also be influenced by interactions between lipid head groups. Graham and Lea (1972) have shown that the permeability of PS films is a function of the pH of the aqueous phase (0.1 M Na Cl). Increasing the pH from 3 to 9 which results in a change in the head group ionization produces an increase in the water permeability.

The addition of cholesterol to membranes results in a 3 to 5 fold reduction in the permeabilities of membranes formed from PC and other lipids (Finkelstein and Cass, 1967).

As well as water permeability, the permeability of non electrolytes such as urea (Lippe, 1969), glycerol (Wood, Wirth and Morgan, 1968), erythritol and formamide (Poznanski et al., 1976) has been studied. For all these substances the permeabilities are considerably lower than that for water but, like the water permeability depend on the lipid composition of the membrane (Lippe, 1969).

The other structural system for studying lipid permeability, liposomes, are composed of phospholipids in aqueous salt solutions which become spontaneously organized to form salt-containing compartments limited by bimolecular membranes.

From comparisons of initial shrinkage rates of liposomes for a wide range of systems Bittman and Blau (1972) reached a number of conclusions concerning permeability to water. For dipalmitoyl PC the water permeability appears to increase more than 20 fold between 38°C and 45°C as the lipid passes through its phase transition temperature. They also found that adding dipalmitoyl PC to dioleoyl PC at 25°C caused a marked reduction in the water permeability which was attributed to the decrease in the concentration of double bonds in the membrane.

De Gier et al. (1968) using liposomes from synthetic PC found that permeability to the non electrolytes, glycerol and erythritol was

enhanced both by the presence of double bonds in the fatty acid residues and by decreasing the fatty acid chain length.

Boggs and Hsai (1972) reported that hydrated egg PC containing unsaturated fatty acids formed a fluid liquid crystalline phase whilst full saturated PC formed a less fluid gel-like phase. In addition to unsaturation, branching in acyl chains appeared to impart fluidity on the membrane.

The nature of the polar head group influences the structure and properties of the phospholipid bilayer. Papakadopoulos and Miller (1967) showed that the structural characteristics of artificial membranes prepared from the purified lipids PC, PE, PS, PA and PI differed as did their permeability characteristics. PE alone formed incompletely sealed liposomes; partial addition of other phospholipids resulted in the formation of closed structures. Cl⁻ ions diffused faster through membranes prepared from the acidic lipids PA and PI.

Hopfer et al. (1970) showed that artificial membranes of lysyl PG were almost completely Cl selective and membranes from PG and DPG were highly cation selective. Membranes prepared from diglucosyl diglyceride (uncharged) or PE (Zwitterionic) showed slight cation selectivity. Schrier-Mucillo et al. (1973) found the neutral glycerides had a restricted ability to form organized liquid crystalline structures. They also found that the presence of a net charge in the head group decreased the degree of order of the bilayers and that bulky head groups favoured the formation of highly organised bilayers.

Other factors able to modify the lipid bilayer also influence the permeability. Cholesterol interacts directly with phospholipid and modified the properties of liposomes (Bangham et al., 1965). Cholesterol and ergosterol decrease the permeability of liposomes to glucose (Demel et al., 1968). Boggs and Hsai (1972) found that cholesterol added up to a certain level decreased the fluidity of bilayers prepared from some natural and synthetic lecithins while the addition of further cholesterol increased the fluidity. Cholesterol acts by inserting between the hydrocarbon chains of lipid bilayers which are already in the liquid

crystalline state condensing the layers and making them less permeable; in addition cholesterol has a liquifying effect on bilayers which are in an ordered gel state making them more mobile (de Kruyff, 1976).

The permeability of liposomes is temperature dependent (De Gier et al., 1968) probably because the increased thermal mobility of the acyl chains in the lipid bilayer enhances permeability.

Lipids affect the activities of membrane bound enzymes (Kotyk, 1977). When the amount of unsaturated fatty acid in yeast mitochondria is low the amount of oxidative phosphorylation is correspondingly low.

Changes in membrane lipids correlate with some changes in physiological properties. Temperature induced phase changes in membrane lipids affect oxygen uptake by yeast (Eletr et al., 1974). Wade et al. (1974) demonstrated phase changes from liquid crystalline to solid gel states in mitochondrial membranes, glyoxysomes and pro plastids from germinating castor bean endosperm and in artificial membranes made from the lipids of these organelles. The phase changes coincided with the temperature range (around 10°C) where chilling sensitive plants suffer from chilling injury.

Summarizing both black lipid films and liposomes yield similar results. The advantage of black lipid films is that they have accurately known areas and therefore precisely calculable permeabilities: the chief disadvantages are that their lipid composition is restricted by the requirement that they form stable membranes, that the amount of the various lipids present in multicomponent membranes is not easy to estimate, and that by necessity they contain residual organic solvent. Liposomes overcome these disadvantages but introduce problems of their own. In osmotic experiments there are incompletely understood geometrical changes such that liposome volume has to be studied by essentially optical methods. Furthermore uncertainty concerning the number of layers of lipid involved in their structure precludes the calculation of accurate permeabilities.

Despite these problems there is broad qualitative agreement concerning the influence of lipid composition on membrane properties, it is in the

precise magnitude of the effects that disagreement exists.

Proteins also contribute to the permeability of membranes in several ways, which can be affected to a greater or lesser extent by the lipids in the membrane. They contribute to the passive movement of solutes by forming channels in the membrane e.g. gramicidin forms pores in membranes which allows the passage of small molecules. In other cases membrane permeable proteins bind to cations and transport them across the membrane passively e.g. valinomycin. These mechanisms are highly dependent on the lipid composition of the membrane. There are other examples of this facultative diffusion system however in most cases the protein has not been identified and can only be assumed to exist by reference to the diffusion kinetics. These systems only work under the influence of a concentration gradient.

In many cases the uptake of solutes goes against the concentration gradient and in these cases active transport systems involving proteins are involved. Group translocation systems involve the chemical modification of the permeant as it is transported. Such systems have been well characterized in micro organisms but are less well established in higher organisms. ATP provides the energy for transporting some permeants and, in the case of the active pumping of Na^+ , K^+ and Ca^{2+} the transporter protein has ATP-ase activity. Many bacterial transport systems are also dependent on ATP, but their mechanisms have not been elucidated. Mitchells chemiosmotic hypothesis provides for the coupling of the potential energy of trans-membrane electrochemical gradients to specific transport systems. Proton gradients particularly are well used in both prokaryotic and eukaryotic cells.

Studies of the inactivation of transport systems in membranes by modification of the lipid environment and their subsequent reactivation by restoration of appropriate lipids has suggested specific lipid requirements for some transport activities e.g. requirement for PG to sustain the activity of certain phosphoenolpyruvate linked phosphotransferases. In the case of Na^+/K^+ ATP ase there is conflicting evidence for on the one hand, a specific requirement for anionic lipids and, on the other simply a requirement for a fluid lipid environment

(Finean, Coleman and Mitchell, 1978). A still more detailed assessment of the influence of lipid environment has been made for the Ca^{2+} transport system where the substitution of a completely artificial membrane environment around the protein molecule has distinguished two requirements, one for the ATP-ase activity and one for sealing the vesicles to achieve effective Ca^{2+} accumulations. In these experiments maximum Ca^{2+} dependent ATP ase activity was achieved with a synthetic dioleoylphosphatidyl ethanolamine but efficient retention of Ca^{2+} in the vesicles also required dioleoylphosphatidylcholine. It is perhaps significant that PC and PE are the principal phospholipids of sarcoplasmic reticulum.

2. 6 Environmental effects on Lipids in plants

2.6.1 Varietal and Manurial effects

Kuiper (1968) compared the lipids of the roots of five grape cultivars which differed in the extent to which they permitted chloride accumulation in the leaves. The MGDG content of roots was positively related to the chloride transport to the leaves and varied from 9.5% to 39% of the total lipid composition. This lipid permits the greatest chloride diffusion in membrane models. PC and PE content was inversely related to chloride accumulation, the levels of these lipids being 13% and 21% respectively in the most resistant cultivar and 4% and 6% in the most sensitive cultivar. Furthermore the most sensitive cultivar had an unusually low sterol content in the roots, 9% compared with 16-22% in the roots of the other four. The importance of long chain saturated fatty acids for chloride transport was also noted. The amount of lignoceric acid present in the roots decreased from 12% in the most resistant cultivar to 1% in the most sensitive one. This fatty acid was mainly observed in the PC and PE fractions. Kuiper (1968) concluded that charged lipids contributed significantly to the observed low chloride transport to the stem and leaves and that the presense of long chain fatty acids also contributed to low permeability of chloride. It is not possible, from these experiments, to state which factor was responsible for this change in chloride permeability.

When barley roots are exposed to increasing concentrations of NaCl the total lipids content and in particular phospholipid content increases. The most marked individual increase in phospholipid is in PC (Ferguson, 1966).

In cotton, salt stress stimulates both flower and boll development. Twersky and Felhändler (1973) found that during the fruiting stage both the total lipid content and the phospholipid content was lower in the brackish water-treated plants. Kuipur (1975) showed that in wheat and oat roots the level of PC is related to the Mg^{2+} and Ca^{2+} stimulated ATP ase activities by increasing the concentration of the nutrient solutions and observing an increase in the level of PC as well as an increase in enzyme

activity.

2.6.2 Temperature effects

Studies looking at the effect of temperature changes on plant lipids have revealed some important information. The structure and permeability properties of membranes are strongly influenced by temperature. Thermal agitation imparts greater molecular mobility on lipids, but this freedom of movement is restrained by the requirement for the polar head group to remain oriented towards the boundary aqueous phase. Consequently it is the hydrocarbon chains which exhibit the greatest responses to change in temperature.

In pure synthetic phospholipids this change from the more ordered gel state to the liquid — crystalline state occurs over a narrow temperature range. However, in natural membranes several different lipid classes exist and within each class a variety of fatty acid permutations may exist. A further complication is that the various lipid classes are unlikely to be distributed evenly. The net implication for naturally occurring lipids is that the transition process (melting) may occupy a temperature range of as much as 10°-40° (Stein, 1972).

When temperature is reduced below 10°C in plants the membrane lipids undergo a change in phase. As well as this change in the physical state of the lipids major changes are also observed in the Arrhenius activation energy (E_a) of a number of key, membrane-associated enzyme systems and in the permeability properties and active transport systems of the membrane. An example of the effect phase changes have on the functioning of membrane proteins can be seen by studying the E_a of each of the enzymes involved in electron transport. Each of these enzymes exhibits an increase in E_a at the same temperature (Raison, Lyons and Thomson, 1971). This change can be abolished by treating mitochondria with detergents while an enzyme which does not show this change, monoamino oxidase, is unaffected by detergents. Electron spin resonance (e.s.r.) spectra showed that there was a membrane fluidity change whose temperature range corresponded with the change in E_a of the enzymes (Melbourn and Keith, 1972). The evidence supports a lipid mediated mechanism for the temperature — induced change in the kinetics

of membrane associated enzymes which is not therefore an intrinsic property of these enzymes. This holds true for most membrane bound enzymes (Raison, 1972).

Changes in the physical properties of membranes induced by lower temperatures can affect the binding of enzymes and the permeability of membranes. Uritani, Hyodo and Kuwano (1971), demonstrated a loss of cytochrome C and malate dehydrogenase from mitochondria isolated from sweet potato maintained at about 1°C. In related studies Yamaki and Uritani (1972) noted a progressive loss of phospholipids such as PE and PC from mitochondrial membranes during storage at chilling temperatures indicating a progressive breakdown of membrane integrity. Thus as well as changing the permeability characteristics of the membrane, changes in the lipid compositions of the membrane also affect the structure and function of the membrane proteins.

As already discussed temperature markedly affects membrane lipid phase characteristics. Lipid composition has an equally dramatic effect and there is evidence that some plants (chilling sensitive) can compensate for the effects of low temperature by modifying the membrane chemistry. For example the amount of each of the principal phospholipids in leaves of cucumber and cotton declines during chilling at 5°C and 85% relative humidity but not in chill resistant cultivars (Wright and Simon, 1973). In alfalfa the fatty acid composition of the roots changes during hardening and there is an accumulation of polyunsaturated lipids (Gerloff et al., 1966). This change in lipid saturation was accomplished in alfalfa by a change in lipid composition. Lipids like MGDG, DGDG, PC and PE which are predominantly esterified with linoleic and lindenic acids increase and replace the more saturated lipids like PG, PI and sulpholipid (Kuiper, 1970).

Wheat seedlings grown at 2°C are capable of developing considerable cold hardiness which is associated with a high level of unsaturated fatty acids (de la Roche et al., 1973). The phospholipid composition of seedlings grown at 2°C and at 24°C was similar indicating that the increased unsaturation at 2°C is the result of an increased desaturation activity and not due to differences in phospholipid synthesis.

Smolenska and Kuiper (1977) examining the effect of low temperature upon the lipid composition of roots and leaves of winter rape showed that transfer of plants to 5°C led to an increase in the level of linolenic acid in the leaves and roots. This increase was most evident in the PC and PE fractions of the leaves and in the neutral lipids. It was concluded that upon transfer of the plants to 5°C a general and non-specific increase in linolenic acid ~~which~~ contributed to the functioning of the plants at low temperature.

In Vicia faba the transition temperature is very high (20°C) so it is normal to grow plants under unsaturation enhancing conditions. Lem et al., (1980) found that for Vicia faba plants grown at temperatures below 20°C there was little effect of frost or chill hardening on the fatty acid levels of galactolipids. Above the optimum growth temperature the level of linolenic acid decreases in the galactolipids suggesting that the change in the linolenic acid is a function of high temperature. When plants were transferred to a lower temperature then desaturation of linoleic to linolenic acid occurred.

Lem et al., (1980) suggested that desaturation of fatty acids in Vicia faba was controlled by both light and temperature. Light mainly affected the levels of palmitoleic acid in PG and it appeared that light intensity and not duration was the important controlling factor. The fatty acid composition did not change significantly during days of continuous darkness suggesting that lipids do not turn over rapidly.

2.6.3 Lipids and Senescence

Several studies on the effect of senescence on lipid composition in plants have been made. Degenerative changes have been observed at the ultrastructural level. Barton (1966) showed in Phaseolus vulgaris leaves chloroplasts decreased in size, thylakoids and stromal ribosomes disintegrated and osmiophilic globules (similar to those found in haustoria) accumulated. It was suggested that these were the result of the accumulation of lipids arising from thylakoid breakdown, although such globules are also a feature of the plastids of normal plants (Greenwood et al., 1963). Butler and Simon (1971) suggested that these globules consist

of both membrane breakdown products and still accumulating lipid precursors or that they could represent a general reservoir of excess lipid not necessarily connected to membrane function and breakdown.

Draper (1969) observed that during the yellowing of cucumber cotyledons the amount of galactolipid, sulpholipid and PG decreased while the levels of PC and PE remained constant suggesting that photosynthetic lipids were being degraded. During the latter stages of senescence there was a decrease in all lipids. During the degradation of chloroplast lipids there was a corresponding increase in linolenic acid in the free fatty acid fraction. This was confirmed by Ferguson and Simon (1973) who also observed that losses from the phospholipid fraction coincided with an increase in the apparent free space and loss from tissues of water and electrolytes.

McKersie et al. (1978) studied the effects of senescence in Phaseolus vulgaris cotyledons on the composition and physical properties of membrane lipids. They found that the phospholipid content of rough and smooth microsomal membrane fractions from cotyledons of germinating bean declined as the tissue became senescent. Both types of membrane contain comparable proportions of three major phospholipids, PC, PE and PI which collectively comprise about 90% of the total which did not change during senescence. The unsaturated: saturated fatty acid ratio for total extracted lipid declined only slightly in both membrane systems. The major difference noted in this study was that with advancing senescence there was a corresponding increase in the sterol: phospholipid ratio by about 50% for rough microsomes and 400% for smooth microsomes, this was thought to be partly although not wholly caused by the decrease in phospholipid.

The physical state of the membrane was assessed by the use of electron spin resonance studies. The Arrhenius activation energy increased during senescence for both rough and smooth microsomes and thus the physical state of the membrane lipid is modified by senescence and this is reflected by an increase in fluidity. It is most likely that this change was caused by the increased proportion of sterols in the membrane.

The conclusions from studies with etiolated and senescent leaves are that there is a net decrease in the amount of membrane lipid and an increase in free fatty acids caused by this (Galliard, 1980). The remaining lipid bound fatty acids show a net increase in fatty acid saturation in the early stages of senescence due to the initial breakdown of chloroplast lipids. Most of the early studies on membrane lipid fatty acid composition during senescence reached the conclusion that there was a decrease in the levels of lipid bound unsaturated fatty acids, however these results only reflected the loss of chloroplast lipids and gave no indication of the effect on the membrane lipids. El Hussein (1977) was able to show that senescence causes a significant increase in the levels of unsaturation in the fatty acids from PC, PE, PI and PS.

This section shows that the environment has a large effect upon the lipid and fatty acid composition of plant tissue. Plants can respond to changes in their nutritional status by altering the composition of the membranes. The temperature that the plant is growing in has a profound effect upon the physical structure of the membrane and thus the working of that membrane. Some plants are able to adapt to growth in different temperatures by altering the composition of the membrane lipids whilst others are unable to do this.

Senescence has a drastic effect on plant membranes causing a general degradation of the membranes and also a change in the saturation of the lipid bound fatty acids.

2.7. The effect of infection on lipids in plants

2.7.1. Permeability

Amongst the earliest investigations of the effect of plant pathogens on the permeability of plant membranes was the work of Thatcher (1942, 1943). From his studies on rusts, powdery mildews, soft rots and dry rots he concluded that, a) cells of fungal pathogens have a higher osmotic potential than their hosts, b) altered permeability of host cells is a regular feature of disease and c) that an increase in host cell permeability is a characteristic of susceptibility whereas a decrease is

found with resistant reactions. While agreeing with the first two conclusions Wheeler and Hanchey (1968) argued that problems with methodology rendered the third conclusion untenable in Thatcher's experiments, the comparisons between resistant and susceptible plants were made long after the important early stages of pathogenesis during which the outcome of infection was decided.

Hoppe and Heitefuss (1974a) in a preliminary study compared the leakage of electrolytes, amino acids and sugars from rust infected leaf halves, adjacent uninfected leaf halves and healthy primary leaves. Infection caused an increase in the leakage of ions, amino acids and sugars which was evident by 4 days after inoculation and increased throughout the course of the experiment. No differences were observed between the uninfected leaf halves and the controls up to 8 days after inoculation, but later (10 and 12 days after inoculation) the leakage from uninfected leaf halves was higher. From these results the authors postulated that the membrane permeability was altered by the pathogen.

These studies indicate that altered permeability of tissues to water and solutes is a characteristic of fungal disease in plants. The precise mechanisms by which this is brought about are however less clear. Studies to reveal the cause have concentrated firstly on ultrastructural changes resulting from infection and secondly on analysis of changes in membrane lipid composition.

2.7.2. Ultrastructural Studies

Ultrastructural studies of victorin-treated oat plants have revealed damage to chloroplast membranes in leaves and to membranes of the nucleus, vacuole, endoplasmic reticulum and other organelles in the root cap cells (Luke, War and Hanchey, 1966). Other ultrastructural studies have resulted in the observations that ribosomes (Williams and Menqola, 1967) mitochondria (Hanchey et al, 1967) and chloroplasts (Luke et al, 1966) undergo modifications in diseased plants.

Hislop et al (1979) studying the effect of pectin lyase from Monilinia fructigena on the ultrastructure of cultured apple cells found that the ultrastructure of healthy cells was modified by enzyme treatment. The

endoplasmic reticulum showed dilation and vesiculation and the staining characteristics of cytoplasm were altered. Many cells treated with lethal amounts of pectic lyase showed pseudo plasmolysis. Some small changes occurred in the plasmalemma of injured cells but the unit structure of the membrane was still in evidence. Mitochondria appeared swollen in injured cells and were always less easily recognised whereas nuclei showed very few changes. The swelling of the endoplasmic reticulum has also been found in tobacco roots infected by Phytophthora parasitica var nicotianae (Hanchey and Wheeler 1971). The hypersensitive response of bean leaves to bacteria involves multi-vesiculation and accumulations of osmiophilic droplets.

One of the earliest manifestations of disease is the change in the nature of cell membranes, measured experimentally as electrolyte release; some interesting work has been undertaken on the release of enzymes from lysosomes. Histochemical methods used by Pitt and Coombes (1968) demonstrated the existence in potato tuber tissues of sub-cellular particles rich in lysosomal acid hydrolases. Infection of potato tubers by Phytophthora erythroseptica was accompanied by swelling and disruption of the host particles, as observed in sections stained for acid phosphatase activity. Particularly interesting was the widespread and heavy cytoplasmic staining of this enzyme in infected tissues. It has not been possible to support these observations biochemically due to the mechanical problems in homogenising the tissue and to biochemical problems due to high activity of endogenous phospholipases and lipoxygenases (Galliard 1971).

Although these results reveal that infection is accompanied by decreased particulate activity of hydrolases with a simultaneous rise in the enzyme activity of the soluble phase, this may not necessarily be due to the release of enzymes from lysosomes. It could be that infection results in specific inactivation of particulate hydrolases by an inhibitor of fungus or host origin. In addition it is known that fungi contain particulate hydrolases (Pitt, 1968) and that these may be secreted and contribute to the soluble enzyme activity.

2.7.3. Phospholipid and Polar Lipids

Various researchers have investigated the vegetative biotrophic phase of mycelial growth by parallel analysis of infected and uninfected host tissue. Muckerjee and Shaw (1962) found increased levels of lipid phosphate in wheat leaves infected with Puccinia graminis. Williams *et al* (1968) recorded increased total lipids in cabbage hypocotyls infected with Plasmodiophora brassicae.

In a significant series of experiments Hoppe and Heitefuss (1974) studied changes in lipid concentration and ^{32}P incorporation into phospholipids and found that the amount of PC, PE, PI and sulpholipid in leaves did not alter as a result of infection but infected plants did exhibit a decrease in the amount of MGDG, DGDG and PG and an increase in PS and PA. These changes were first noticed 4 days after infection which corresponded with the increase in leakage observed in their previous experiments (1974a). The differences were restricted to the region around the pustule and were not observed in the surrounding mycelium-free tissue. The amount of all phospholipids and glycolipids in the uninfected leaf halves was slightly lower than in the controls. Neither germinated nor ungerminated uredospores of the parasite contained MGDG, DGDG, sulpholipid or PG indicating that the decrease in these lipids could have been the result of an increasing proportion of fungal lipid in the extracted tissue. However, the extent of these decreases rendered such an explanation unlikely. The infected leaf halves showed an increase in ^{32}P incorporation into cell phospholipids especially PS, but differences in ^{32}P incorporation between the uninfected leaf halves and the controls was only small. From these results the authors proposed that the increase in PS content could be involved in the increased leakage of ions across the membrane. That this increase could be accounted for by the presense of fungal material in the leaves was ruled out as it was calculated that the fungus would have to contribute 40% of the dry matter of the host:parasite complex if the PS increase was to be explained solely by fungal lipids and they concluded that the parasite could not contribute to this extent. However Brennan and Lösel (1978) suggested that this conclusion may not have been accurate as Lösel and Lewis (1974) had shown that this amount of fungal material was found in Puccinia poarum-infected Tussilago as

indicated by chitosan estimations and microscopic observations. The work of Lösel and Lewis was, however, on isolated aerial pustules whereas that of Hoppe and Heitefuss was on leaf halves of a different species and so it is not possible directly to compare these experiments. From their experiments Hoppe and Heitefuss (1974b) concluded that the infection caused a decline in chloroplast lipids in bean leaves but the levels of PS and PA increased.

Lösel reported that there were increased amounts and altered proportions of PE, PC and PA in infected tissues of the alternate hosts of Puccinia poarum. These were allied to an increase in the amount of PS which tended to be more important in fungal tissue and in green plant tissue corresponding to the area of dense fungal growth in the rust infected leaf.

Hoppe and Heitefuss (1974c) found that the PC and PE from infected leaf halves showed a higher degree of unsaturation than the lipids from uninfected leaf halves and uninfected leaves. The PE and PC of fungal uredospores were more unsaturated than the lipids from uninfected leaf halves and uninfected leaves. The PE and PC of fungal uredospores were more unsaturated than the lipids from bean leaves. The changes in the fatty acid composition in PG, MGDG, and DGDG was small and insignificant. These differences in fatty acid composition were restricted to the area of the pustule, the adjacent fungus-free host tissue was affected only to a small extent. They concluded that these higher levels of unsaturation in diseased leaves might be responsible for the increase in leakage of non-electrolytes as both began 4 days after inoculation. The increased unsaturation was unlikely to result solely from the fungal contribution since an increase was also found to a smaller extent, in uninfected leaf halves.

Schipper and Mirocha (1970) also investigating the total fatty acid composition of Phaseolus vulgaris infected by Uromyces phaseoli found an increase in synthesis of palmitic, stearic and oleic acids between 6 and 9 days after infection. This result, however, is not comparable with that of Hoppe and Heitefuss (1974c) because the individual phospholipid fatty acid components were not studied and the total fatty acid analysed

included neutral lipids.

Hoppe and Heitefuss (1975a) looked at phospholipids and phospholipid fatty acids in healthy and rust infected bean leaves of cultivars resistant or susceptible to rust. In susceptible cultivars, as expected, infection resulted in an increase in PS and a decline in PG, and PC and PE contained higher amounts of unsaturated fatty acids than did lipids from control leaves. This did not occur in resistant cultivars whose lipid profile was the same as the control plants. There were also no significant differences in the lipid or fatty acid composition between resistant and susceptible cultivars.

Sedrina, Askerova and Khartina (1981) studied the changes in bean root mitochondrial membranes during infection by Rhizoctonia solani and showed that the content of phospholipids and neutral lipids per unit of membrane protein nitrogen increased following infection. However, these changes occurred unevenly in different lipid classes. Thus PC and PE in the phospholipid fraction increased by 15-18%, PG by more than 60%, the triglycerides by 100%, diglycerides by 20% while the monoglyceride content declined. LPC was also found in mitochondrial membranes in infected plants only and this was mirrored by an increase in the activity of phospholipase A. Infection also caused a decrease in fatty acid unsaturation in mitochondrial lipids agreeing with the data of Koshanova et al (1977). Sedrina et al (1981) also found that these changes were paralleled by a decrease in the activity of mitochondrial enzymes. The rigidity of the membrane was further increased by a decline in the phospholipid:sterol ratio. There are some doubts regarding the experimental method employed in this study since the infected tissue had to be taken from a part of the root from which the pathogen was absent. The reason for this was twofold, firstly to avoid problems with fungal lipid contamination and secondly because the parasite concerned in this study causes a necrotrophic disease and so the infected tissue is dead. The effects observed could therefore be the result of senescence and care should be taken in interpreting the results.

Lewis and Lösel (1974) studying the effect of Puccinia poarum on both its pycnial-aecial host Tussilago farfara and its uredial-telial host Poa

pratensis reported an increase in the lipid content of infected tissues which corresponded to a diversion of metabolites from uninfected parts of the same leaf or from part of the host plant remote from infection sites as well as to changes in the destination of current photosynthate.

Comparison of the distribution of $^{14}\text{CO}_2$ in infected and healthy hosts of Puccinia poarum immediately after exposure to the label in the light and following a subsequent dark chase showed that much of the activity initially incorporated into sugars was lost during the dark period, while label incorporated into lipids in all infected tissues rose several times (Brennan and Lösel, 1978). The ^{14}C activity in lipids of healthy leaves from diseased plants hardly changed during this period. In rust infected tissue during the "chase" period, the initially higher proportion of activity in polar lipids of diseased tissue decreasing as a greater amount of label accumulated in neutral lipids. Immediately following photosynthesis the ^{14}C activity of lipid fractions from both healthy and diseased plants was mainly in diacylglycerides chloroplast glycolipids, PG and PA. During the dark period, loss of the initially high activity of diacylglycerols and polar lipids adjacent to pustules was accompanied by increases in other classes of neutral lipid and membrane lipids within the fungal pustule tissues.

2.7.4. Storage and Neutral Lipids

Lipid inclusions have been found both in plant tissues infected by biotrophic fungi and in hyphae or spores of the fungal partner. They have been reported from the mycelium, mature haustoria and conidia of Erysiphe graminis, from the haustoria of E. cichoracearum (McKeen et al, 1966) and from E. pisi (Manners, 1979). A list of lipid inclusions found in plant tissues infected by biotrophic fungi can be found in Brennan and Lösel (1978).

Differences have been seen in the distribution of stainable lipid in the rust Puccinia poarum and leaf tissue of its two hosts Tussilago farfara (Lösel and Lewis, 1974) and Poa pratensis (Lösel, 1978). Haustoria are rare in the aecial host, Tussilago farfara but a dense fungal platenchyma fills the intercellular spaces of the mesophyll. Oil drops are

found in host cells near developing intercellular mycelium only during the early stages. As the aecial pustule develops, oil drops become abundant in the fungal tissue, from which they later disappear. In Poa pratensis almost all the cells in the region of the uredosorus are penetrated by haustoria with which one or more oil drops are associated in the host cell (Lösel, 1978). These oil droplets remain abundant for as long as the parasite is actively growing. Thus plants respond, in some instances, to the presence of fungal hyphae by producing lipid bodies (Brennan and Lösel, 1978).

The four major component sterols in healthy bean leaves are campesterol, cholesterol, stigmasterol and β sitosterol. In infected susceptible cultivars two unknown additional sterols are found (Hoppe and Heitefuss, 1975b) one of which is the major compound of germinated uredospores, 7-stigmasten- 3β -ol.

The changes in sterol content occurring in the infected susceptible leaves can be partially accounted for by fungal sterols. The presence of 7 stigmasten- 3β -ol and the increase in stigmasterol content in the infected leaves appeared to be correlated with the amount of fungal tissue in that host, but an increase in campesterol and the presence of a second unknown sterol cannot be explained by the presence of fungal sterols and therefore some plant mediated changes must also be occurring.

2.7.5. Enzymes and Toxins

Various workers have investigated the possibility of phospholipase being an important factor in pathogenesis. The observations of Lumsden and Bateman (1968) of phospholipase activity in Phaseolus vulgaris infected with Thielaviopsis basicola was followed by Lumsden's (1970) demonstration of phospholipase production by Sclerotinia sclerotium both in culture and in bean tissue. Hoppe and Heitefuss (1974b) found increased phospholipase activity in rust-infected halves of bean leaves compared with uninfected half leaves and control leaves, the enzyme reaching a maximum 6-8 days after infection, a time corresponding to maximum sporulation by the fungus. They speculated that increased phospholipase activity might damage mitochondria in infected leaf tissue but had to

conceed that in their system damage to mitochondria and uncoupling of respiration occurred at a much later stage of infection than the phospholipase maximum.

Studies on the effects of fungal toxins on membrane structure have revealed some interesting results. When plants are treated with syringomycin the cells show a detergent-like lysis of cellular membranes. Tissues treated with ^{14}C labelled toxin show a localization of the label over the plasma and nuclear membranes. These effects are reversed by sterols and phospholipids (Bachmann and De Vay, 1971). Other pathogenic metabolites which affect membrane permeability include the enniatins A and B. These compounds uncouple oxidative phosphorylation and stimulate an energy dependent uptake of potassium (Gottlieb, 1972). The induction of water soaked lesions by high molecular weight lipomucopolysaccharides from Pseudomonas lac rymans was explained by formation of a couple with proteins which rendered the plasma membrane more permeable to water (Williams and Keen, 1971).

Several phytotoxic compounds cause an increase in membrane permeability: ascochitine (Oku and Nakaniski, 1966), citrinin (Gottlieb, 1972), helminthosporal (White and Taniguchi, 1972), isomanticin (Dorn, 1974), ophiobolin A and B (Chain et al, 1971) and Zinniol (White and Starratt, 1967).

Phaseotoxin from Psuedomonas phaseolicola causes a decrease of electrolyte leakage accompanied by an increase in osmotic value. It was suggested that this effect counteracts an increase in membrane permeability which is common for the resistant reaction (Zeller and Rudolph, 1972). This hypothesis would explain the fact that the toxin can break the resistance of bean cultivars to halo blight (Rudolph, 1972). Zeller et al (1976) infiltrated young swiss chard leaves with this toxin and this led to changes in fatty acid composition namely a decrease in the levels of unsaturation. This effect was most pronounced in the chloroplast lipids (MGDG, DGDG and PG). Similar but lesser changes were observed in the typical membrane lipids, PC and PE, and it was concluded that these changes were closely connected with the toxin-induced chlorosis. Swiss chard leaves which had been etiolated for 14 days showed a slight but not

significant increase in membrane permeability. Since etiolated leaves are generally characterized by changes in the lipid bound fatty acids similar to those found in toxin-treated leaves, it was concluded that the decrease in membrane permeability was caused by mechanisms other than changes in the lipid composition of the plasma membrane.

Lupu et al, (1980) using tobacco leaves infected by Erysiphe cichoracearum found an increase in lipoxygenase activity in infected plants compared with uninfected plants and they suggested that, as lipoxygenase activity was associated with membrane damage, this enzyme was one of the primary factors leading to enhanced leakage. Lupu et al (1980) speculated that endogenous antioxidants found in leaf homogenates were produced by the plants to prevent the disruption of cell membrane integrity. This hypothesis was supported by the evidence that exogenous application of α tocopherol, a natural antioxidant, suppressed both lipoxygenase activity and mildew growth on leaves. However, they did not show lipid peroxidation in vivo, nor did they demonstrate the source of the lipoxygenase, so this hypothesis although attractive must remain unproven.

3. Materials and Methods

3.1 Plants

The following pea cultivars were used at different stages during the experimental work: Onward, Feltham First and Hurst Green Shaft. Before germinating the seeds were sterilised for 5 minutes in 10% (W/V) sodium hypochlorite. After rinsing in sterile distilled water, the seeds were germinated inside perspex boxes on moist tissue paper. After 3 days at 25° the germinated seeds were planted in John Innes No. 2 potting compost in 5" square pots with 5 seeds in each pot. The plants were grown in a Fisons growth cabinet model 600G3 THTL at 18°C under a light intensity of 150 micro einsteins m⁻² sec⁻¹ with a 16 hour day photoperiod.

Four week old plants were inoculated by dusting with spores shaken from 14 day old infections of a previous batch of plants. Newly inoculated plants were isolated in a separate cabinet and grown under the same conditions as before. Mildew was allowed to develop for the period required by the experimnts. Uninfected plants of the same age grown under identical conditions were used as controls.

3.2 Inoculum source

Erysiphe pisi colonies were maintained on the susceptible pea cultivar, Onward, which were supplied on a fortnightly basis by the University of London Botanic Garden. They were placed in a greenhouse compartment and infected by shaking plants bearing 14 day-old sporing colonies over them.

3.3 Experimental Material

When the leaves were used for lipid analysis the 3rd, 4th and 5th leaf pairs were used. Otherwise stem epidermis was used and was stripped from plants after plasmolysis in 0.5 M mannitol for 1 hour.

When infected tissue was required for analysis the spores were dislodged by shaking and mycelium was removed by rubbing the surface of the plant with forefinger and thumb while wearing surgical gloves. The

mycelium so obtained was analysed for lipids separately.

3.4 General Precautions during lipid analysis

At all points during lipid extraction and analysis precautions were taken to prevent the oxidation of the lipid. Butylated hydroxy toluene (BHT) at a concentration of $50 \mu\text{g l}^{-1}$ was routinely added to all solvents used in extraction, storage and chromatography to prevent oxidation. BHT is extremely volatile and is therefore lost during solvent evaporation. The other routine precaution was the use of oxygen free nitrogen for the evaporation of final traces of solvents.

Lipids were stored in solutions containing BHT under a layer of oxygen free nitrogen at 4°C in the dark.

All glassware used in the lipid extraction and analytical work was washed with chromic acid, rinsed initially with dilute HCl and then with deionised water.

3.5 Chemicals

All chemicals used routinely were 'Analar' grade and were obtained from British Drug Houses (BDH) Ltd, Poole, Dorset.

The phospholipids and fatty acid pure samples were obtained from Sigma (London) Chemical Company.

3.6 Extraction of plant lipids

The various extraction methods assessed are described in section 3.1.8. This section describes the method regarded as optimal and was used routinely unless otherwise specified. 0.5g tissue (fresh weight), 0.052(0.012g, dry weight equivalent) was placed in 15 ml of boiling isopropanol for 2 minutes to deactivate lipid-degrading enzymes. The isopropanol was retained and the tissue removed and placed in 15 ml of chloroform: methanol (2.1, V/V). The tissue was ground with a mortar and pestle for 5 minutes, the homogenate was then filtered under a vacuum through Whatman No. 1 filter paper. The filtrate was added to the

isopropanol and stored under nitrogen. The residue was re-extracted twice with further 15 ml volumes of chloroform methanol.

3.7 Removal of non-lipid material

The method was based on that of Folch, Lees and Sloane-Stanley (1957). The water-soluble non-lipid contaminants were removed from the extract by shaking the chloroform: methanol (2.1, V/V) sample with 0.2 volumes of 0.1 M NaCl in a test tube. The mixture was allowed to equilibrate, after which the upper phase was removed with minimal disturbance by Pasteur pipette. Usually a small residue of the upper phase remained in the tube, in which case sufficient methanol was added to unify the two phases.

3.8 Removal of water from the extract

Extracts were dried by the addition of anhydrous Na_2SO_4 both directly to the extract and also by filtration through a further quantity of the dry salt held in a fluted filter paper. Organic solvent was then removed by rotary evaporation under reduced pressure in an all glass apparatus.

3.9 Column chromatography

The residue from rotary evaporation was washed with 2 x 5 ml volumes of diethyl ether, 3 x 5 ml volumes of chloroform, 3 x 5 ml volumes of chloroform methanol (2:1 V/V) and finally 3 x 5 ml volumes of methanol. All the washings except the 2 x 5 ml volumes of diethyl ether were combined and stored under nitrogen. The diethyl ether washings were chromatographed as described below on a column of silica gel (60-120 mesh 5cm^3) activated for 1 hour at 100°C , suspended in diethyl ether and packed in a 20 ml syringe body fitted with a silicone rubber-tube closed by a clamp. The sample, dissolved in diethyl ether, was then added to the top of the column and eluted with five column volumes (5 ml) of diethyl ether, 5 column volumes of chloroform, 5 column volumes of chloroform methanol 2:1, V/V) and 20 column volumes of methanol at a flow rate of 2 ml min^{-1} .

The diethyl ether eluted fraction was taken to dryness under vacuum at 35°C , redissolved in 0.5 ml of diethyl ether and used for the separation

of neutral lipids by t.l.c.

The chloroform and chloroform methanol (2:1 V/V) fractions were taken to dryness separately, re-dissolved in 0.5 ml chloroform and chloroform: methanol (2:1 V/V) respectively and used for the separation of polar lipids by t.l.c.

The methanol fraction was divided into two, one containing the first 15 column volumes and the other containing the last 5 column volumes of methanol. The two fractions were taken to dryness and redissolved in 0.5 ml methanol which was used for the separation of polar lipids by t.l.c. These fractions were kept separate to check whether all the lipid was being eluted from the column.

3.10 Thin Layer Chromatography

Lipids were separated by ascending t.l.c. using pre-coated tlc plates (Whatman LPK5 silica plates 20 cm x 20 cm, 500 μ m thick). The samples from section 2.9 were applied to the tlc plates in bands using a micropipette. To ensure that no lipid was lost at this stage, the tubes containing the lipid extracts were washed twice with 0.5 ml of the appropriate solvent and these were applied to the tlc plates on the same streaks.

Chromatograms were developed at room temperature in a Shandon Chromatank after equilibration in a solvent saturated atmosphere for 1-2 hours. Two different solvent tank mixtures were used:-

- a) To separate polar lipids — Chloroform : methanol : acetic acid : water (65:25:10:8, V/V)
- b) To separate neutral lipids — Diethyl ether : toluene : ethanol : acetic acid (40:2:0.2, V/V) followed by diethyl ether : hexane (6:94, V/V) (Freeman and West, 1966)

The plates were developed until the solvent front reached 3 cm from the top.

3.10.1 Detection of lipids on tlc plates

Three general detection methods were used

a) Iodine vapour (Mangold and Malins, 1960)

A few iodine crystals were placed on the bottom of a chromatography tank into which chromatograms were placed. After a few minutes lipids appear as brown spots on a pale yellow background. As little as $3\text{ }\mu\text{g}$ of lipid can be detected in this way (Mangold and Malins, 1960).

b) Rhodamine 6G (Mangold 1961)

0.01% Rhodamine 6G was sprayed onto the chromatogram and viewed under UV light (270 nm). The lipids appear as yellow fluorescent spots. The lower detection limit is $1\text{ }\mu\text{g}$ lipid (Mangold, 1961).

c) Dichlorofluorescein (Mangold, 1961)

0.01% $2^{17}1$ dichlorofluorescein in methanol was sprayed onto the chromatogram and viewed under UV light (270 nm). Lipids appear as dark spots on a fluorescent background. The lower detecting limit is $1\text{--}5\text{ }\mu\text{g}$ lipid (Mangold, 1961).

The following specific tests were used.

a) Phosphate Stain (Wagner et al., 1961)

Reagent: Solution (a) 8g of ammonium molybdate in 60 ml of water; Solution (b) To 20 ml concentrated HCL, 5 ml of mercury and 40 ml of solution (a) was added, this was agitated for 30 minutes and filtered.

Spray Reagent: To the remainder of solution a) 100 ml 18 M H_2SO_4 and all of solution b was added. This was allowed to cool and then diluted to 500 ml with water.

The reagent was sprayed onto the chromatogram and the phospholipids appeared as blue spots on a white background after a few minutes. In this way it was possible to detect as little as $1\text{ }\mu\text{g}$ lipid (Wagner et al., 1961).

b) Ninhydrin stain (Skipski et al., 1962)

0.2% ninhydrin in butanol saturated with water was sprayed onto the chromatogram which was then heated at 100°C for 5 minutes. Lipids with a free amino group appear as red spots on a white background. The limit to detection was 10 μ g (Mangold, 1961).

c) Stain for sterol and sterol esters

18 M sulphuric acid : glacial acetic acid (1:1, V/V) was sprayed onto the chromatogram which was then heated at 90°C for 15 minutes. Sterol and sterol esters appear as red spots on a white background.

3.10.2 Elution from lipids of silica gel

a) Polar Lipids

The area to be eluted was marked on the tlc plate and moistened with water. This enables the silica gel from the whole of this area to be removed. The silica gel was placed in a tapered centrifuge tube, 5 ml chloroform was added to the tube and this was agitated on a vortex mixer for 1 minute. The tube was then centrifuged at 200g for 5 minutes to sediment the silica gel. The supernatant was removed and stored under nitrogen at 0°C. The silica gel was washed with 2 further 5 ml volumes of chloroform, 3 washings with 5 ml chloroform:methanol (2:1, V/V) and finally 3 washings with 5 ml methanol. All washings were bulked together.

b) Neutral Lipids

The neutral lipids were eluted from silica gel as described above except that the solvents used were as follows: 3 x 5 ml volumes of diethyl ether followed by 2 x 5 ml volumes of chloroform:methanol (2:1, V/V).

3.10.3 Quantitative analysis of lipids from tlc plates

All these methods can be used in the presence or absence of silica gel. Lipids in solution were first dried under reduced pressure at 35°C.

a) Reduction of dichromate (Amenta, 1964)

For samples containing 40-250 μg of lipid, 2 ml of dichromate solution (prepared by dissolving 2.5g $\text{K}_2\text{Cr}_2\text{O}_7$ in 1.1 18 M H_2SO_4) was added to the dried lipid samples in glass stoppered test tubes. If silica gel was present the test tubes were agitated on a vortex mixer until it formed a fine suspension. The tubes were heated in a water bath at 100°C for 45 minutes and well mixed 3 times during this period. The tubes were allowed to cool and then centrifuged at 200g for 5 minutes. 1 ml of supernatant was pipetted into a test tube and diluted to 10 ml with water. Absorbance was read at 350 nm on a Beckman model 35 spectrophotometer against a distilled water blank. The amount of lipid in an unknown mixture was estimated against calibration curves obtained using pure lipid samples. This method depends upon the reduction of dichromate and is sensitive down to 10 μg of lipid if the amount of dichromate is reduced to 0.5 ml. The response is linear up to 1 μg lipid if 5 ml reagent is used.

b) Method of Marsh and Weinstein (1966)

2 ml of 18 M H_2SO_4 was added to samples in test tubes. If silica gel was present the test tubes were mixed on a vortex mixer until the silica gel formed a fine suspension, if none was present the tubes were mixed for a standard 1 minute.

The tubes were placed in an oven at 180°C for 20 minutes after which they were allowed to cool and 3 ml of distilled water was added. After standing to disperse air bubbles any silica gel present was sedimented by centrifugation the absorbance of the supernatants was read at 375 nm on a Beckman model 35 spectrophotometer against a blank treated as above but with no lipid present. Amount of lipid in an unknown mixture was estimated against calibration curves obtained with a series of appropriate standards. The detection limit was 5 μg and the plots remain linear up to 200 μg (Section 4.1.5).

c) Phosphate analysis (Bartlett, 1959)

1 ml of 60% perchloric acid was added to the samples of phospholipids in test tubes. Phospholipids were digested in 1 ml of 60% perchloric acid

by refluxing at 170°C for 30 minutes using a dry heating block. The test tubes were cooled and 8.3 ml of water was added followed by 1.5 ml of 2.5% (W/V) aqueous ammonium molybdate solution and 0.2 ml reducing solution (10%, W/V, ascorbic acid). The tubes were left at room temperature for 30 minutes to allow the colour to develop. If silica gel was present the samples were centrifuged at 200g for 5 minutes and the supernatants were removed prior to reading the absorbance at 695 nm on a Beckman model 35 spectrophotometer. The amount of phospholipid present was estimated against calibration curves produced with either standard phospholipid samples or inorganic phosphorous.

3.11 Gas Liquid Chromatography

3.11.1 Preparation of fatty acid methyl esters (FAMES)

Areas corresponding to each phospholipid on tlc plates were scraped onto boats and transferred to glass tubes fitted with teflon-lined screw caps. 0.5 ml of 14% Boron trifluoride in methanol was added to each tube. The tubes were flushed with nitrogen, sealed firmly and placed in a water bath at 100°C for 30 minutes. The tubes were allowed to cool and 0.1 ml of an internal standard, methyl pentadecanoate (0.039 μ moles in 0.1 ml hexane) and 2 ml hexane was added. The contents of the tubes were mixed thoroughly, and centrifuged at 200g for 2 minutes. At this stage two phases were present, the upper phase containing the FAMES dissolved in hexane was removed and transferred to white soda-glass screw neck vials with aluminium foil-lined caps. The lower methanolic phase was washed with a further 2 ml of hexane which was removed and pooled with the first extract. The FAMES samples dissolved in hexane were taken to dryness under a stream of nitrogen on a dry heating block at 35°C. The samples were redissolved in 10 ml of hexane in preparation for glc.

This method was used for monoglycerides, diglycerides and free fatty acids as well as for polar lipids. In order to methylate triglycerides 25% toluene and 14% boron trifluoride in methanol replaced the 14% boron trifluoride in methanol and for sterol esters 30% toluene and 14% boron trifluoride in methanol was used.

3.11.2 Separation of fatty acid methyl esters

FAMES were separated in a Perkin Elmer Sigma 2b gas chromatograph equipped with a flame ionisation detector and a Sigma 10b data station. 1 μ l of the FAMES sample dissolved in hexane was injected into a 2 M x 3 mm i.d. column of 15% Reoplex 400 on chromosorb W. HP 100/120 mesh, run isothermally at 195°C. Injectors and detectors were at 210°C. Nitrogen at a flow rate of 35 ml min⁻¹ was used as a carrier gas. The fatty acids methyl esters were recognised by comparing the retention times with those of authentic standards.

A response factor for each fatty acid was calculated by analysing known amounts of each fatty acid and calculating the ratio between the peak area of interest and that of the standard, methyl pentadecanoate. Amounts of fatty acids present were calculated from the following equation:-

$$\frac{\text{Area under the peak for individual fatty acids}}{\text{Area under the peak for methyl penta-decanoate (0.039 } \mu\text{mol in 0.1 ml)}} \times \text{Relative response factor for individual fatty acid} \times 0.039$$

This gives a value in μ moles per sample.

To separate both short chain (14 carbon atoms) and long chain (18 carbon atoms) fatty acids 1 μ l of the FAMES sample dissolved in hexane was resolved on a 2 M x 3 mm i.d. column of 15% DEGS on chromosorb W temperature programmed at 150°C for 15 minutes increasing 4°C⁻¹ to 190°C and finally isothermally for a further 15 minutes. Injectors and detectors were at 210°C and nitrogen at a flow rate of 35 ml min⁻¹ was used as carrier gas. The retention times, relative response factors and amounts of each fatty acid were calculated as previously described.

The amount of each lipid present was calculated from the fatty acid data.

Figure 7

2 2 2
4

Figure 7: Flow diagram of Lipid extractions, purifications and separation techniques

0.5g fresh weight tissue (leaves, epidermal strips) boiled in isopropanol for 2 minutes.

Isopropanol retained. Tissue removed and placed in 15ml chloroform :methanol (2:1, V:V) and ground with a mortar and pestle. The homogenate filtered through Whatman No. 1 filter paper under vacuum. The filtrate added to isopropanol and stored, the residue placed in 15ml chloroform: methanol (2:1, V:V) and the process repeated twice.

LIPID EXTRACTION

PROTEOLIPID COMPLEXES BROKEN DOWN The combined extract taken to dryness and resuspended in 10ml of chloroform:methanol (2:1, V:V).

NON-LIPID MATERIAL REMOVED 2ml 0.1 M NaCl added to the extract, mixture allowed to equilibrate after which upper phase removed.

WATER REMOVED Lower phase filtered through rosetted Whatman No. 1 filter paper containing dry $(\text{NH})_2\text{SO}_4$ into rotary evaporator flask.

Extract taken to dryness in rotary evaporator and resuspended

SEPARATION OF NEUTRAL LIPIDS FROM POLAR LIPIDS in 2 x 5ml volumes diethyl ether
3 x 5ml volumes chloroform
3 x 5ml volumes chloroform:methanol (2:1, V:V)
3 x 5ml volumes of methanol.

Washings, with the exception of the diethyl ether washings were combined and stored at 4°C under nitrogen

Column chromatography: The diethyl ether washings were placed in a column of silica gel and the lipids were separated using the following solvents and a flow rate of 2ml min^{-1} .

5 Column volumes Diethyl ether
5 Column volumes Chloroform
5 Column volumes Chloroform: methanol (2:1, V:V)
20 Column volumes Methanol

The diethyl ether fraction was taken to dryness, redissolved in 0.5ml diethyl ether and used for the separations of neutral lipids by tlc.

The other fractions were taken to dryness, redissolved in 0.5ml; chloroform, chloroform methanol (2:1, V:V) or methanol and used for the separation of phospholipids by tlc.

3.12 Extraction of fungal lipid

40-50 mg dry weight of mycelium was placed in a glass stoppered test tube to which 5 ml of boiling isopropanol was added and left for 2 minutes. 10 mls of chloroform: methanol (2:1, V:V) was added to the tube which was left at room temperature for 4 hours with intermittent shaking. The sample was ground in a ground glass homogeniser 3 times in chloroform:methanol (2:1, V:V) and then filtered through Whatman No. 1 filter paper, taken to dryness under reduced pressure at 35°C and the residue was resuspended in a small volume of chloroform: methanol (2:1, V:V). Lipids from haustorial complex-enriched fractions were also extracted in this way.

3.13 Extraction of tissue for amino acid and chlorophyll estimation

Samples for analysis consisted either of 5 leaf discs (0.25 cm diameter) punched from each of 4 leaves (the third and fourth leaf pairs) or 0.1 g fresh weight (0.015 g dry weight equivalent) of epidermis. The samples were placed in 5 ml of methanol and boiled for 15 minutes, cooled and stored at 0°C. Chlorophyll was estimated directly by measurement of the absorbance at 670 nm.

3.14 Amino acid estimation (Yem and Cocking, 1955)

0.5 ml of 0.2 M citrate buffer pH5 was mixed with 1 ml of sample containing 0.05 — 5.6 g amino nitrogen. 0.2 ml of 5% (W/V) ninhydrin in 2 methoxyethanol and 1 ml of 0.01 M KCN was then added. The tubes were heated for 15 minutes at 100°C and then cooled for 15 minutes in tap water. The solution was made up to 5 ml with ethanol and the absorbance was read at 570 nm.

The amount of amino nitrogen was estimated from a standard curve prepared by using dilutions of an equimolar mixture of alanine, glutamine and lysine.

3.15 Protein estimation

Protein was measured using Folin and Ciocalteu reagent following the method of Lowry, Rosborough, Farr and Randall (1951).

Reagents

Solution A 2% (W/V) Na_2CO_3 , n 0.1 M NaOH

Solution B 0.5% (W/V) $\text{CuSO}_4\cdot\text{SH}_2\text{O}$ in 1% (W.V) trisodium citrate

Solution C was made by mixing 50 ml of A with 1 ml of B just before use.

Folin and Ciocalteu's phenol reagent was diluted 1:1.5 with distilled water to make a 1 M acidic solution. The quantity of water necessary was determined by titration against 1M NaOH with phenolphthalein as indicator.

Procedure

5 ml of solution C was mixed with 1 ml of sample and incubated at room temperature for 10 minutes. 0.5 ml of diluted Folin and Ciocalteu's reagent was added with mixing and the tubes incubated for a further 30 minutes at room temperature in the dark. The absorbance at 500 nm was then measured on a Beckman model 35 spectrophotometer and the protein concentration estimated from a standard plot prepared using bovine serum albumin (Sigma, London). The response was linear from 0.200 g ml^{-1} of bovine serum albumin.

3.16 Measurement of cell viability

Plant tissue was placed in neutral red solution (0.1% neutral red in 1 M KNO_3 , n 0.1 M phosphate buffer pH7) for 20 minutes and then washed in the same solution minus neutral red. The neutral red remains in the vacuoles of viable cells and percentage viability was assessed by counting 10 groups of 100 cells using an Olympus binocular light microscope at x400 magnification.

3.17 Estimation of the frequency of haustoria

The numbers of haustoria per cell were estimated by peeling lower epidermes and mounting them in water and observing at x400 on an Olympus binocular light microscope.

3.18 Isolation of haustorial complexes

The method used for isolating haustorial complexes was that of Manners (1979) given in outline in fig. 9. The composition of the isolation medium was 0.2 M sucrose, 3 mM Mg SO₄ and 0.1 M phosphate buffer pH 6.8.

In an attempt to reduce the level of chloroplast contamination in the final extract, fraction 2 was syringe filtered through 5 μ m nylon mesh and the haustorial complexes were washed from the nylon mesh after filtration. At this stage an estimate of the contamination was made.

3.19 Isolation of membrane fractions (after Leonard and Van Der Woude, 1976)

10-20 g of leaves or stem segments were rinsed in cold distilled water; this and all subsequent operation taking place at 4°C. The rinsed tissue was cut into 2 cm segments and ground in a pre-chilled mortar and pestle. The grinding medium consisted of 250mM sucrose, 3mM EDTA and 25mM Tris - MES (pH 7.2) and was used at 4 ml: 1 g fresh weight of tissue.

The homogenate was filtered through 4 layers of cheesecloth and successively centrifuged on a Beckman LB55 centrifuge using an 8 x 50 ml angle rotor, at 1000 g for 10 minutes, 8,000 g for 15 minutes, 13,000 g for 15 minutes, 40,000 g for 30 minutes and 80,000 g for 30 minutes. Pellets from these centrifugations were resuspended in the buffered sucrose and assayed for protein (Section 3.10) and various enzymes (Section 3.20). When lipid extractions were required the pellets were placed in boiling isopropanol and the lipids were extracted using the method of Section 2.12.

Figure 8: Flow diagram for the isolation of haustorial complexes from Pisum sativum infected with Erysiphe pisi (After Manners, 1979)

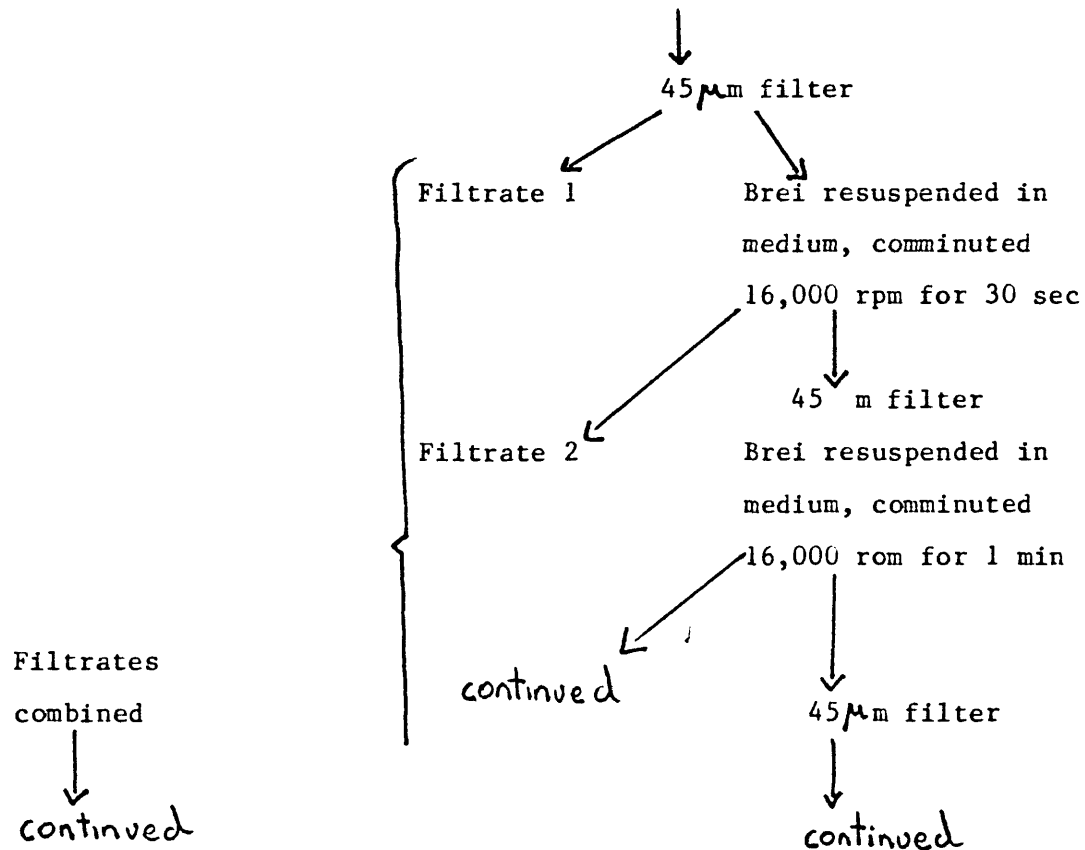
Pea plants removed 7 days after inoculation, kept in dark for 30 h.

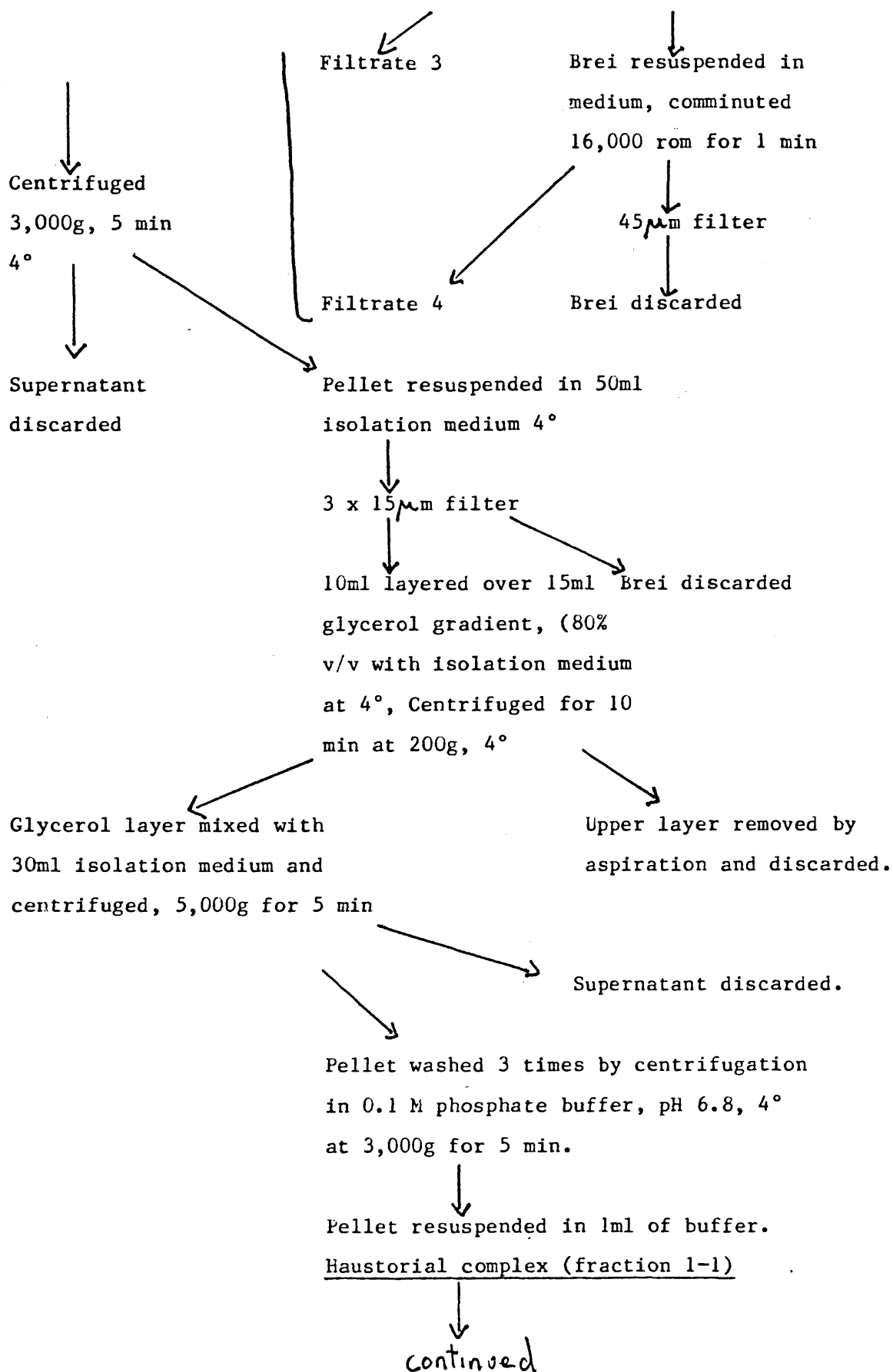
↓
Infected leaves (40-100 g.)

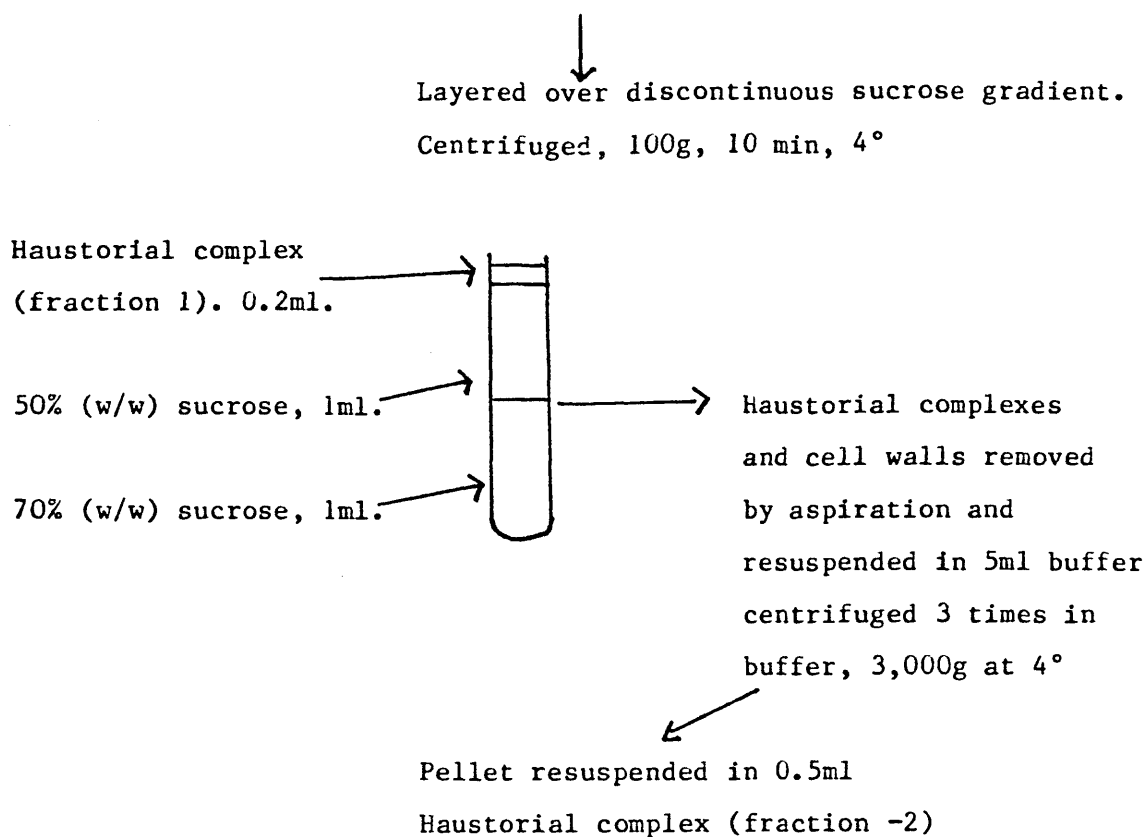
Leaves brushed with an electric rotary cotton mop and mycelium discarded.

↓
Leaves washed, 3 times in tap water at 4°. 3 times in distilled water at 4°.

10g batches of leaves comminuted in 200ml isolation medium, 4° at 8,000 rpm for 30 seconds in Sorvall Omnimixer.







3.20 Enzyme Assays

a) ATP ase (Hodges and Leonard, 1974)

All ATP ase assays were performed at 38°C in a total volume of 1 ml. The pH of the assay was either 6 or 9.

The disodium salt of ATP (sigma) was converted to the tris salt so that ion-stimulated activity could be measured. This conversion was accomplished by adding sufficient Dowex 50-w ion exchange resin (H^+ form) to the ATP disodium salt to give a pH between 1 and 2. The resin was removed by filtration on a Millipore filter. The solution was brought to the desired pH (either 6 or 9) by the addition of tris crystals the substrate was frozen until required.

Reagents

Substrate solution: 3.33 mM ATP tris salt (pH 6 or 9), 1.67 mM $Mg SO_4$, 55.5 mM KCl (if K^+ stimulated ATP ase measured), 36.7 mM Tris-MES buffer at pH 6 or 9.

Termination reagent: 1% (W/V) ammonium molybdate in 1 M H_2SO_4

Reducing reagent: 1 amino - 2 naphthol - 4 sulphuric acid (Fiske and Subba Row, 1925)

0.1 ml of membrane fraction containing 10.50 mg protein was added to 0.9 ml of the appropriate substrate solution. The reaction was allowed to proceed for 30 minutes (depending on the amount of substrate to be hydrolysed) and then stopped by adding 2 ml of ice cold stopping reagent and placing the tubes on ice. To determine the amount of phosphate released 0.4 ml of reducing agent was added, the reaction mixture was mixed thoroughly and then was incubated for 35 minutes at room temperature.

The absorbance was read at 660 nm and the amount of phosphorous was estimated from a standard curve obtained using inorganic phosphate.

b) Cytochrome C oxidase (Hodges and Leonard, 1974)

Cytochrome C oxidase was assayed by measuring the oxidation of reduced cytochrome C. Cytochrome C was chemically reduced by adding a few crystals of sodium dithionite, the excess dithionite being removed by passing air through the solution for a few minutes. The reaction mixture consisted of 0.1 ml enzyme sample (10-50 mg protein), 0.1 ml of 0.3% (W/V) digitonin and 2.7 ml of 50 mM phosphate buffer pH 7.5. The reaction was started by the addition of 0.1 ml of reduced cytochrome C. One drop of saturated potassium ferricyanide was added at the end of the assay to determine the absorbance of the completely oxidised cytochrome C.

The rates of cytochrome C oxidation were determined from the initial linear rates and the amount of cytochrome C utilised was measured using an extinction coefficient for cytochrome C of $18.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at 550 nm.

Statistics

Unless otherwise stated in the results section all values presented are the means from four replicates and the confidence limits quoted are 95% confidence limits calculated by using Students t test. All data in percentage form were subjected to arc sin transformation prior to

statistical analysis.

4. Results Section

4.1 Development of lipid extraction and analytical procedures

Method of development has been included to illustrate the problems encountered in lipid analysis and to show how the quantitative analytical procedures used in this study were derived. This section has been written in chronological order to demonstrate the problems as they arose and to show the measures undertaken to overcome them. Although lipid analysis is routine for many biochemists, few of the techniques have been especially developed for plant tissues and virtually none are applicable without modification to the very small amounts of tissue it was desired to analyse for this project.

4.1.1. Separation of lipids by thin layer chromatography

An initial study was conducted using authentic phospholipid standards, to determine which tlc solvent system gave the most reliable separation of all the major phospholipid classes. Both one and two dimensional chromatography was tested, for although a one dimensional system would be preferable due to ease and speed of use, there was some doubt as to whether a single solvent could give the degree of separation desired. Initially the solvent systems tested were those used by previous workers.

In the original experiments a sample of each phospholipid was chromatographed along with a mixture containing all the phospholipids. This enabled Rf values to be determined and any interference between compounds to be detected. When leaf extracts were analysed some lipids in the extracts seemed to affect the Rf values of others. Several of the solvent systems that were used initially were therefore retested with leaf extracts (Section 3.3) and constituents were recognised by using specific sprays (Section 3.10.1).

The results of these experiments are shown in table 2 and figure 9. The two dimensional systems in general gave better separation. The best separations with the one dimensional systems employed Chloroform: Methanol : Acetic acid: Water (65 : 15: 10 : 4 V:V) (system 3) (65:25:10:4) (system 5) and (65:25:10:8, V:V) (System 6). The first of

these systems would be suitable but for the closeness of the PG and PE bands (Rf values 0.49 and 0.51 respectively). Resolution was improved by the addition of more methanol as in system 5, however this did not solve the proximity of PC and MGDG (Rf values 0.33 and 0.28 respectively). The separation was improved by the addition of more water as in system 6 which was the system finally chosen for routine use. Although PG, DGDG and MGDG are predominantly chloroplast lipids and therefore were largely absent from epidermal extracts, initial experiments did involve whole leaves, and the possibility that these lipids may be present in final extracts meant that this system was the most suitable overall.

The number of plates that could be chromatographed before the Rf values were altered was tested by using a series of plates carrying phospholipids chromatographed consecutively with the same solvent mix. The results from this experiment (table 3) show that by the time the fourth plate was chromatographed PI, PS and DGDG were scarcely separating as were PG and PE. These results were obtained by chromatographing the tlc plates with freshly mixed solvent after one hours equilibration in the tank.

The maximum amount of material that could be chromatographed whilst retaining good separation was equivalent to 2g fresh weight of leaf material.

For routine analysis of lipid non-destructive detection methods were used. Chemical selectivity was unnecessary because the order of the lipid bands was already known. The three non-destructive reagents used regularly were iodine vapour and sprays of dichlorofluorescein and rhodamine 6G. The detection limit for each method was $1\mu\text{g}$, which meant that they were all of sufficient sensitivity for routine use, in quantitative work dichlorofluorescein and rhodamine 6G were used.

Table 2: Thin Layer chromatography of Polar Lipids

Lipid	Rf Value in Solvent Systems					
	1	2	3	4	5	6
PC	0.33	0.27	0.34	0.23	0.33	0.37
PE	0.83	0.45	0.51	0.46	0.67	0.74
PG	0.76	0.41	0.49	0.57	0.59	0.61
PI	0.49	0.15	0.10	0.26	0.20	0.25
PS	0.58	0.19	0.27	0.12	0.25	0.31
PA	0.90	0.63	0.64	0.04	0.72	0.80
LPC	0.14	0.10	0.05	0.08	0.01	0.10
MGDG	0.85	0.86	0.72	0.85	0.90	0.86
DGDG	0.35	0.36	0.20	0.51	0.28	0.29

All the Rf values are the means of 3 replicates

Solvent systems

1. Chloroform: Methanol: Acetic acid: Water 25:15:40:2 V:V
2. Chloroform: Methanol: Water 65:25:4 V:V
3. Chloroform: Methanol: Acetic acid: Water 65:105:10;4
4. Chloroform: Methanol: Ammonium hydroxide 75:25:3
5. Chloroform: Methanol: Acetic acid: Water 65:25:10:4
6. Chloroform: Methanol: Acetic acid: Water 65:25:10:8

A similar procedure was adopted for neutral lipids except that higher plate loadings were required to recognise some of the classes. The results are shown in table 4. The major problem with the separation of the neutral lipids was to differentiate the diglycerides from the sterols. None of the single solvent systems tested gave a suitable separation so the double solvent system devised by Freeman and West (1966) was used for routine analysis as this gave a reasonable separation of all the neutral lipid classes.

The maximum amount of lipid for efficient separation was not tested as the 0.5g fresh weight equivalent of leaves that was found in the case of the polar lipids did not cause any overloading of the neutral lipid plates.

The reproducibility of the sequential solvent system was also tested (Table 5), four plates could be developed consecutively using this system before the Rf values were affected.

Table 3: The effect of chromatography on consecutive plates on the Rf values of polar lipids using Solvent system Chloroform: Methanol: Acetic acid: Water (65:25:10:8, V:V)

Lipid	Rf Value after run number			
	1	2	3	4
PC	0.37	0.38	0.41	0.50
PE	0.74	0.73	0.77	0.85
PG	0.61	0.64	0.68	0.81
PI	0.25	0.20	0.24	0.34
PS	0.31	0.31	0.34	0.34
PA	0.81	0.85	0.86	0.91
LPC	0.10	0.08	0.15	0.24
MGDG	0.86	0.88	0.88	0.90
DGDG	0.29	0.27	0.31	0.35

All Rf values are the means of 3 replicates

Table 4: Thin Layer Chromatography of Neutral Lipids

Lipid	Rf Value in Solvent System			
	1	2	3	4
Sterol esters	0.87	0.92	0.93	0.83
Fatty acid methyl esters	0.08	0.63	0.74	0.74
Triglycerides	0.13	0.37	0.62	0.59
Fatty acids	0.04	0.18	0.40	0.25
Sterols	0.00	0.09	0.18	0.40
Diglycerides	0.00	0.09	0.20	0.51
Monoglycerides	0.00	0.00	0.02	0.12

All the Rf values are means of three replicates

Solvent Systems

1. Heptane: Toluene 9:1
2. Light Petroleum (B.Pt 60.70): Diethyl ether: Acetic acid 90:10:1
3. Light Petroleum (B.Pt 60.70) Diethyl ether: Acetic acid 80:20:1
4. Diethyl ether: Toluene: Ethanol: Acetic acid 40:50:2:0.2
followed by Diethyl ether: Hexane
(Freeman & West, 1966)

Table 5: The effect of developing consecutive plates on the Rf values of neutral lipids using Solvent system: Diethyl ether: Toluene: Ethanol: Acetic acid (40:50:2:0.2, V:V) followed by Diethyl ether: Hexamine (6:94, V:V)

Lipid	Rf Value after Run number				
	1	2	3	4	5
Sterol esters	0.83	0.83	0.73	0.73	0.58
Fatty acid methyl esters	0.74	0.74	0.68	0.68	0.47
Triglycerides	0.59	0.59	0.57	0.57	0.35
Fatty acids	0.25	0.25	0.25	0.25	0.15
Sterols	0.40	0.40	0.41	0.41	0.31
Diglycerides	0.51	0.51	0.45	0.45	0.30
Monoglycerides	0.12	0.12	0.14	0.14	0.10

All the Rf values are the means of three replicates

Plates 1 & 2 and 3 & 4 were developed together therefore the Rf values are the same.

Table 6: Solubility of lipid in solvent washings of dried crude lipid extract.

Wash	Total Lipid (μ g)	% Composition
Chloroform 1	841 $\pm$ 22.3	39.35
Chloroform 2	131 $\pm$ 12.7	6.13
Chloroform 3	11 $\pm$ 3.8	0.51
Chloroform 4	11 $\pm$ 0.4	0.05
Chloroform: Methanol (2:1, V:V)	643 $\pm$ 13.7	30.09
Chloroform: Methanol (2:1, V:V) 2	298 $\pm$ 12.9	13.94
Chloroform: Methanol (2:1, V:V) 3	8 $\pm$ 2.1	0.37
Chloroform: Methanol (2:1, V:V) 4	0	-
Methanol 1	194 $\pm$ 15.1	9.08
Methanol 2	10 $\pm$ 0.7	0.47
Methanol 3	0	-

These results are the means of 4 replicates $\pm$ 95% confidence limits

4.1.2 Removal of non-lipid contaminants from lipid extracts

When lipids are extracted from pea leaves in chloroform: methanol (2:1, V:V) the resultant extract taken to dryness and resuspended in chloroform: methanol (2:1, V:V) does not separate into clearly discernable bands when examined by t.l.c. with a polar solvent system. This is due to non-lipid components in the extract, whose presence results from the use of methanol in the extractant. Methanol is an essential part of the extraction procedure due to the need to break hydrogen bonds and electrostatic charges between proteins and lipids in membranes. Methanol also extracts

sugars, amino acids and salts and it is these which have to be removed before further analysis is attempted.

One method for removing these non-lipid components is to take the samples to dryness under vacuum in a rotary evaporator at 40°C. The residue is then suspended in chloroform in which only the lipids are soluble. This method relies upon the rupture of lipid-protein complexes during solvent removal, so rendering the lipid soluble in chloroform. The non-lipid components that are insoluble in chloroform would then be left undissolved. This method was tested by making successive 5 ml chloroform washes of the residue until no further lipid was extracted. The flask was then washed with successive 5ml volume of chloroform:methanol (2:1, V:V) until no lipid was left in the washes and finally with successive 5ml volumes of methanol. The total lipid content of each wash, measured by the method of Amenta (1964) is shown in table 6.

Only 46.04% of the total lipid redissolved in chloroform, solubilization of the remainder required additional methanol. Once methanol is used the non-lipid components are also partly redissolved. Although selective solvent solubility does not provide sufficient purification, the drying procedure is still beneficial as it does breakdown the lipid: protein complexes so that in the next phase separation stage these complexes do not aggregate at the interface and thereby hinder recovery of lipids.

The standard method for removing the non-lipid contaminants from the lipid extracts is a phase transition technique (Folch, Lees and Sloane-Stanley, 1957). This can be done in two ways either as described in the methods section or combined with the extraction technique as in section 4.18. This section only deals with the former.

To investigate the possibility that lipid was lost in the upper phase with the non-lipid contaminants, both the upper and lower phases were dried and analysed for total lipids using the method of Amenta (1966). Although only $4.92 \pm 0.84\%$ of the lipid was present in the upper phase when analysed by class the loss for some of them was more marked. Thus although there was no difference in the fatty acid composition between the two

phases (table 7) up to 28.9% of the total PI was present in the methanol phase.

To eliminate this loss the method of Folch et al (1957) was followed and the effect of different concentrations of NaCl solutions in the separation procedure rather than water was tested. Figure 10 shows that between 0 and 0.50 M there is an approximately linear and inverse relationship between salt and lipid in the aqueous phase. At 50mM the lipid lost in the upper phase is 0.93%. It was decided that this was an acceptable loss and this salt concentration was used routinely. A substantial amount of non-lipid material remained in the lower phase which still affected the efficiency of the tlc separation. By adding 0.05M NaCl dissolved in methanol: chloroform: water (3:48:47 by volume), which corresponds to the composition of the upper phase (Folch et al, 1957), it was possible to remove a substantial amount of this non lipid material. The method is described in section 3.7. The use of these subsequent washes did not increase the amount of lipid lost in the upper phase and after the second wash it was possible to separate all the major lipid classes clearly.

Figure 9: Two dimensional thin layer chromatogram of total lipid from pea leaves

Figure 9: Two dimensional thin layer chromatogram of total lipid from pea leaves

Solvent System: Vertical direction Chloroform: Methanol: Ammonium hydroxide (75:25:3, V:V)
Horizontal direction Chloroform: Methanol: Acetic acid: Water (170:25:25:4, V:V)

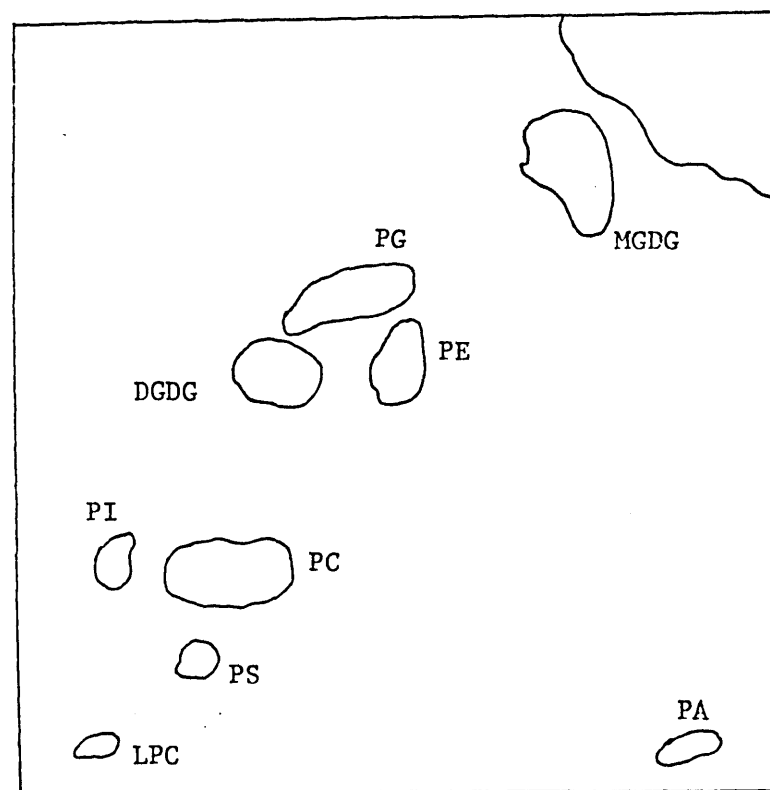


Figure 10: The effect of NaCl concentration on lipid retention in the aqueous phase during solvent partitioning

Figure 10: The effect of NaCl concentration on lipid retention in the aqueous phase during solvent partitioning

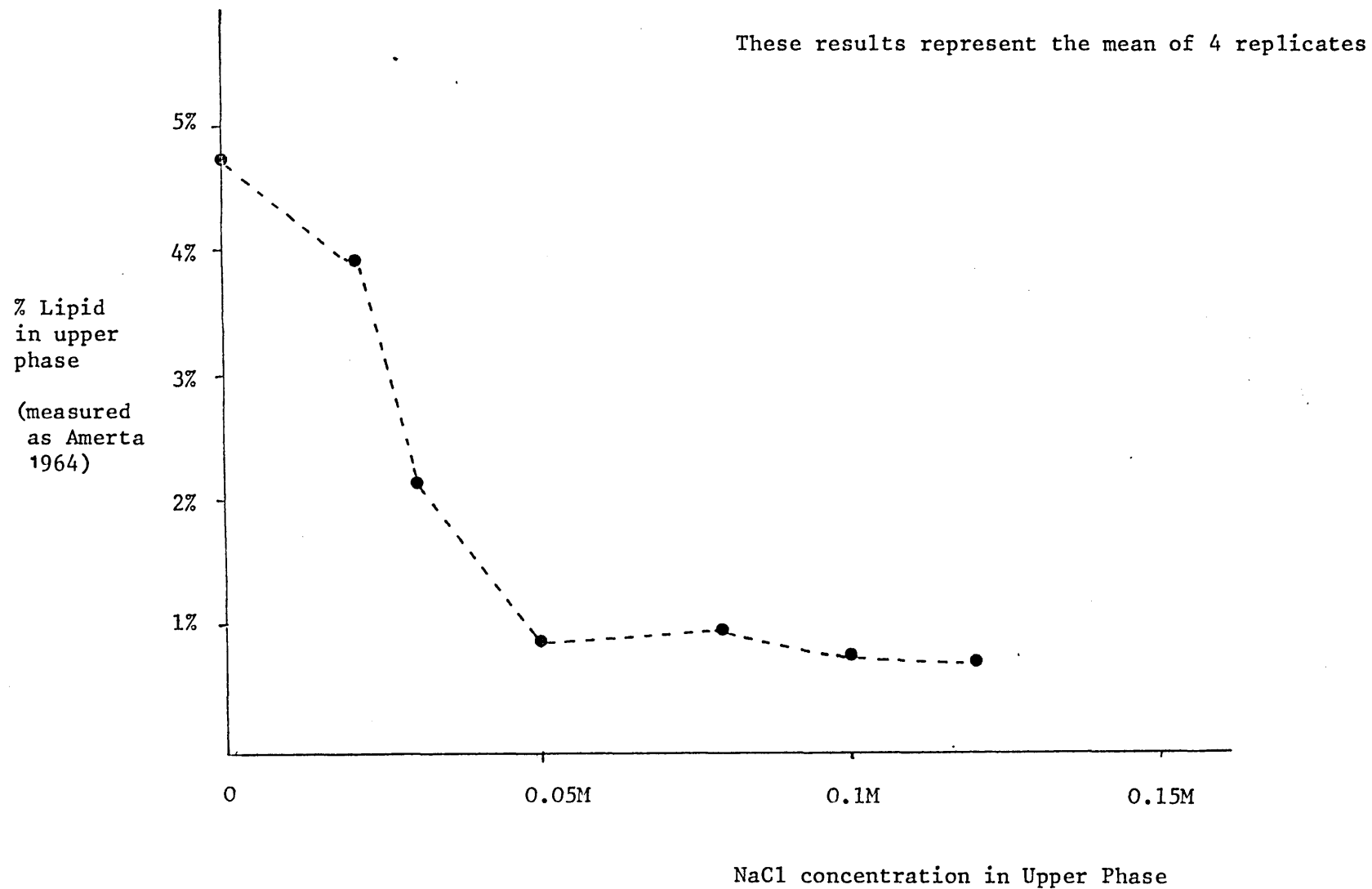


Table 7a: Lipid composition of the lower and upper phases in the phase separation procedure.

Lipid	Lower Phase		Upper Phase	
Phosphatidyl choline	361.58 ± 16.71	13.40%	17.51 ± 1.30	12.98%
Phosphatidyl ethanolamine	195.07 ± 10.10	7.23%	9.62 ± 0.71	7.13%
Phosphatidyl glycerol	206.62 ± 12.13	7.66%	8.16 ± 0.68	6.06%
Phosphatidyl inositol	87.13 ± 4.21	3.23%	35.49 ± 1.78	26.31%
Phosphatidyl serine	15.62 ± 1.21	0.58%	0.82 ± 0.12	0.61%
Phosphatidic acid	11.14 ± 1.05	0.41%	0.00	0.00%
Monogalactosyl diglyceride	779.50 ± 21.09	28.89%	33.01 ± 1.01	24.47%
Digalactosyl diglyceride	558.80 ± 18.31	20.71%	23.36 ± 1.34	17.32%
Lysophosphatidyl choline	10.01 ± 0.65	0.37%	0	0.00%
Neutral Lipid	331.66 ± 12.31	12.29%	0	0.00%
Sulpholipid	140.87 ± 7.18	5.22%	6.85 ± 0.74	5.08%
Total lipid	2698 ± 37.04	95.18%	134.82 ± 2.51	4.92%

All these results are the means of 4 replicates

Separation by tlc using: chloroform: Methanol: Acetic acid: water
(65.25:10:8, V:V)

Table 7b Percentage composition of fatty acids recovered from the lower and upper phases in the phase separation procedure.

Fatty Acid	Lower Phase	Upper Phase
Palmitic acid	11.7 ± 1.2	12.5 ± 1.0
Palmitoleic acid	6.9 ± 0.8	5.8 ± 0.6
Stearic acid	3.2 ± 0.7	3.7 ± 0.7
Oleic acid	3.4 ± 0.7	2.9 ± 0.4
Linoleic acid	14.3 ± 1.1	14.7 ± 1.3
Linolenic acid	56.4 ± 4.8	60.4 ± 4.1

The figures after the means are 95% confidence limits calculated by Students t test

At each stage in this phase separation process the upper phase was removed by pasteur pipette. Due to the difficulty of total phase separation using this technique the final traces of the last upper phase wash were dispersed in the lower phase by addition of methanol. This has the effect of raising the water content of the lower phase (equilibrium composition was already 86:41:1 by volume chloroform: methanol: water) and prior to further analysis removal of this water was required. This was achieved by adding dry Sephadex G25 to the extract which was then dried on a rotary evaporator under a vacuum at 35°C to remove the organic solvents in whose presense Sephadex G25 absorbs water only feebly. Chloroform was then added to the flask and evaporated again to ensure that total

absorption of the water by the Sephadex.

The method for recovery of lipid absorbed by the Sephadex (section 3.9) was tested by analysing samples of known lipid and fatty acid compositions using tlc and glc. There was a $97.21 \pm 0.31\%$ recovery of lipid from the original extract and this was consistent both in phospholipid and fatty acid composition.

4.1.3 Examination of chromatographic systems used to separate neutral lipids and polar lipids

Lipid extracts, once freed from non-lipid components were examined by tlc using solvents suitable for polar lipids. However, resolution was poor due to drag effects on the plates caused by the presence of large amounts of pigments and neutral lipids migrating with the solvent front. Two different methods were employed to remove these neutral lipids, firstly column chromatography on silica gel and secondly pre running the tlc plates with a strongly hydrophobic solvent mixture.

a) Column Chromatography

The method outlined in section 3.7, is dependent on lipids binding to silicilic acid by polar and ionic forces and to a limited extent by non-polar and Van der Waals forces. Separation takes place by elution using a series of solvents of increasing polarity, complete separation of the polar phospholipids by single column chromatography is impossible because of overlapping polarities.

Columns were prepared as in section 3.9 and initially three solvent systems were tested, loosely based on the methods of Nichols (1963), Rouser, Kritcsevksy and Yamamoto (1967) and Lepage (1966) respectively (table 8).

A lipid extract from 0.5g fresh weight equivalent of whole pea leaves was used to test these systems. Once the separate fractions had been collected they were analysed for their lipid composition by tlc, but not quantified.

With the first system the chloroform eluted all the neutral lipid and trace amounts of PE. The first chloroform: methanol fraction contained small amounts of neutral lipid and most of the important polar lipid classes, whereas the following chloroform: methanol and methanol fractions contained polar lipids alone.

With the second solvent system the chloroform: acetone fraction eluted some PE, PC and MGDG. Acetone alone eluted more PE, PC and DGDG and methanol eluted the rest of the phospholipids.

With the third system all neutral lipid was eluted by diethyl ether and none of the polar lipids were found in these fractions, whereas polar but no neutral lipids were found in the chloroform: methanol fractions.

Table 8 Solvent Systems used to separate neutral from polar lipids.

System 1

100% Chloroform	5 Column Volumes
80% Chloroform 20% Methanol	2 Column Volumes
50% Chloroform 50% Methanol	2 Column Volumes
20% Chloroform 80% Methanol	2 Column Volumes
100% Methanol	5 Column Volumes

System 2

100% Chloroform	5 Column Volumes
50% Chloroform 50% Acetone	5 Column Volumes
100% Acetone	5 Column Volumes
90% Acetone 10% Water	5 Column Volumes
100% Methanol	5 Column Volumes

System 3

100% Hexane	5 Column Volumes
90% Hexane 10% Diethyl ether	2 Column Volumes
50% Hexane 50% Diethyl ether	2 Column Volumes
100% Diethyl ether	2 Column Volumes
100% Chloroform	5 Column Volumes
90% Chloroform 10% Methanol	5 Column Volumes
50% Chloroform 50% Methanol	5 Column Volumes

These results suggested a system that would satisfactorily separate the neutral lipids from the phospholipids. Further trials showed that all neutral lipids could be eluted using 5 column volumes of diethyl ether. Previously all the samples had been added to the column dissolved in chloroform so this new method necessitated changes in the procedure. Dried lipid extract was first dissolved in 2 x 5ml volumes of diethyl ether and this was retained for column chromatography. The evaporator flask was then washed with 3 x 5ml volumes of chloroform, 3 x 5ml volumes of chloroform:

methanol (2:1, V:V) and finally 3 x 5ml volumes of methanol. These washings were not subjected to column chromatography since although diethyl ether washings contained no neutral lipid and also when combined with the diethyl ether washing gave a $100.3 \pm 0.7\%$ (from 4 replicates) return in the amount of lipid in the extract.

After 5 column washes of diethyl ether had passed through the column the procedure was completed by eluting with 5 column volumes of chloroform, 5 column volumes of chloroform: methanol (2:1, V:V) and 4 x 5 column volumes of methanol. These last fractions were kept separately to check by tlc that the last methanol fractions contained no phospholipid. This ensured virtual total recovery.

b) Thin Layer Chromatography

Neutral lipids were separated from polar lipids by development of plates with a mixture of acetone: light petroleum (3:1, V:V) in which neutral lipids migrate with the solvent front. This was tested and found to occur, however the subsequent separation of polar lipids on tlc was affected on some occasions. It was due to this inconsistency that column chromatography was used to separate neutral from polar lipids.

4.1.4 Elution of lipids from silica gel

On many occasions during analysis it was necessary to elute lipids from silica gel, the general method for which is described in section 3.10.2. In arriving at this procedure two protocols were tested, a modified version of that used by Skipski and Barclay (1969) and a new development. In the first method elution is successively with 5ml of the solvent mixture used for tlc (chloroform: methanol: acetic acid: water; 65:25:10:8, by volume), 5ml of methanol and finally 5ml methanol: acetic acid: water (94:1:5, by volume). In the second method chloroform is used for the first three elutions, for the next three 5ml volumes of chloroform: methanol (2:1, V:V) are employed and finally three 5ml volumes of methanol (as used in section 4.1.2).

These methods were tested on phospholipid standards and the results are shown in table 9. The second method gave slightly though not statistically

significant better recovery rates and is also simpler and hence was employed as routine.

For the elution of neutral lipids from silica gel the method described by Skipski and Barclay (1969, Section 3.10.2) proved adequate. Table 10 shows the recovery of lipid obtained when standards were eluted from plates.

Table 9: Recovery of phospholipids eluted from silica gel after tlc

Phospholipid	Amount applied (μg)	Amount recovered (μg)	% Recovery (Amenta, 1964)
Phosphatidyl choline	250	243.75 ± 9.5	97.5 ± 3.8
Phosphatidyl ethanolamine	250	247.25 ± 4.2	98.9 ± 1.7
Phosphatidyl inositol	100	100.50 ± 4.7	100.5 ± 4.7
Phosphatidyl serine	100	97.90 ± 1.2	97.9 ± 1.2
Phosphatidyl glycerol	100	100.00 ± 4.9	100.0 ± 4.9
Lysophosphatidyl choline	250	249.00 ± 7.0	99.6 ± 2.8

All the results are the means of 4 replicates $\pm$ 95% confidence limits

Table 10: Recovery of neutral lipids from silica gel after tlc

Neutral Lipid	Amount applied (μg)	Amount Recovered (μg)	% Recovery (Amenta, 1964)
Tripalmitin	250	254.95 ± 8.4	101.98 ± 3.37
Dipalmitin	250	245.57 ± 5.3	98.23 ± 2.14
Palmitic acid	250	250.37 ± 4.9	100.15 ± 1.96
Cholesterol oleate	250	249.82 ± 9.1	99.93 ± 3.66
Cholesterol	250	247.12 ± 9.9	98.85 ± 3.95

All the results are the means of four replicates $\pm$ 95% confidence limits

4.1.5. Assessment of quantitative analyses involving spectrophotometry

This section evaluates the spectrophotometric methods used to quantify lipids. They give no indication of the type of lipid or of its structure. The methods listed were those of Amenta (1964), Marsh and Weinstein (1966) and Bartlett (1959) all of which have been used extensively in quantitative leaf lipid analysis. These methods are all described in section 3.10.3. Using standard lipid samples the lower limits of detection and the upper limits to linearity were estimated.

Reduction of dichromate (Amenta, 1963)

Using the procedure, described in section 3.10.3 the limits of detection for all the lipids lies between 40 and 250 μ g, however if the amount of dichromate reagent used in the assay is decreased to 0.5ml then it is possible to detect as little as 10 μ g of lipid (figure 12). Similarly if the amount of dichromate is increased to 5ml it is possible to detect accurately up to 1000 μ g of lipid.

Figure 11: Calibration curves for different lipids using the reduction of dichromate method using 2ml dichromate

Figure 11: Calibration curves for different lipids using the reduction of dichromate methanol using 2m| dichromate

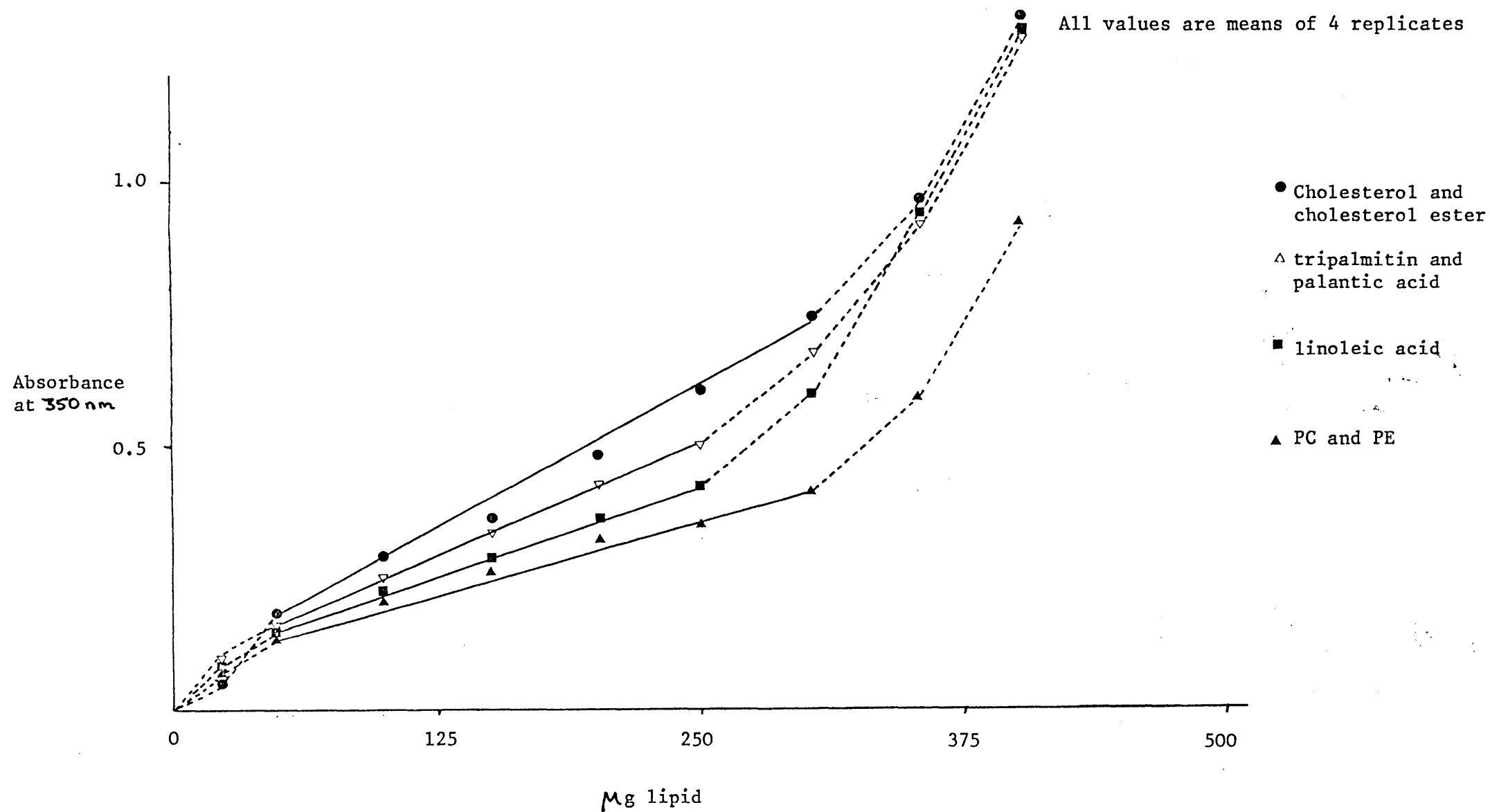


Figure 12: Calibration curves for lipids using the reduction of dichromate method (0.5ml of reagent)

Figure 12: Calibration curves for lipids using the reduction of dichromate method (0.5ml of reagent)

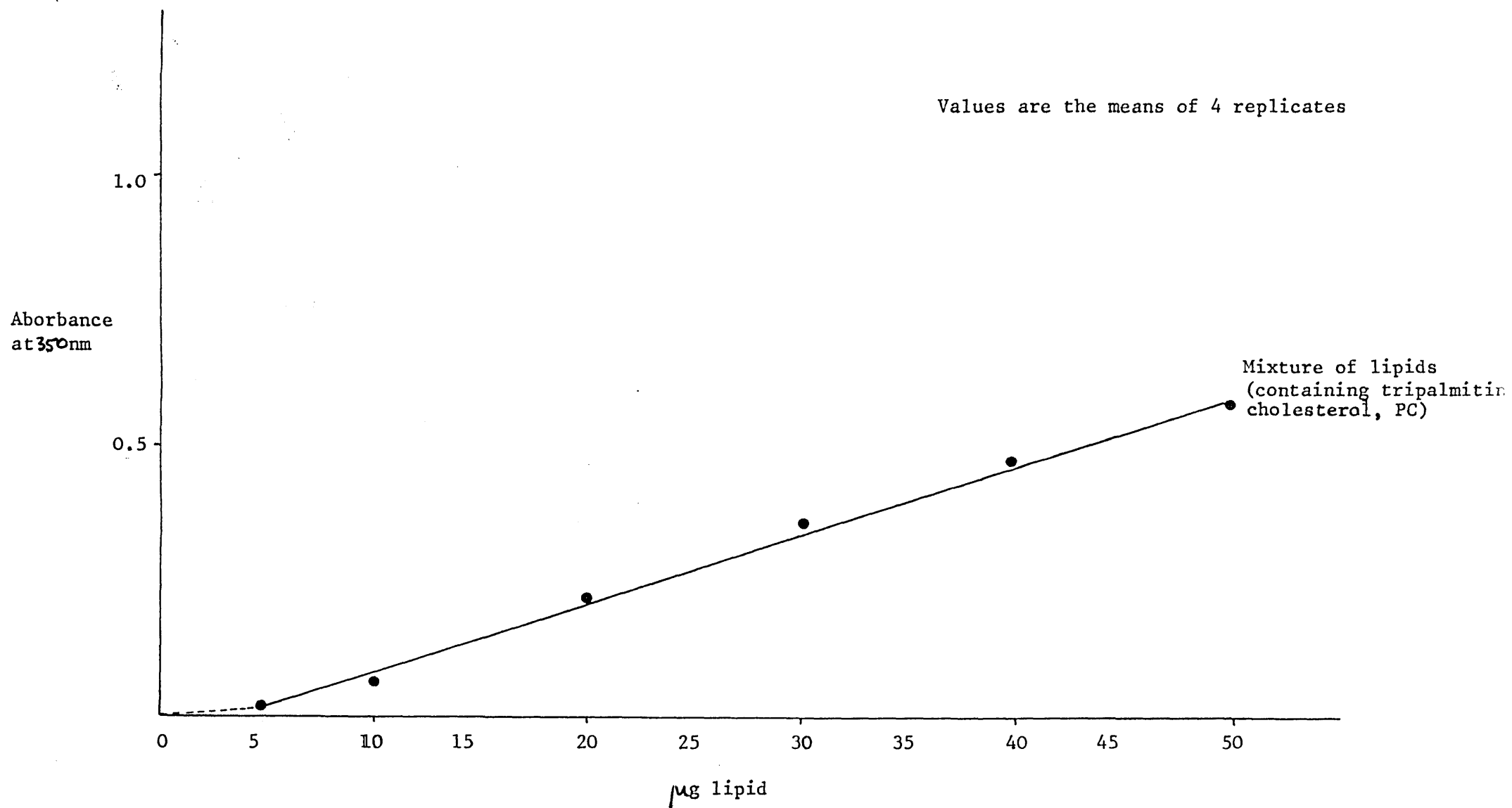
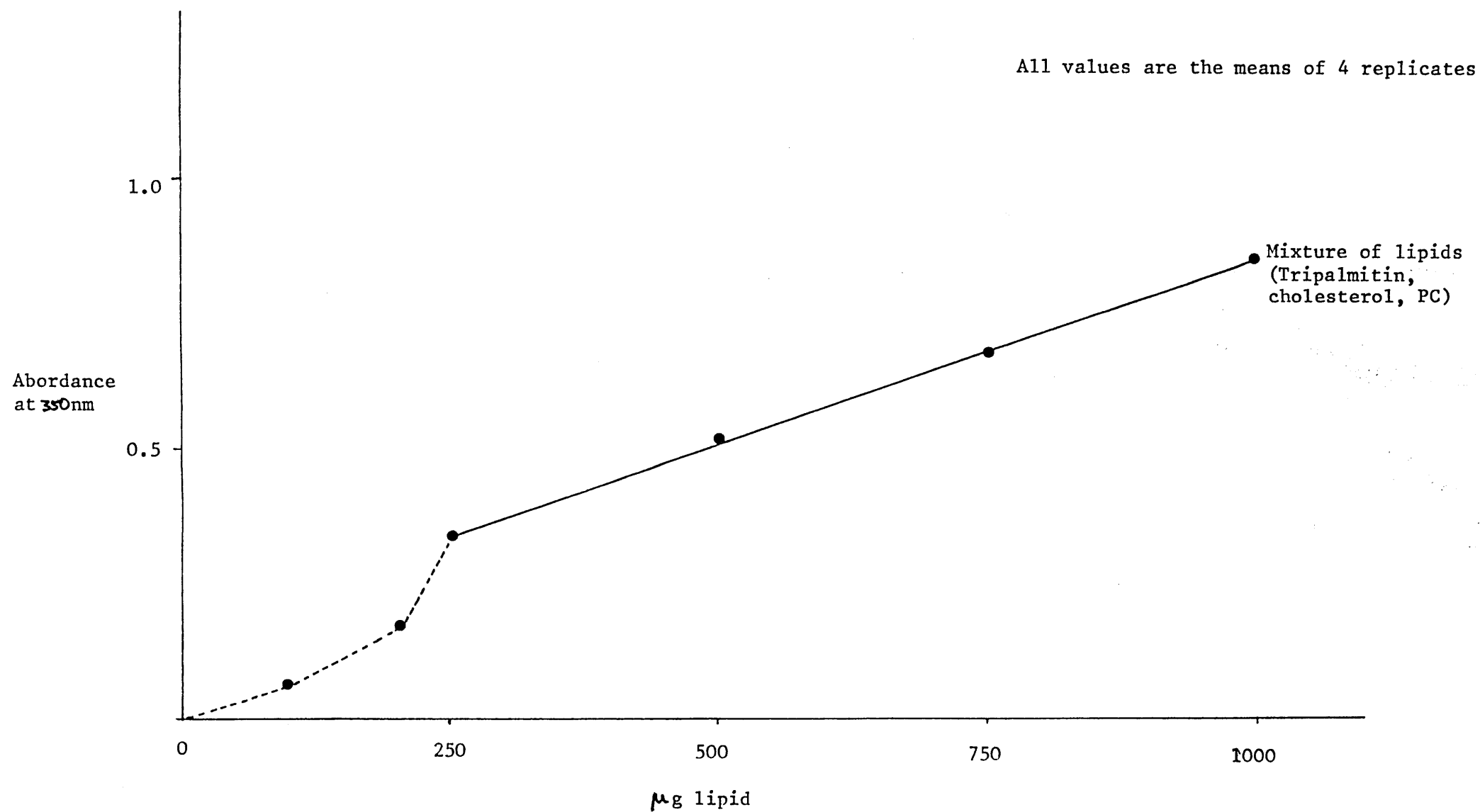


Figure 13: Calibration curve for lipids using the method of Amenta (1964)
employing 5ml dichromate

Figure 13: Calibration curve for lipids using the method of Amento (1964) employing 5ml dichromate



Figures 12 and 13 show the calibration curves obtained when a lipid mixture was used, whereas figure 11 shows the calibration curves obtained using different lipid standards. All lipids give a linear response over the same range, however the curve for each lipid has a different gradient. Consequently when analysing a natural sample of unknown composition a standard curve obtained using a mixture of standards should give the most accurate results. The degree of fatty acid unsaturation also has an effect upon the slope of the curve, which means that standard curves for individual lipids are impossible to obtain due to the variation in saturation in natural lipids. This method was only used where such approximation could be tolerated.

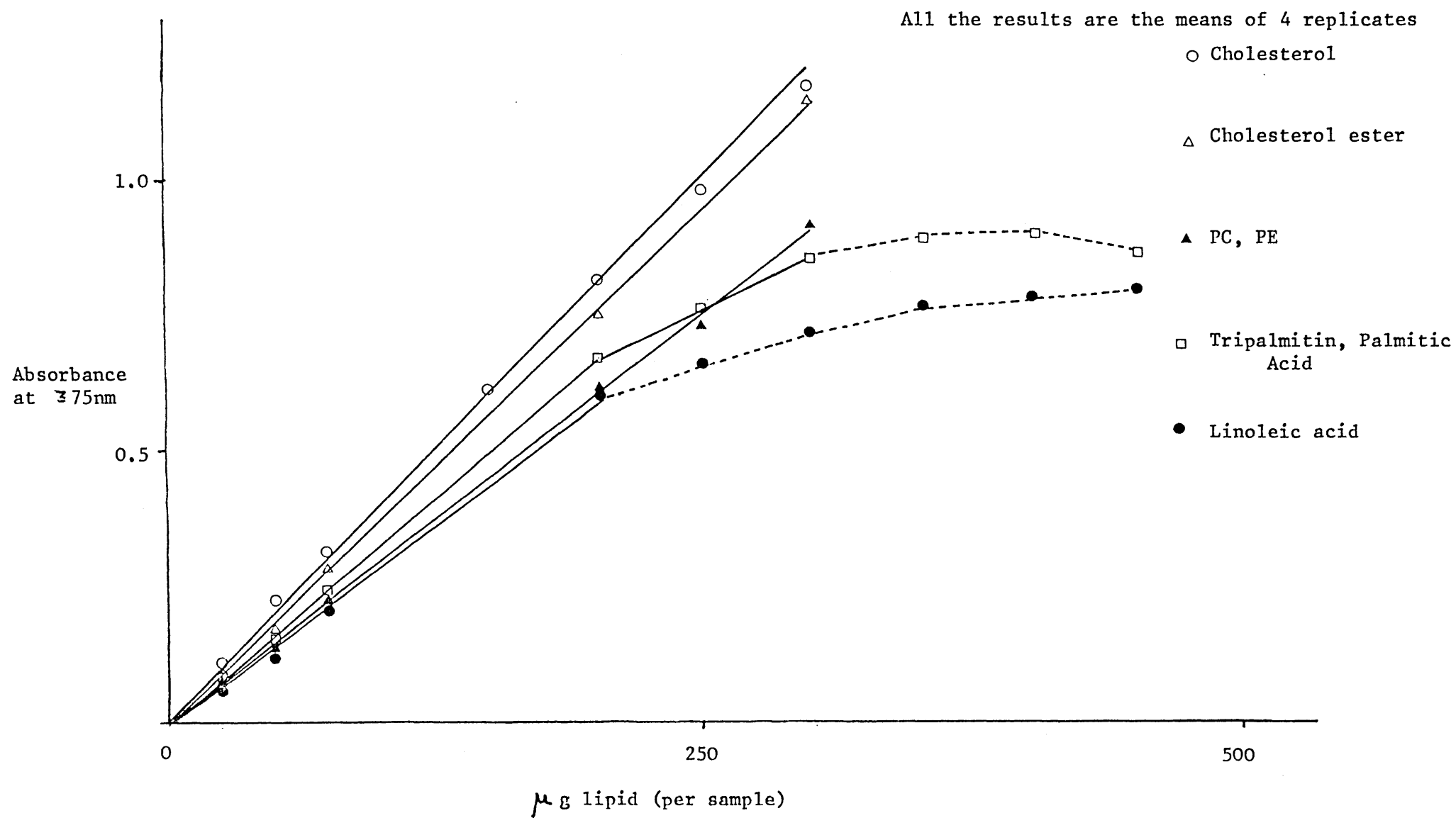
Another problem with this procedure was that the methods used to locate lipids following tlc increase the absorbance readings, in the case of iodine by $10.3 \pm 1.8\%$ and dichlorofluorescein by $9.4 \pm 2.1\%$ (values taken from four replicate analyses of total lipids). This problem can be overcome by using as standards samples which have been separated by tlc and detected using the same reagents as the unknown samples.

Marsh and Weinstein

This method, described in section 3.10.2, relies upon charring of lipids by sulphuric acid and like the previous one is not specific. Figure 14 shows the standard curves obtained using a range of different lipids. The detection limit for this method was $5\mu\text{g}$ and the plots remained linear up to $200\mu\text{g}$, after which a semilinear relationship became apparent for some of the samples.

Figure 14: Calibration curves for various lipids using the method of Marsh and Weinstein (1966)

Figure 14: Calibration curves for various lipids using the method of Marsh and Weinstein (1966)



All the problems associated with the previous method were also found with this method, however the method has the advantage over that of Amenta (1964) in that it has a lower detection limit and sensitivity does not rely upon changing the amount of reagent used, an awkward complication for routine analysis.

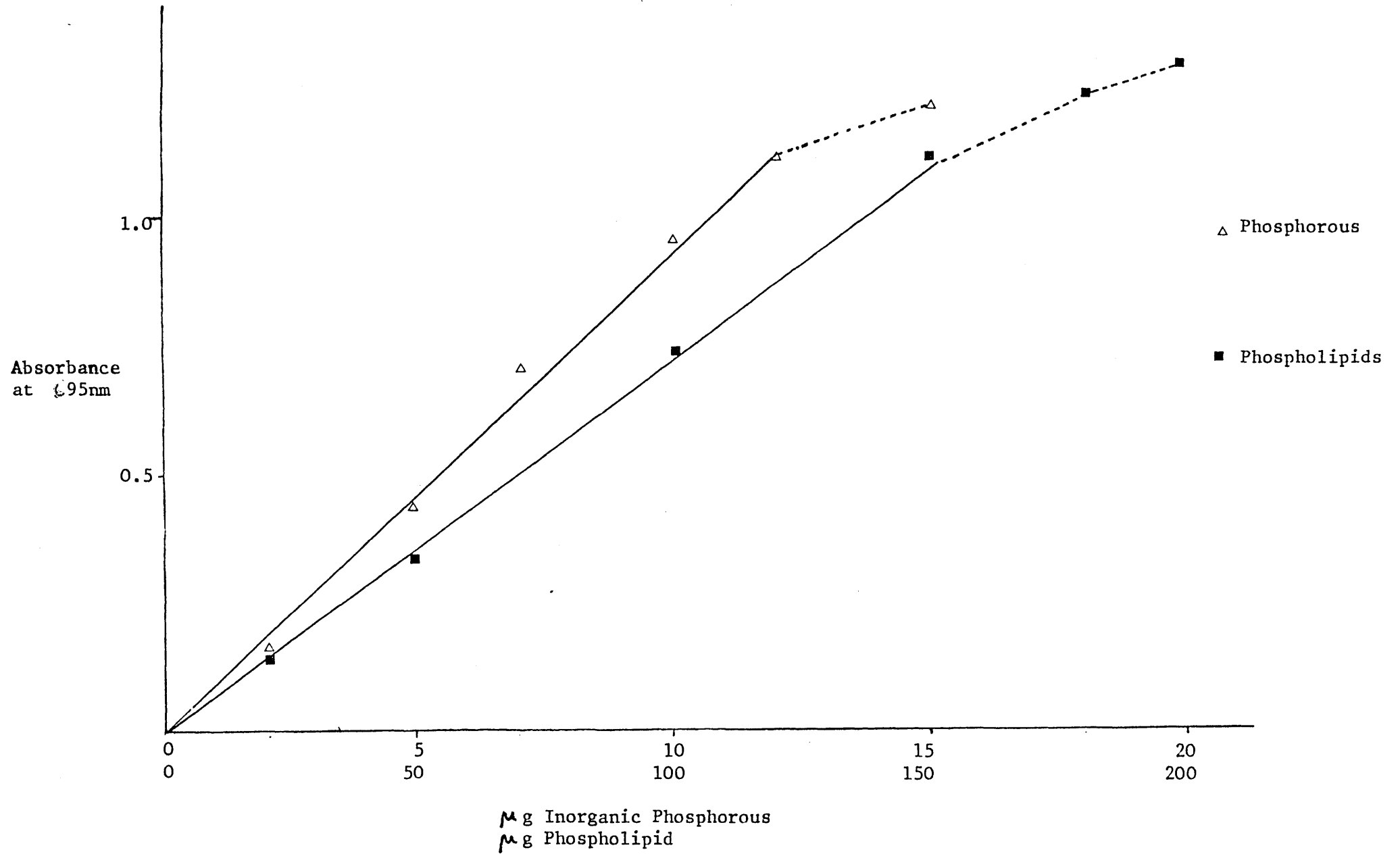
This method could be used both with and without silica gel providing that it was removed prior to spectrophotometry.

Phosphate determination (Bartlett, 1959)

Phospholipids were determined by measuring their phosphorous content, the method is described in section 3.10.3. Figure 15 shows standard curves for known phospholipids and inorganic phosphorous.

Figure 15: Calibration curve for phosphate analysis

Figure 15: Calibration curve for phosphate analysis



The main problem with this method, other than safety aspects involved using perchloric acid on a routine basis, was the development of spurious colours in particular when iodine was used for lipid identification. Iodine being volatile, should sublime from the tlc plates when exposed to air however, inevitably on occasions some iodine is left on the plates and this causes the high absorbance readings. The other lipid recognition methods such as dichlorofluorescein also cause similar problems.

The other cause of variation with this method was the perchlorate — sensitivity of colour development. Figure 16 shows the effect of varying the amount of perchloric acid used in the digestion on the absorbance readings for different levels of inorganic phosphorous. Small changes in the levels of perchloric acid can give great changes in the apparent level of phosphorous. These variations are well within the level of experimental error and could explain the spurious colours obtained on occasions using this method.

The method is linear for 0.15~~µg~~ P or 0.125~~µg~~ of phospholipid. Tichy (1973) found that when inorganic phosphorous from phospholipid was determined in the presense of silica gel, higher activities were required to prevent autoreduction of molybdate which occurred about pH1 and for which silica gel acted as a buffer. However, this did not apply in these experiments as the pH never went above pH1 and the presence of silica gel had no effect on colour development.

This method was only used while the extraction and analytical procedures were being tested. Once the lipid determination methods were optimised, quantification by GLC and the methods of Amenta (1964) and Marsh and Wernstein (1966) were used in preference, for although these latter methods are not specific for phospholipids they do give more consistent results and are easier, quicker and safer to use.

4.1.6. Experiments to optimize the the conditions necessary to produce fatty acid methyl esters (FAMES) from lipid samples

In order for the fatty acids in lipids to be analysed successfully by gas chromatography they must first be converted to their methyl esters. This is generally achieved by using 14% boron trifluoride in methanol.

However, before routine use the optimal conditions for methyl esterification were sought. The method is described in Section 3.11.1. The following standards were examined, tripalmitin, dipalmitin, palmitic acid, phosphatidyl choline dipalmityl and cholesteryl oleate. The standards were applied to tlc plates and separated, the amount of each lipid applied being adjusted to give 100 μ g of fatty acid.

Figure 16: The effect of varying amounts of perchloric acid on phosphorous determination by the Bartlett method

Figure 16: The effect of varying amounts of perchloric acid on phosphorous determination
by the Bartlett method

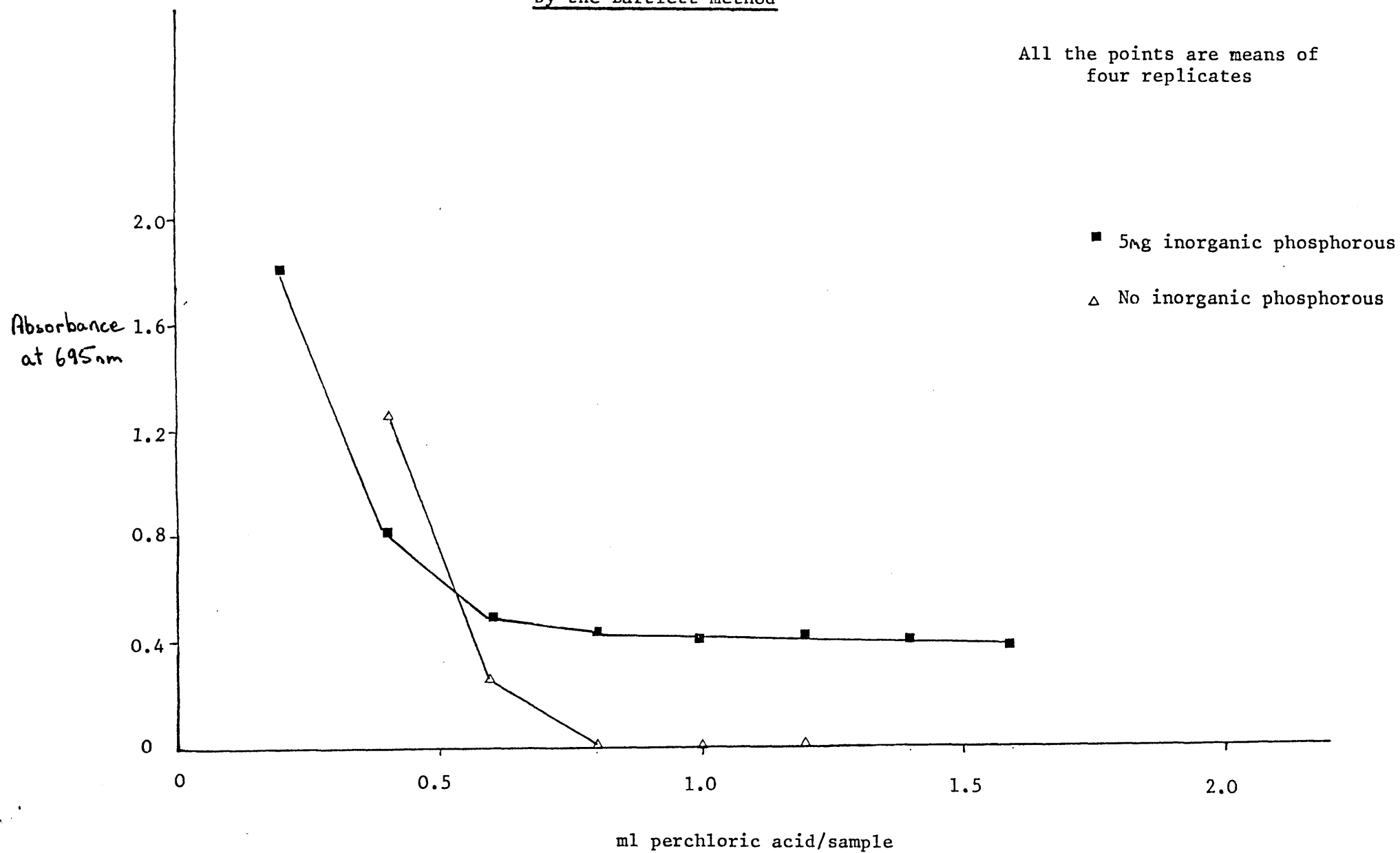
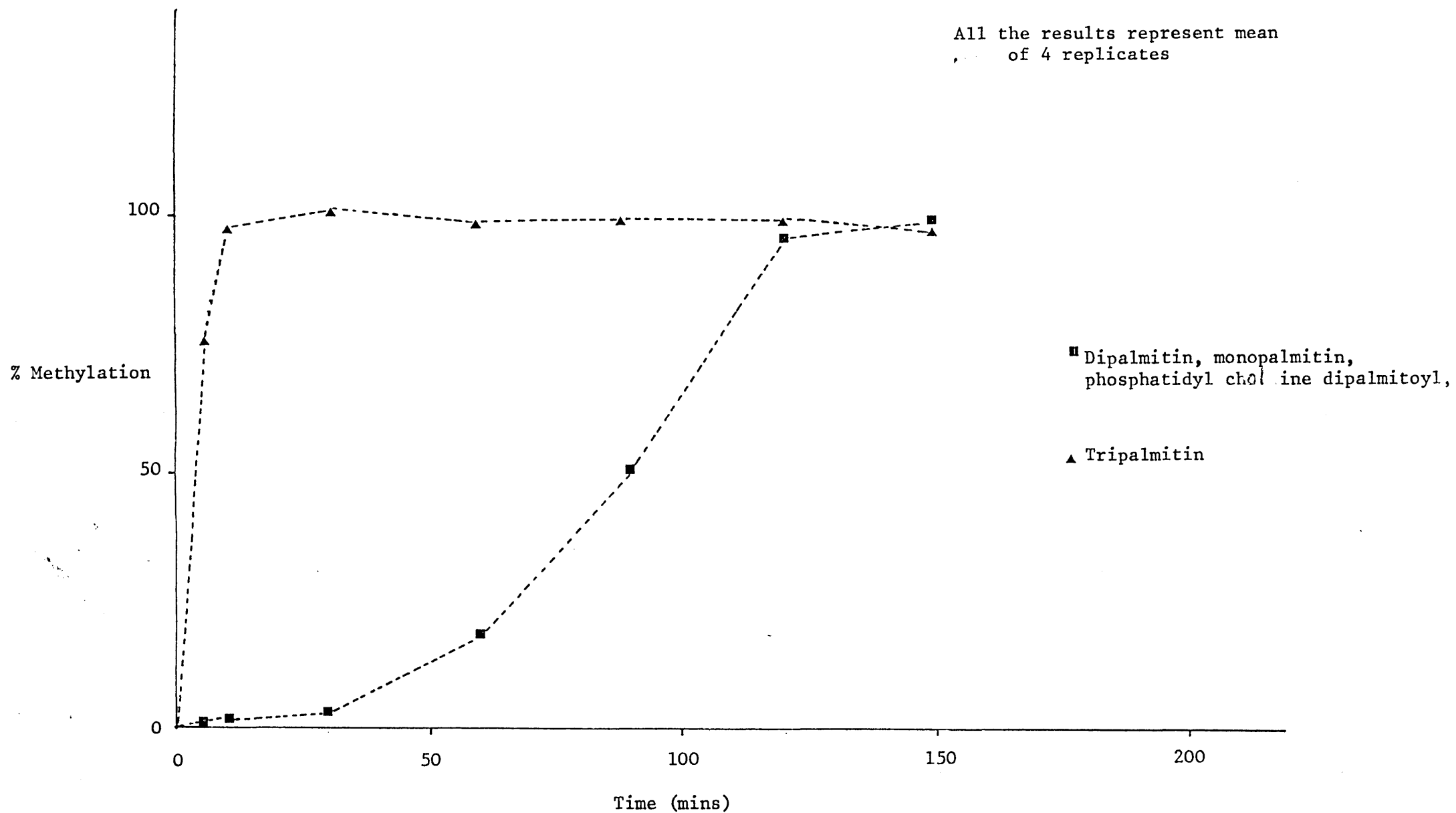


Figure 17: Methylations of fatty acid from different lipids

Figure 17: Methylations of fatty acid from different lipids



Sterol esters were not methylated

The results of these experiments are found in figure 17. The methylation of monoglycerides, diglycerides, phospholipids and fatty acids is complete in approximately 10 minutes. The samples remain stable if they are kept for at least 2.5 hours, however, for triglycerides esterification requires two hours.

In the original description of this method Morrison and Smith (1964) found that if the 14% boron trifluoride in methanol contained 20% (V/V) benzene the time required for methylating was reduced to 25 minutes. In the present study toluene replaced benzene due to the toxicity of the latter. The influence of a proportion of toluene on methylation is shown in figure 18. The shortest time for 100% methylation of tripalmitin was 20 minutes in the presence of 25% toluene.

Sterol esters are not methylated at all by 14% boron trifluoride in methanol because of insolubility. If toluene is added to the reaction the sterol esters are solubilized and can be methylated. Using cholesteryl oleate as a standard and varying the concentration of toluene in the reaction as before an optimum time for methylation of 45 minutes was found with 30% toluene (Figure 19).

Figure 18: The effects of different concentrations of toluene on triplamitin methylation

Table 18: The effects of different concentrations of toluene on tripalmitin methylation

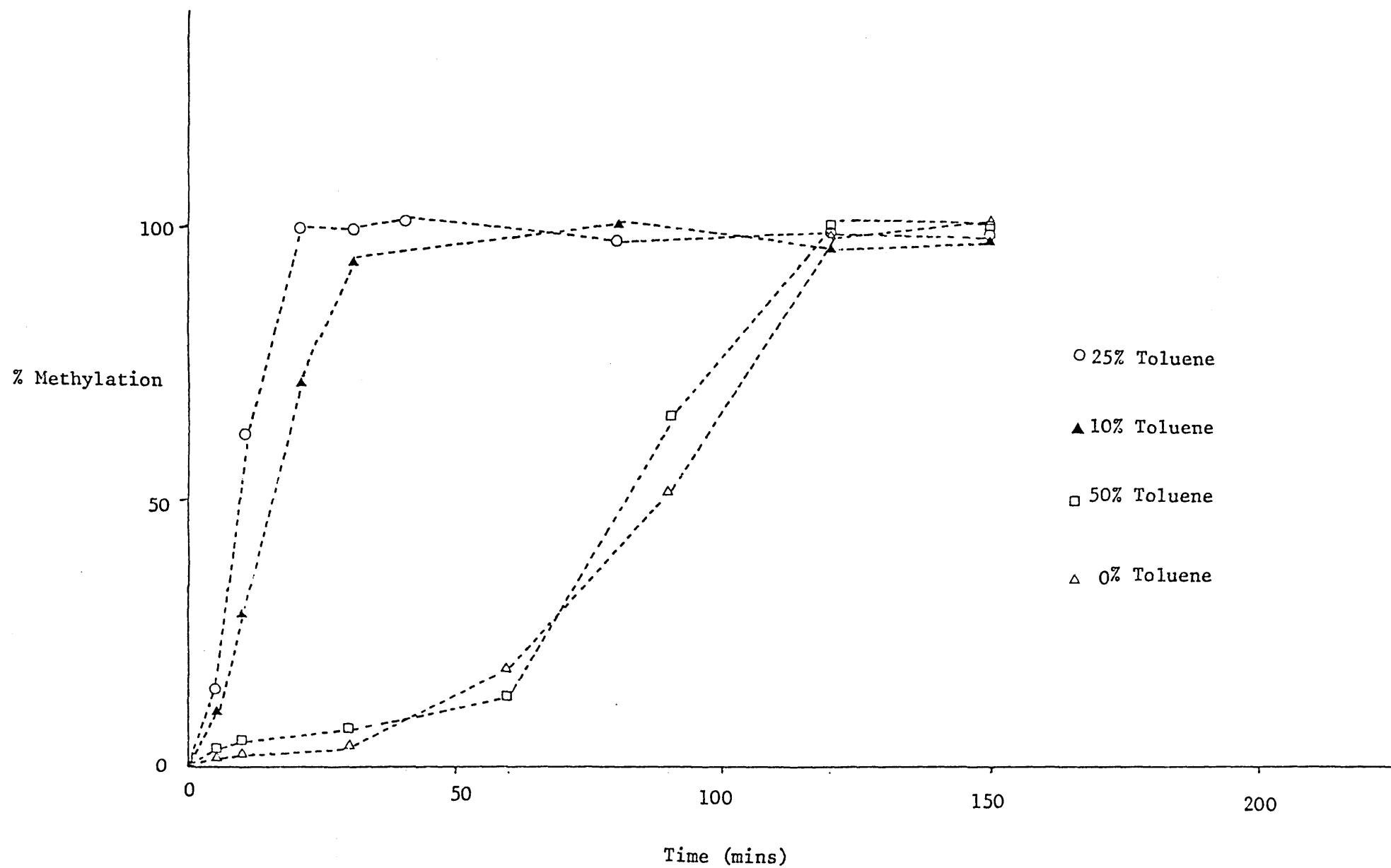
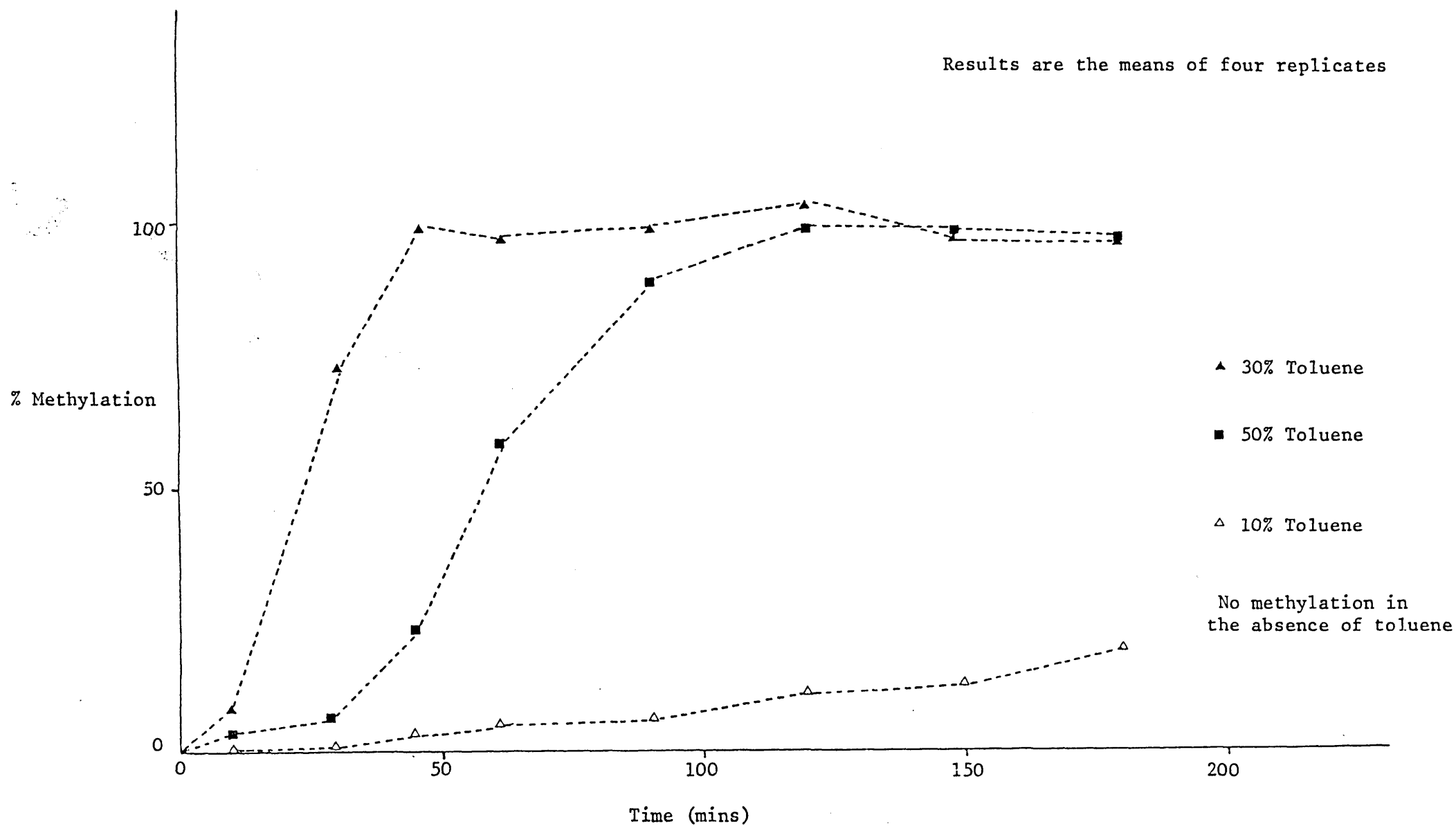


Figure 19: The effects of different concentrations of toluene on cholesteryl oleate methylation

Figure 19: The effects of different concentrations of toluene on cholesteryl oleate methylation



FAMES must be partitioned into hexanes in preparation for analysis by GLC (Section 3.11.1). Extraction efficiency of the method was tested with the following standard fatty acids; palmitic acid, stearic, oleic, linoleic and linolenic in a mixture containing 50 μ g of each. These were methylated and the two separate 2ml volumes of hexane used for partitioning the fatty acid and methyl esters were tested for their fatty acid composition by GLC. The mean fatty acid return was $99.3 \pm 1.2\%$ and the returns for the two hexane volumes were $78.2 \pm 1.9\%$ and $21.1 \pm 1.4\%$.

Deterioration upon storage could have caused problems with the analysis of FAMES. To investigate this a mixture of fatty acids; as above, was stored at 4°C under nitrogen with 0.05% BHT for a month. Samples were removed at weekly intervals. None showed any change in composition up to one month after esterification and as none of the samples used for analysis were stored this long fatty acid deterioration at this stage was not a problem.

The degree of fatty acid saturation might effect the methylation yield. Table 11 shows that there is a significant ($p \approx 0.01\%$) difference between fatty acids, the times and their interactions when the full analysis is made for the data up to two hours. For the final 30 minutes, however, there are no significant differences between fatty acids, which confirms the suitability of this shorter period for methylation. Sterol esters have a methylation time of 45 minutes, however if there are any differences in the fatty acid composition caused by methylation for this time it is relatively unimportant as sterol esters are not major components of the extracts under study. A further point is that the degree of accuracy achieved in the pilot studies is in excess of routine requirement. Because the main study involves a comparison of different extracts and providing that analytical errors are constant no overall bias should be introduced into the results.

Table 11: Recovery rates of fatty acid methyl esters after differing times of methylation at 100°C

Fatty Acid Methyl Ester	Times				
	10	30	60	90	120
Palmitic acid	98.3	99.2	98.7	97.5	98.3
Stearic acid	99.2	98.6	99.1	98.5	97.9
Oleic acid	98.7	98.7	98.3	95.9	93.6
Linoleic acid	98.9	99.6	97.6	91.7	90.4
Linolenic acid	99.3	98.8	95.4	91.8	89.6

All the results are the means of four replicates

Analysis of Variance of the whole table

Item	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Probability
Fatty Acid (F)	4	278.07	69.52	69.52	0.01
Times (T)	4	427.31	106.83	106.83	0.01
F x T	16	230.50	14.41	14.41	0.01
Replicates	75	75.19	1.00		
Totals	99	1011.07			

Analysis of Variance of the 10-30 minute data

Item	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Probability
Fatty Acid (F)	4	0.72	0.18	0.60	0.5
Times (T)	1	0.04	0.04	0.13	0.5
F x T	4	3.07	0.77	2.57	0.5
Replicates	30	9.04	0.30		
Total	39	12.87			

4.1.7. Calibration of the gas chromatograph

Gas chromatography was used for the routine analysis of leaf phospholipid fatty acids (Section 3.11.2). This method had been devised to give optimum separation and analysis of the standard fatty acids found in leaf phospholipids (D. Chapman pers. comm.). The retention times for each fatty acid were measured and their relative response factors were calculated. The integrator was then programmed to recognise each peak, calculate the area under the peak, amend this area by multiplying the area obtained by a relative response factor for each fatty acid and then obtain a value for the amount of the fatty acid present by comparison with an

internal standard.

This method proved suitable for the analysis of both whole leaf and epidermis phospholipid fatty acids, but could not be used for the analysis of the neutral lipid fractions or the fungal lipids. This was because these lipids contained both short chain fatty acids e.g. lauric acid and also long chain fatty acids e.g. arachidonic. It was necessary to use an entirely new column and method to analyse these extracts as it was not possible to adopt the previous method for this purpose.

The conditions on the new column were optimized for efficient separation of the fatty acids in the shortest possible time and the relative response factors for each fatty acid were calculated (Table 13). In all these experiments the following fatty acid methyl esters were used as a mixture of 100 μ g of each in 100 μ l of hexane, lauric, myristic, pentadecanoic, palmitic, stearic, oleic, linoleic, linolenic, arachidic and arachidonic.

Initially the following conditions were used; injection temperature 210°C, detector temperature 210°C column temperature 150°C, carrier gas flow rate 35mls/min, the integrator was programmed to analyse the peak areas of each fatty acid. No time limits were programmed into either the integrator or the gas chromatograph. The process was stopped when all fatty acids had eluted from the column. The results (Table 12) show that although the separation of the short chain fatty acids was extremely effective, the long chain fatty acids while separating well had an unacceptably long retention time. By varying the operating parameters a two stage system involving a temperature programme was eventually chosen as the best compromise between speed and accuracy.

The response factors relative to the standard used in later experiments (pentadecanoic acid) were calculated, table 13.

The glc was tested to assess whether increasing the sample size had any effect on the area of the fatty acid peaks. This was tested by injecting samples containing the same compositions of fatty acid standards but with increasing amounts of the fatty acids. Table 14 shows that there was no significant difference in the response in the range 0-750 μ g of fatty acid

and as these values far exceed the amounts of fatty acids analysed in the later experiments, this showed that erroneous values would not be found for this reason.

Table 12: Retention times of fatty acids on the gas chromatographs using different oven temperatures and carrier gas flow rates

Fatty Acid	Retention Time Using System				
	1	2	3	4	5
Capric Acid	2.18	-	-	-	2.18
Lauric acid	3.17	2.32	-	-	3.70
Myristic acid	6.57	3.41	3.10	4.14	6.57
Pentadecenoic acid	9.63	8.20	5.16	6.73	9.63
Palmitic acid	11.90	9.95	7.91	8.81	11.90
Stearic acid	20.03	18.87	14.31	16.54	18.78
Oleic acid	28.21	26.14	17.42	19.99	24.32
Linoleic acid	37.86	34.82	22.35	28.67	28.76
Linolenic acid	47.61	43.01	27.89	34.76	31.93
Arachidic acid	42.38	40.21	25.00	31.55	30.04
Arachidonic acid	55.34	51.01	34.17	47.81	36.14

All figures represent the means of 3 replicates, no confidence limits are quoted as the retention times all remain stable once the conditions are set. A dash in the column means that the fatty acid could not be separated.

System 1: Column Temperature 150 C°; carrier gas flow 35ml/min
 System 2: Column Temperature 150 C°; carrier gas flow 40ml/min
 System 3: Column Temperature 190 C°; carrier gas flow 35ml/min
 System 4: Column Temperature 170 C°; carrier gas flow 35ml/min
 System 5: Column Temperature 150 C°; carrier gas flow 35ml/min
 for 15 mins, temp. increased 4 C° a minute for
 10 mins and finally allowed to run at 190 C° for
 15 minutes.

Table 13: Relative response factors for individual fatty acids using Gas Chromatography column

Fatty Acid	Relative response factor
Capric Acid	0.987
Lauric acid	1.215
Myristic acid	1.015
Pentadecanoic acid	1.000
Palmitic acid	1.058
Stearic acid	0.958
Oleic acid	0.868
Linoleic acid	0.981
Linolenic acid	1.086
Arachidic acid	1.011
Arachidonic acid	0.984

All these values were the means of four replicates, confidence limits were not calculated as for practical purposes there was no variation.

Table 14: The effect of increasing sample size on chromatograph response

16:0		18:0		Fatty Acid 18:1		18:2		18:3	
Area of Peak	%	Area of Peak	%	Area of Peak	%	Area of Peak	%	Area of Peak	%
2.24	24.7	0.81	9.8	1.13	15.2	2.12	25.2	2.34	25.1
10.49	24.8	4.06	10.6	5.59	16.1	10.90	27.8	10.81	24.9
35.44	25.4	12.76	10.1	15.57	13.6	32.21	26.9	37.24	26.0
57.48	24.2	19.14	8.9	30.01	15.4	55.50	25.2	64.12	26.3
83.14	26.2	26.15	9.1	39.43	13.4	75.32	25.6	83.71	25.7

4.1.8. Optimization of lipid extraction procedures

The aim of the lipid extraction procedure was to obtain total extraction of a sample which was both uncontaminated and undegraded. Within plant cells lipids are bound by a range of weak forces, Van der Waals, hydrogen bonds, electrostatic and hydrophobic associations, to a range of other cellular constituents. Additionally some lipids are covalently linked as esters to carbohydrate as in lipopolysaccharides of bacterial cell walls.

Lipids that are hydrophobically associated may be extracted with non-polar solvents such as chloroform. Membrane-associated lipids require additional polar solvents such as methanol to disrupt the hydrogen bonding and electrostatic forces between the lipids and proteins. Covalently

bound lipids cannot be extracted directly by any solvents but must first be cleared from the complexes by acid or alkaline hydrolysis. It is this use of an alcohol in the extraction procedure which causes the problems with non lipid contaminants because aqueous methanol (containing water from the tissue) is an effective solvent for sugars, amino acids and some electrolytes (Section 4.1.3).

Lipid-degrading enzymes such as phospholipase D are activated by the organic solvents used in the extraction procedure. Phospholipase D is an extremely stable enzyme and to deactivate it treatment with boiling alcohols such as isopropanol, methanol etc. is necessary. The efficiency of the standard procedure was tested by comparing the amounts of PA found in the final extracts with and without an isopropanol boiling stage. Provided that this stage is included only $2.4 \pm 0.8\%$ PA is detected whereas if omitted up to $49.7 \pm 14.7\%$ may be present. It is therefore extremely important to include this initial stage.

The extraction procedure

Using the work of Fishwick and Wright (1977) as a starting point the following extraction methods were tested:-

Method 1

The tissue was washed 3 times with chloroform: methanol (2:1 V:V) allowing 5 minutes between washes.

Method 2

The tissue was blended in a "Sorval Omnimixer" with 20ml chloroform: methanol: water (5:10:4 by volume) for 2 minutes, after which 5ml chloroform and 0.1ml concentrated HCl was added and blended for a further 2 minutes. The extract was then filtered through Whatman No. 1 filter paper and the filtrate retained. The residue was rinsed with 20ml chloroform: methanol (2:1 V:V), the washings being added to the filtrate.

Method 3

As for method 2 but omitting the HCl.

Method 4

The tissue was ground with a mortar and pestle three times in chloroform: methanol (2:1 V:V) and the extract filtered through Whatman No. 1 filter paper, the filtrate was retained. The process was repeated twice and all filtrates were combined.

Method 5

As for method 4 except that 0.1ml concentrated HCl added each time.

Method 6

The tissue was homogenised three times in a "Sorval omnimixer" in 15ml chloroform: methanol (2:1, V:V). After each homogenisation the extract was filtered, the filtrate retained and the residue returned to the omnimixer.

The samples used for testing these methods were 0.5g fresh weight (0.052 ± 0.012 g dry weight) pea leaf samples which were boiled in 15ml isopropanol prior to extraction. (The isopropanol was kept and added to the final extract since a small amount of lipid is extracted at this stage). The rationale behind these methods is as follows: treatment 1 is the mildest procedure, treatments 4 and 6 are of increasing severity, treatments 2 and 3 combine an extraction procedure with a "clean up" procedure as in Bligh and Dyer (1959). Treatments 2 and 5 include HCl to see if there is any gain in the amount of lipid extracted by breaking covalent bonds between fatty acids and polysaccharides.

Initially each treatment was kept separately and analysed individually to ensure that all possible lipid had been extracted. The extracts were analysed for their total lipid content by the method of Amenta (1964), using as standard an equimolar mixture of cholesterol, tripalmitin, dipalmitin, cholesterol oleate, phosphatidyl choline dipalmitoyl and

phosphatidyl ethanolamine.

Table 15 shows the total lipid extracted for each method and also the split fractions. Methods 4, 5 and 6 extract the largest amounts of total lipid. The lack of significant differences between these methods shows that mortar and pestle and mechanical homogenizer are broadly equivalent in their abilities to disrupt the tissues. This is important as when epidermal strips are used in the analysis the amount of tissue is too small for effective use of the Sorval omnimixer.

The addition of HCl (methods 2 and 5) into the extraction media results in no increase in total lipid extracted, showing that there is no significant amount of lipid covalently bound to other polymers.

Table 15: Amount of lipid extracted using different methods

For method details see test.

Method Number	Amount of Lipid present after extraction no. (g)				Total Lipid (g)
	1	2	3	4	
Method 1	424.5 $\pm$ 26.8	410.2 $\pm$ 78.9	232.7 $\pm$ 46.4	231.6 $\pm$ 54.0	1299.7 $\pm$ 179.2
Method 2	1060.7 $\pm$ 262.7	465.2 $\pm$ 66.6	246.8 $\pm$ 46.0	24.0 $\pm$ 40.9	1840.7 $\pm$ 187.2
Method 3	1038.0 $\pm$ 238.8	491.2 $\pm$ 55.5	327.0 $\pm$ 48.4	19.0 $\pm$ 31.4	1875.2 $\pm$ 137.4
Method 4	1514.5 $\pm$ 95.6	626.2 $\pm$ 81.7	291.0 $\pm$ 60.7	18.5 $\pm$ 21.2	2450.2 $\pm$ 254.7
Method 5	1684.0 $\pm$ 82.8	608.7 $\pm$ 42.2	206.6 $\pm$ 48.7	2.7 $\pm$ 7.6	2502.2 $\pm$ 193.0
Method 6	1519.7 $\pm$ 80.6	586.5 $\pm$ 45.9	300.0 $\pm$ 50.6	2.5 $\pm$ 2.9	2408.7 $\pm$ 268.0

The figures are the means of four replicates and are quoted with 95% confidence limits

The modified Bligh and Dyer methods 2 and 3 had lower yields than 4, 5 and 6 partially because 5.3 $\pm$ 0.8% of the lipid was lost in the upper phase. This was reduced to 0.9 $\pm$ 0.2% by incorporation of 0.5M NaCl into the extraction media instead of water, but the yields were still not as high as methods 4, 5 and 6. It was assumed that these lower yields were the result of lipid lost as proteolipid complexes which were not broken down because at no stage in the process was the sample taken to dryness.

The extraction procedure adopted as standard throughout the experimental section is described in section 3.6. The method involves filtering organic solvents through paper, but analyses of the washed

papers revealed no loss of lipid by binding to the paper.

4.1.9. Recovery Rates during analysis

Because of the complexity of the procedures for extraction, separation and quantification of the lipids, it was felt necessary to check the efficiency of the process and calculate recovery factors. The possibility of sample deterioration was also examined and precautions to prevent oxidation were evaluated.

Treatment 1

A standard mixture as follows: total 1000 μ g consisting of 200 μ g each of PC, PE, PI, tripalmitin and cholesterol oleate with a fatty acid composition of; 43.1% palmitic acid, 2% palmitoleic acid, 4% stearic acid, 23.9% oleic acid, 11.8% linoleic acid and 15.2% linolenic acid, was dissolved in 45ml of chloroform: methanol (2:1, V:V). This solution was then subjected to the various analytical procedures described in section 3. All the precautions to prevent oxidation were observed i.e. storage under nitrogen and the use of 0.005% BHT in all solvents. The samples separated by tlc were analysed both by GLC and the method of Marsh and Weinstein.

Treatment 2

As for treatment 1 but omitting anti-oxidation precautions i.e. no BHT in the solvents and no storage under nitrogen.

Treatment 3

As for treatment 1 but with no BHT in the solvent mixture.

Treatment 4

As for treatment 1 but with no storage under nitrogen.

The results (Table 16) show that there are no treatment differences as regards total lipid recovery or the amounts in individual lipid classes.

However, when the fatty acids were analysed, treatment 2 and 3 were found to give significantly lower recoveries of total fatty acids than treatments 1 and 4. When the fatty acids were studied individually it was found that this reduction was caused by a small decrease in the amount of oleic acid and a large decrease in the amounts of linoleic and linolenic acid.

The main conclusions to be drawn from this experiment are that recovery factors of 80% can be consistently achieved and that this is not biased towards any one lipid class or group of fatty acids, provided that appropriate precautions are taken to prevent oxidation of unsaturated fatty acids. As a preventative for the latter, the use of a chemical antioxidant in solvents was clearly preferable to reliance upon a nitrogen atmosphere. There were considerable differences in the use of the latter during certain stages of sample handling. Once the efficiency of BHT was established the use of nitrogen was reserved mainly for sample storage.

Table 16: Effectiveness of precautions to prevent oxidation of selected lipids — For treatment details see text

a) The effect on lipid composition

Lipid	Original Amounts (μg)	Amount after treatment number			
		1 (μg)	2 (μg)	3 (μg)	4 (μg)
Phosphatidylcholine	200	161 $\pm$ 7.9	158 $\pm$ 11.9	163 $\pm$ 10.4	167 $\pm$ 8
Phosphatidylethanolamine	200	159 $\pm$ 12.3	157 $\pm$ 14.4	164 $\pm$ 14.1	160 $\pm$ 7
Phosphatidylinositol	200	161 $\pm$ 14.1	161 $\pm$ 8.7	153 $\pm$ 11.0	158 $\pm$ 14
Tripalmitin	200	154 $\pm$ 8.1	159 $\pm$ 12.0	165 $\pm$ 8.9	159 $\pm$ 10
Cholesterol oleate	200	163 $\pm$ 6.1	160 $\pm$ 10.4	157 $\pm$ 12.4	156 $\pm$ 8
Total Lipid	1000	798 $\pm$ 15.6	795 $\pm$ 16.9	802 $\pm$ 13.4	

There are no significant differences in lipid class between the treatments

b) The effect on fatty acid composition

Fatty acids	Original amounts (μg)	% Original amount after treatment number			
		1	2	3	4
Palmitic acid	336.3	78.9 $\pm$ 2.6	75.9 $\pm$ 1.8	76.7 $\pm$ 1.9	77.9 $\pm$ 2.9
Palmitoleic acid	15.5	81.4 $\pm$ 3.2	81.4 $\pm$ 2.0	82.7 $\pm$ 3.9	85.9 $\pm$ 3.4
Stearic acid	31.2	77.6 $\pm$ 1.6	82.0 $\pm$ 2.1	79.5 $\pm$ 1.2	73.1 $\pm$ 5.4
Oleic acid	187.2	80.0 $\pm$ 3.7	72.3 $\pm$ 2.7	70.6 $\pm$ 3.4	81.0 $\pm$ 2.0
Linoleic acid	93.6	77.7 $\pm$ 1.9	53.3 $\pm$ 4.3	53.1 $\pm$ 2.2	72.5 $\pm$ 6.1
Linolenic acid	117.0	81.4 $\pm$ 4.4	47.5 $\pm$ 5.6	46.7 $\pm$ 1.7	77.6 $\pm$ 1.6
Total	780.9	79.4 $\pm$ 2.2	68.4 $\pm$ 1.7	68.1 $\pm$ 1.0	77.8 $\pm$ 3.4

The results are the means of 4 replicates and are quoted with 95% confidence limits

4.2. Lipids in infected and uninfected pea leaves

For this series of experiments four different susceptible pea cultivars were used Onward, Feltham First, Hurst Green Shaft and Pilot. The plants were grown and infected as described in Section 3.1. The extent of infection on the leaves was expressed in two ways; the time after initial infection and the number of haustoria per cell. Figure 42 shows that there were no significant differences between the cultivars in the number of haustoria per cell during the time course of this investigation and that the time after initial infection was thus a valid indicator of the degree of infection.

4.2.1. Total Lipids

The mean total lipid values for each cultivar were as follows:- Onward $50.4 \mu\text{g lipid g}^{-1}$ dry weight, Feltham first $51.2 \pm 5.1 \mu\text{g lipid g}^{-1}$ dry weight, Hurst Green Shaft $51.2 \pm 5.1 \mu\text{g lipid g}^{-1}$ dry weight and Pilot $54.7 \pm 4.5 \mu\text{g lipid g}^{-1}$ dry weight. There was thus no significant difference ($p = 0.05$) between the cultivars with respect to total lipids. Figure 20 shows the changes in total lipids during the development of infection over a 24 day period. This shows that there were no significant changes ($p = 0.05$) in the total lipid levels in any of the cultivars.

4.2.2. Polar Lipids

The mean polar lipid amounts in each cultivar at the beginning of the experiment were as follows ($\mu\text{g g}^{-1}$ dry weight): Onward 45.3 ± 5.3 , Feltham First 41.5 ± 3.1 , Hurst Green Shaft 43.2 ± 1.7 and Pilot 45.2 ± 4.3 , there was thus no significant difference ($p = 0.05$) between the cultivars polar lipid values. Figure 21 shows that there were no significant changes ($p = 0.05$) in the polar lipid contents in any of the cultivars over the 24 day period of disease development.

4.2.3. Neutral Lipids

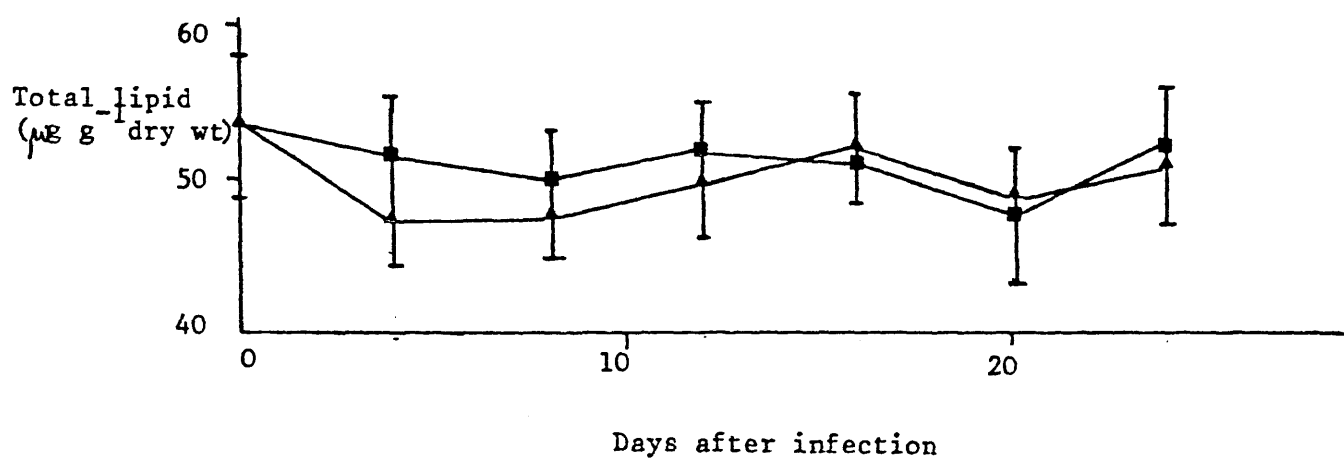
The mean neutral lipid amounts in each cultivar at the beginning of the experiment were as follows ($\mu\text{g g}^{-1}$ dry weight): Onward 8.4 ± 1.0 , Feltham First 9.7 ± 1.5 , Hurst Green Shaft 8.5 ± 2.2 and Pilot 8.8 ± 1.7 . There

was thus no significant difference ($p = 0.05$) between the neutral lipid values in each cultivar. Figure 22 shows that there were no significant changes ($p = 0.05$) in the neutral lipid levels in any of the cultivars during the 24 day period of disease development.

Figure 20: The effect of infection with E. pisi on the total lipids in the leaves of 4 different pea cultivars

Figure 20

a) Onward



b) Feltham First

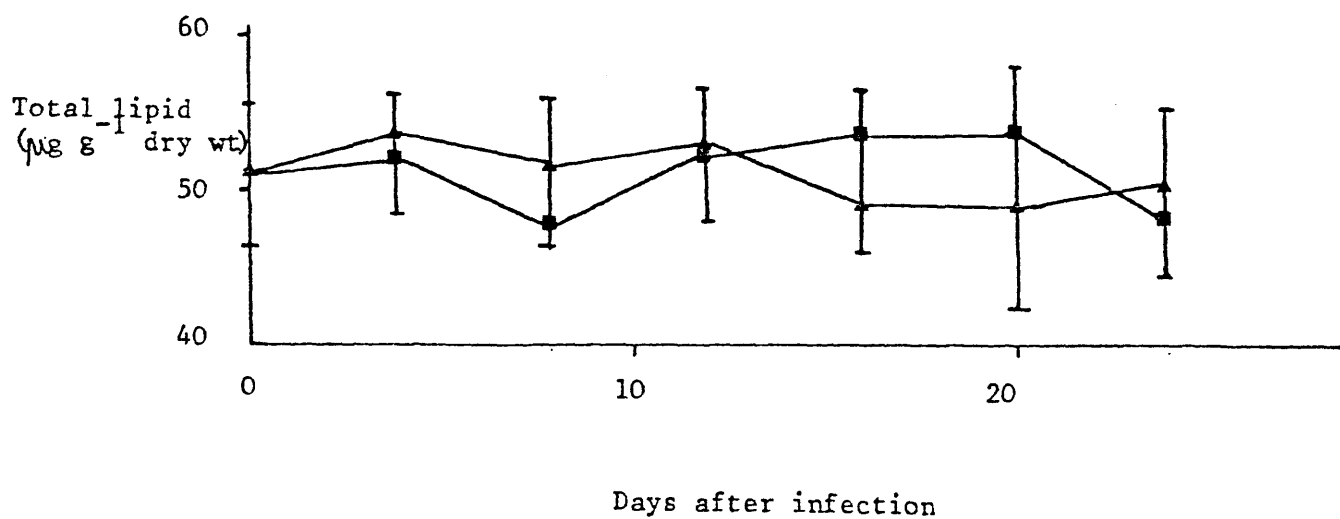
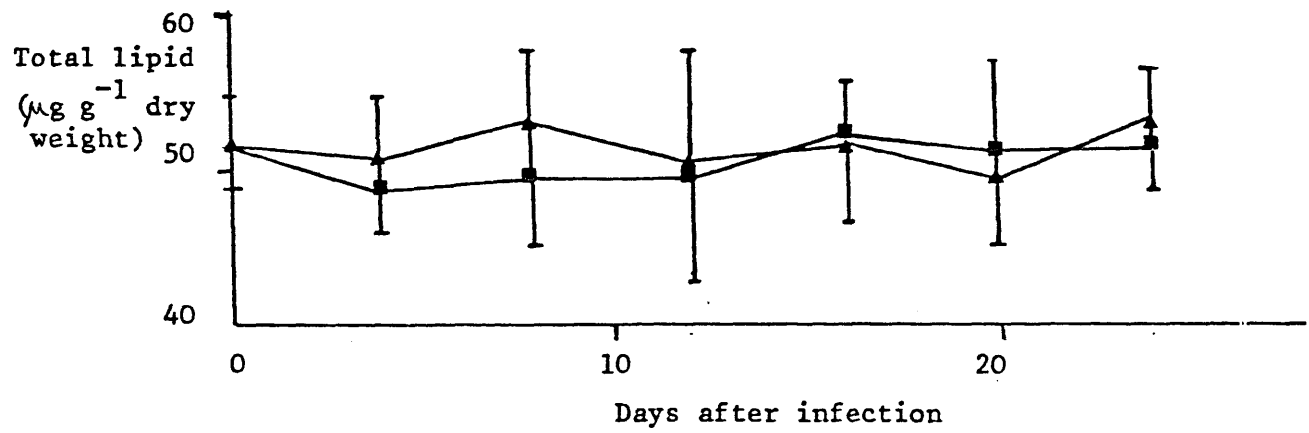
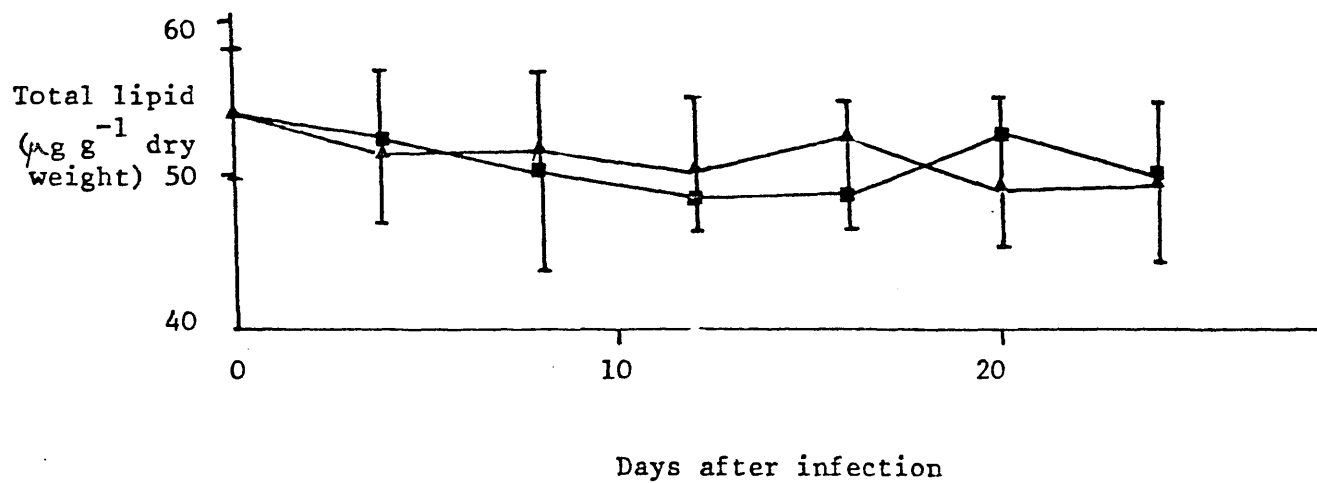


Figure 20 (cont)

c) Hurst Green Shaft



d) Pilot

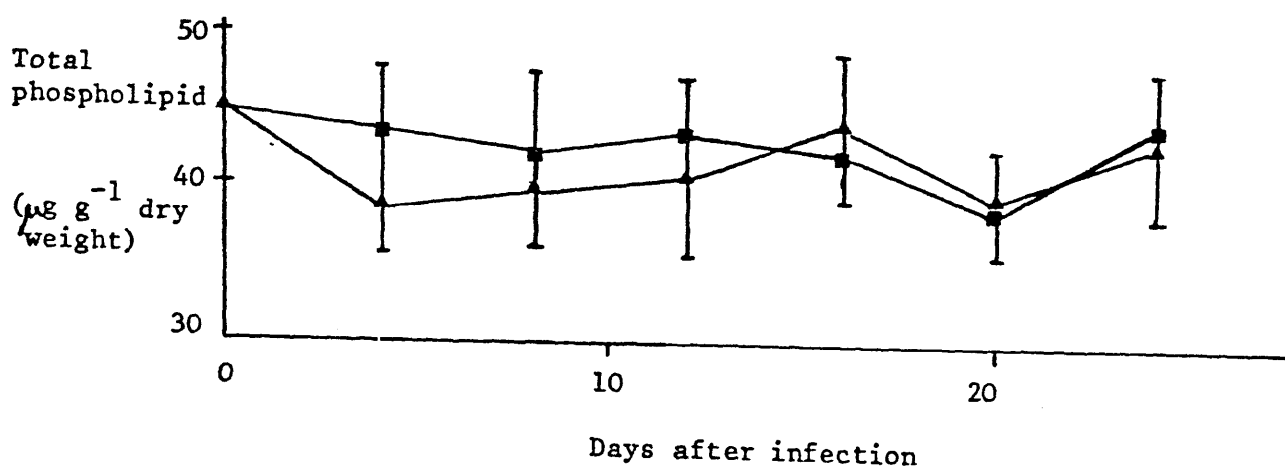


- ▲ Infected plants
- Uninfected plants
-] 95% confidence limits

Figure 21: The effect of infection with E. pisi on the total phospholipids in the leaves of 4 different pea cultivars

Figure 21

a) Onward



b) Feltham First

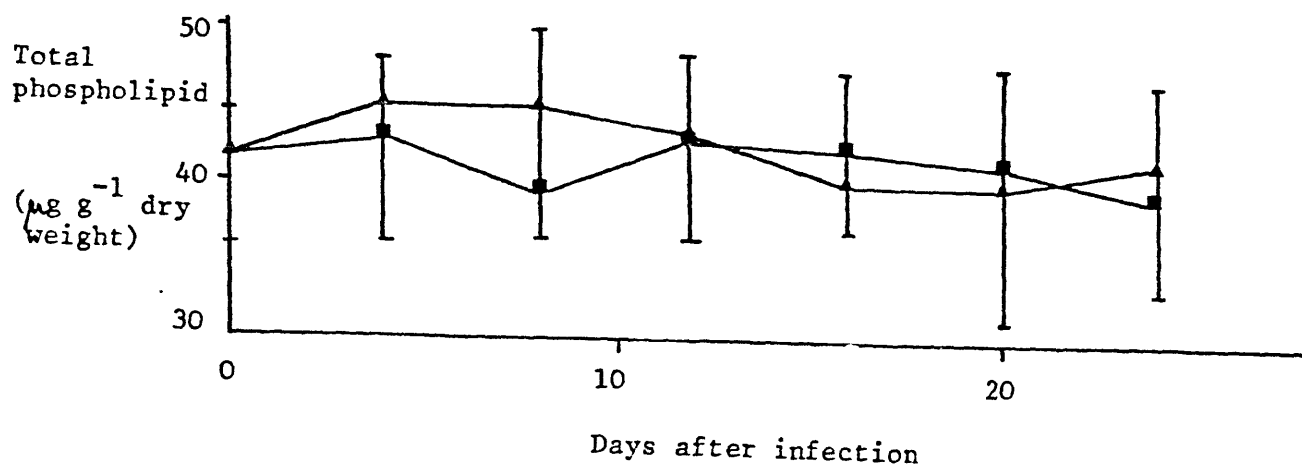
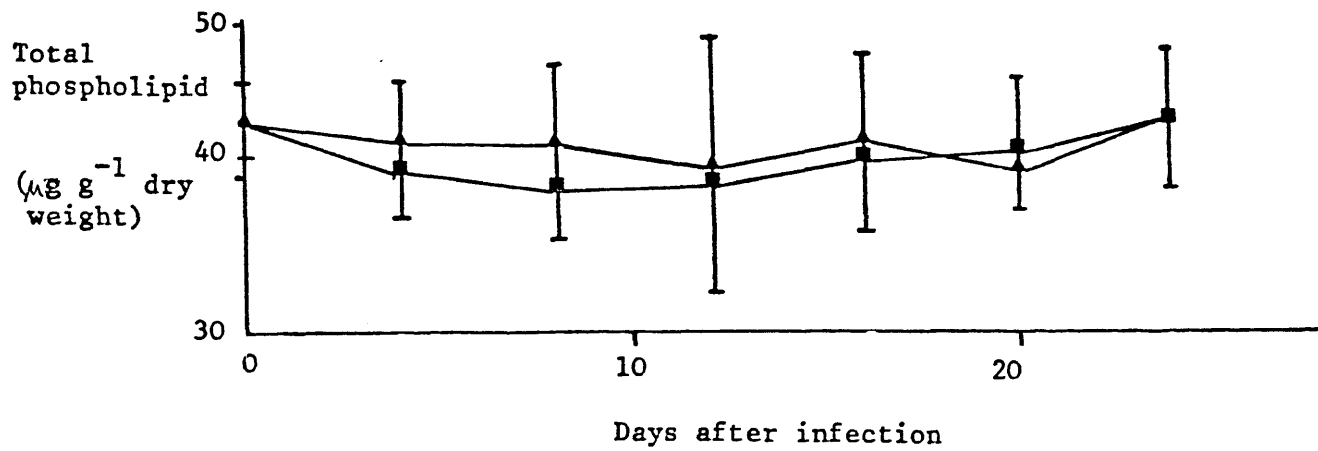
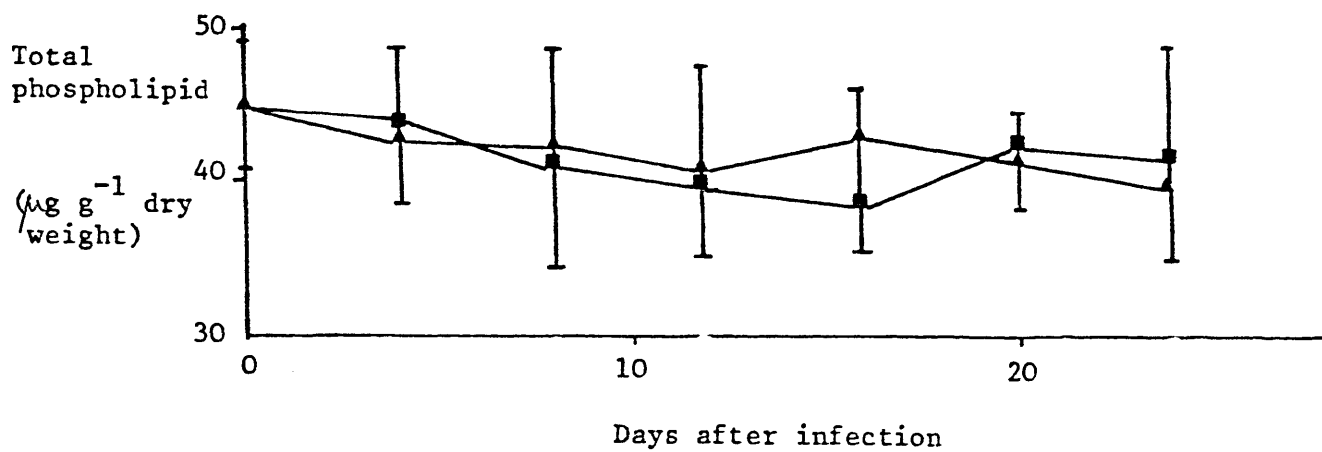


Figure 21 (cont)

c) Hurst Green Shaft



d) Pilot



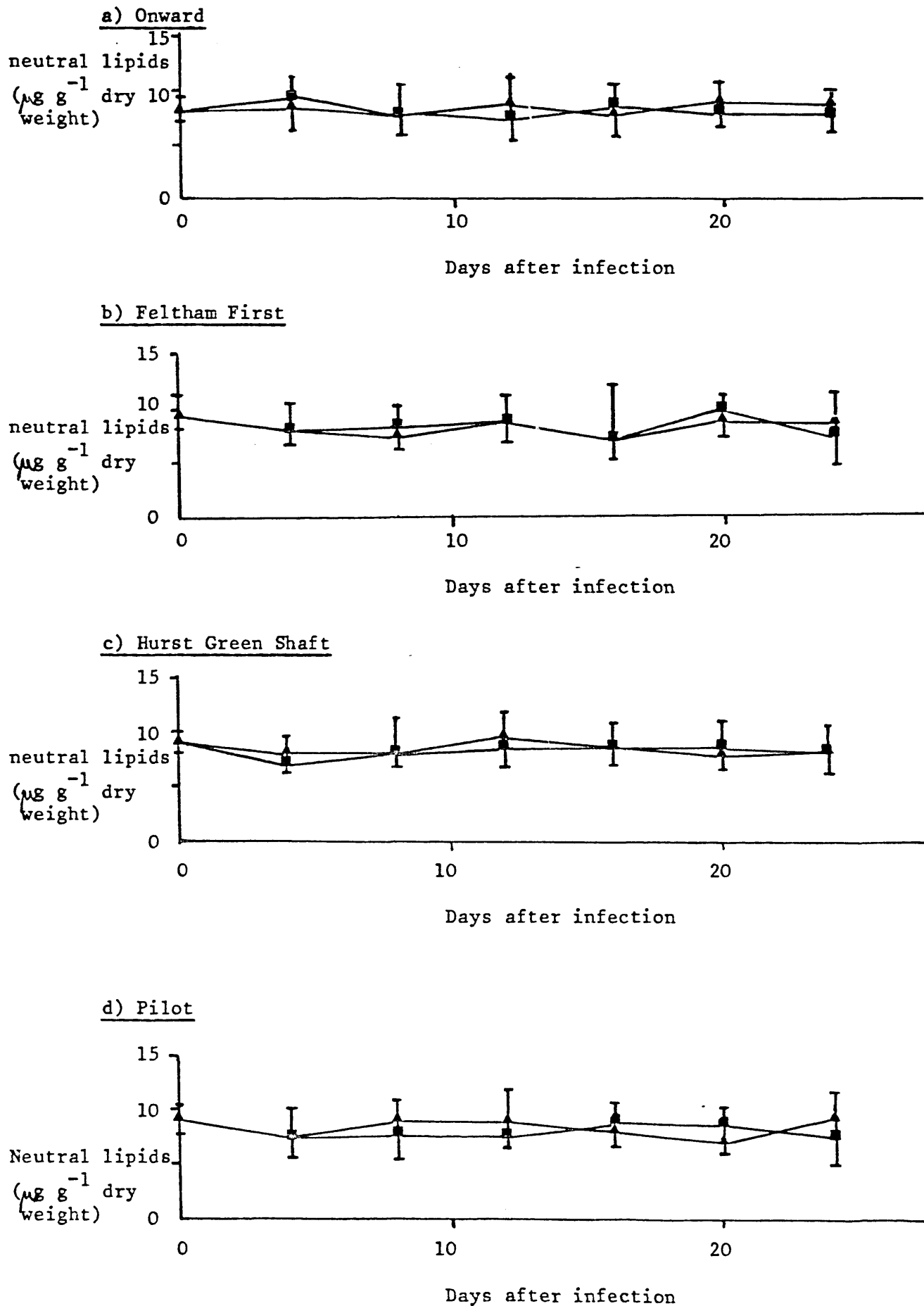
▲

■

I

Figure 22: The effect of infection with E. pisi on the total neutral lipids in leaves of 4 different pea cultivars

Figure 22



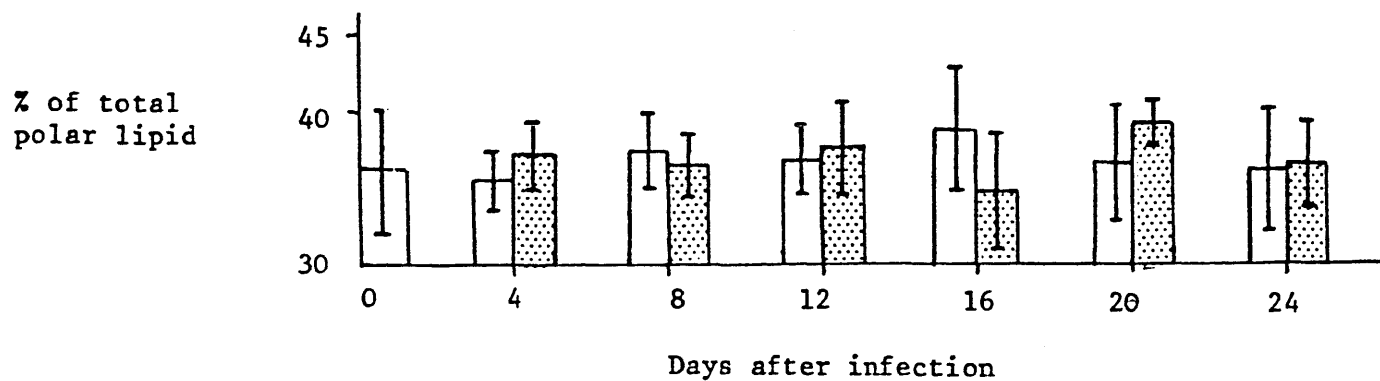
4.2.4. Individual Polar Lipids

The percentage composition of the individual polar lipids in uninfected leaves at the beginning of the experiment in the cultivar Onward was as follows: MGDG $36.2 \pm 3.2\%$, DGDG $25.4 \pm 2.9\%$, PC $16.2 \pm 1.8\%$, PG $9.7 \pm 0.4\%$, PE $8.1 \pm 1.3\%$, PI $2.5 \pm 0.6\%$, PS $1.1 \pm 0.5\%$ and PA $0.6 \pm 0.5\%$. Figures 23, 24, 25 and 26 show that there was no significant difference ($p = 0.05$) in the amount of each polar lipid as a percentage of the total polar lipid composition in the four cultivars tested at the beginning of the experiment nor were there significant changes ($p = 0.05$) in these values during the course of the 24 day infection period whether the plants were infected or not. (Figures 23, 24, 25, and 26).

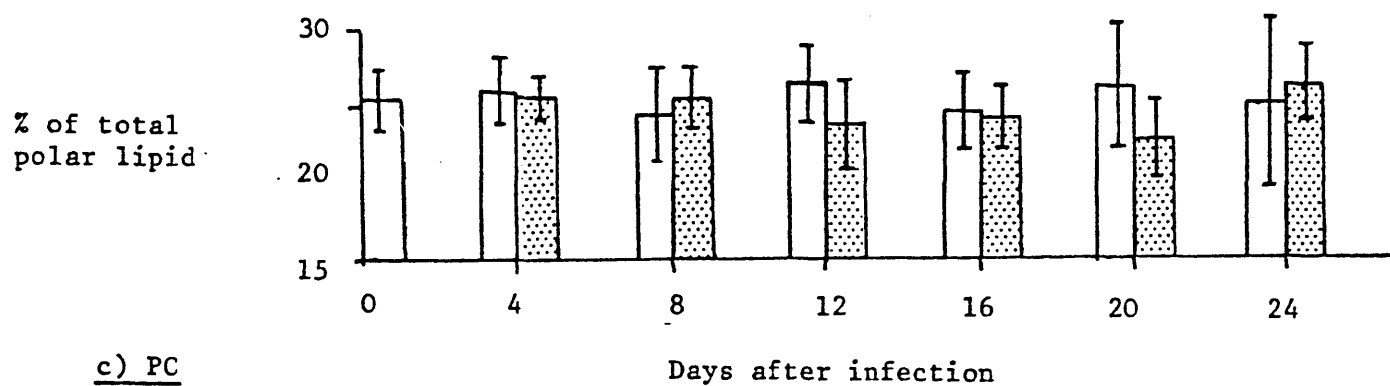
Figure 23: The effect of infection with E. pisi on the individual polar lipid composition in the leaves of the pea cultivar Onward

Figure 23

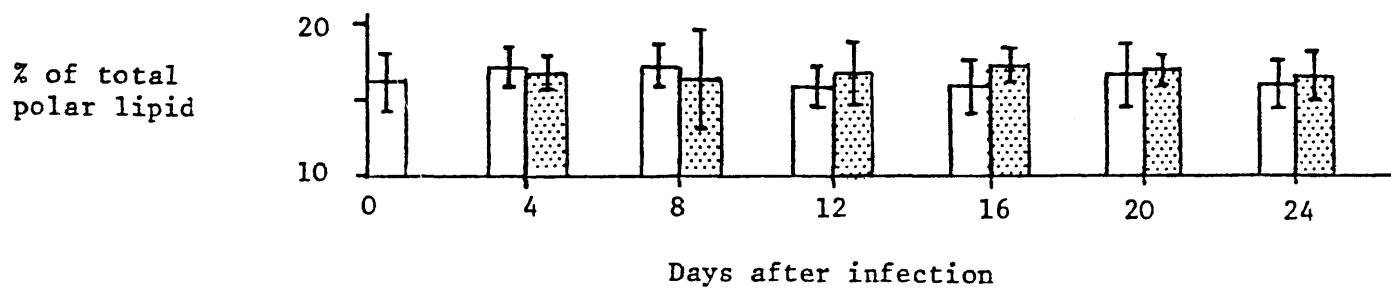
a) MGDG



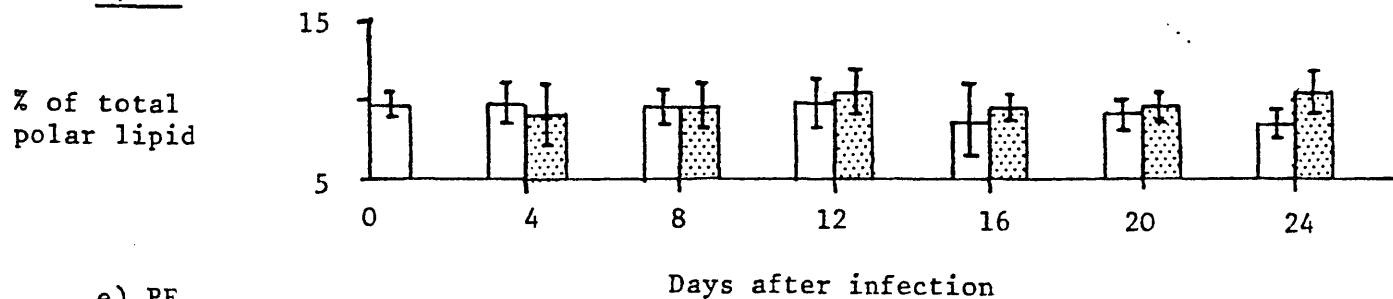
b) DGDG



c) PC



d) PG



e) PE

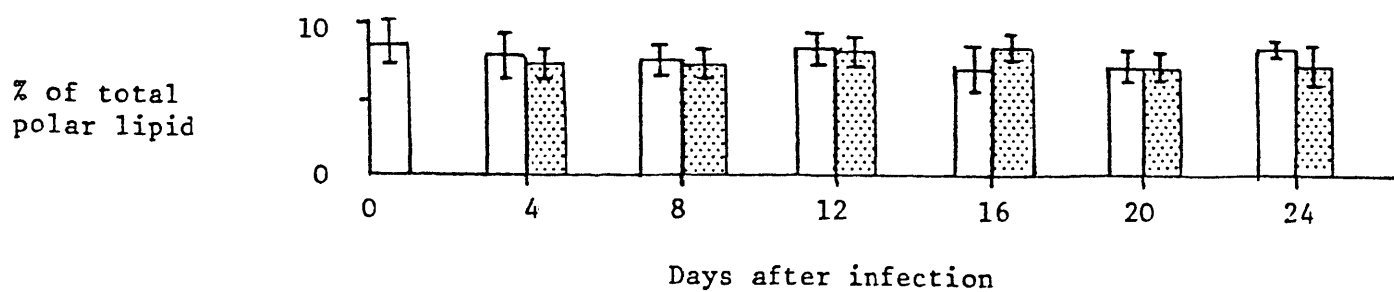
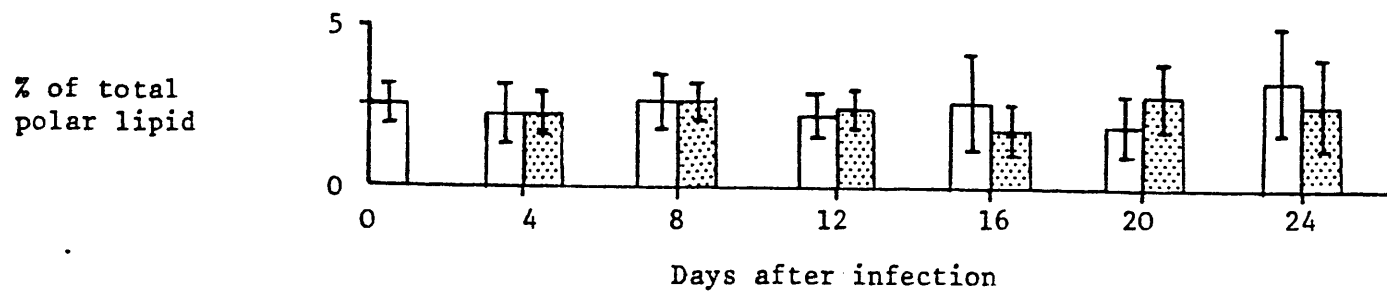
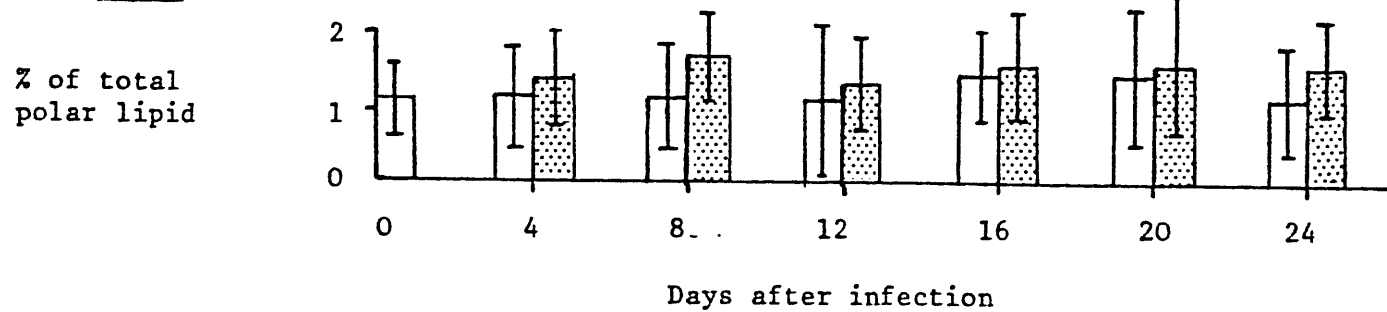


Figure 23 (cont.)

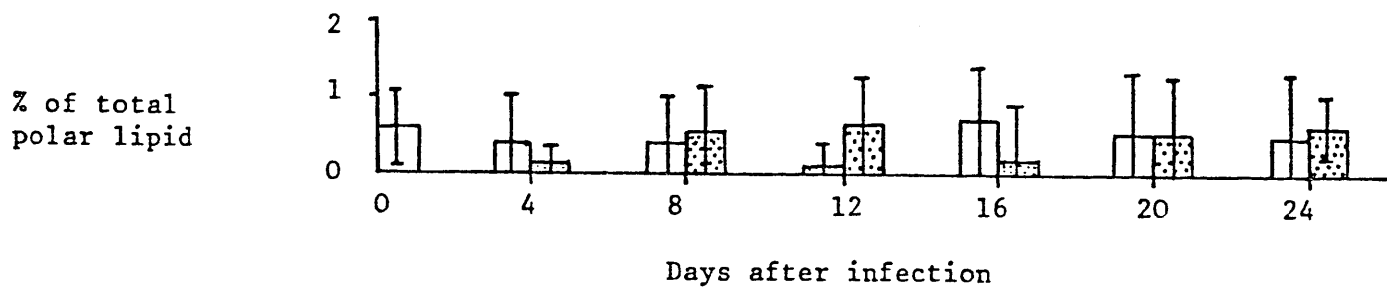
f) PI



g) PS



h) PA



Unshaded Uninfected Plants

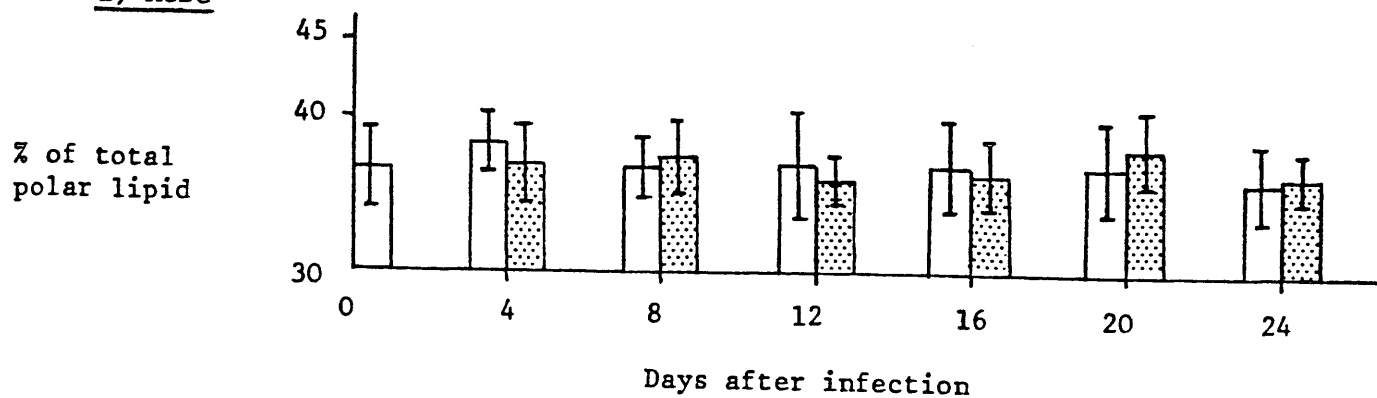
Shaded Infected Plants

I 95% Confidence Limits

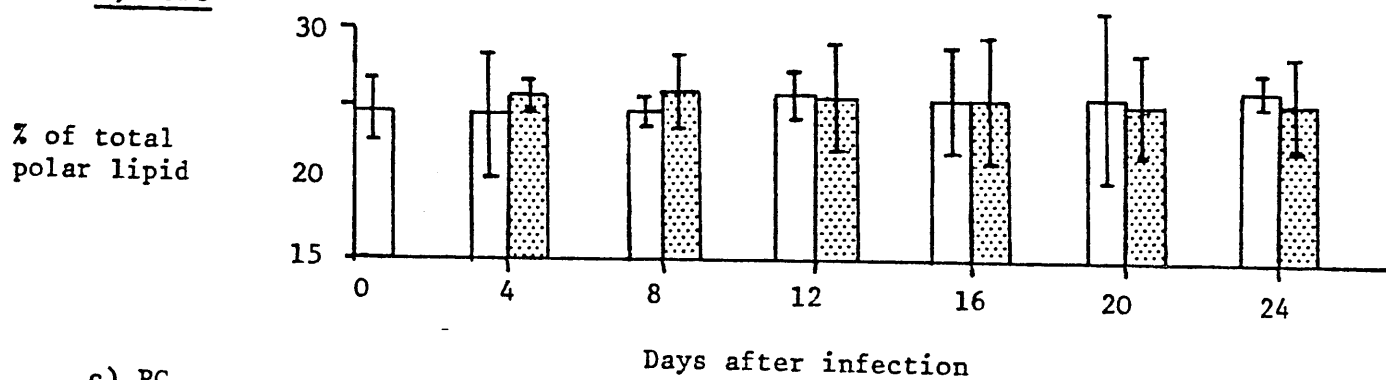
Figure 24: The effect of infection with E. pisi on the individual polar lipid composition in the leaves of the pea cultivar, Feltham First

Figure 24

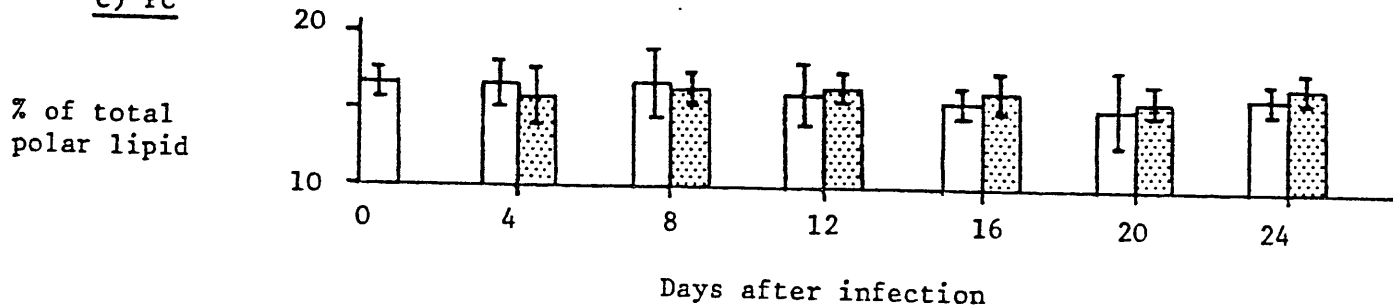
a) MGDG



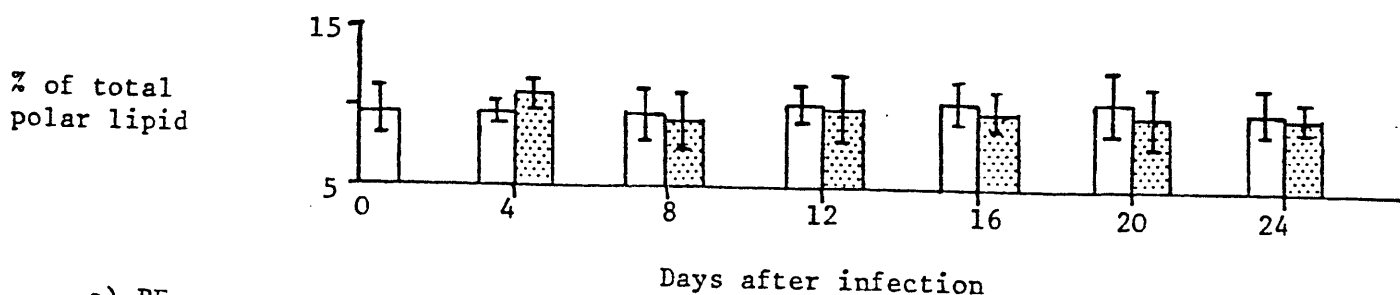
b) DGDG



c) PC



d) PG



e) PE

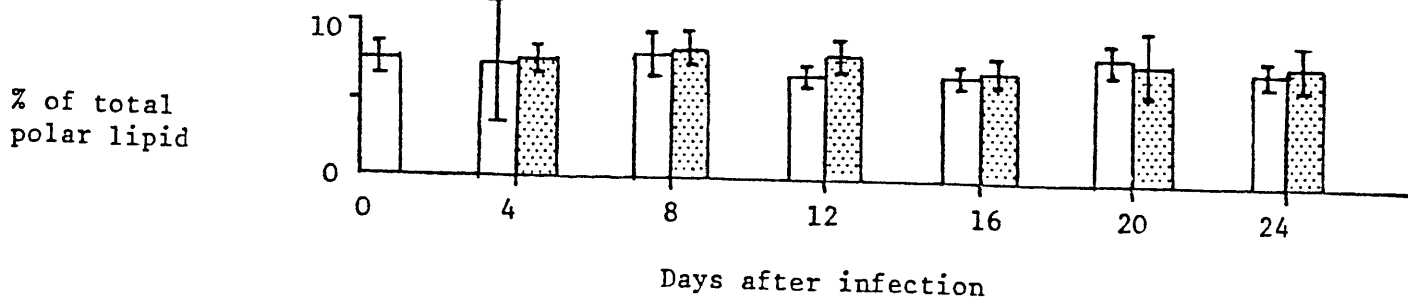
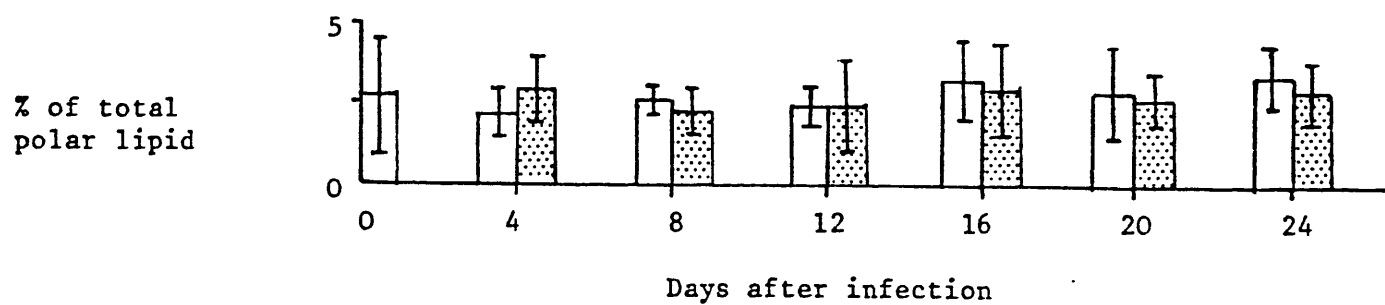
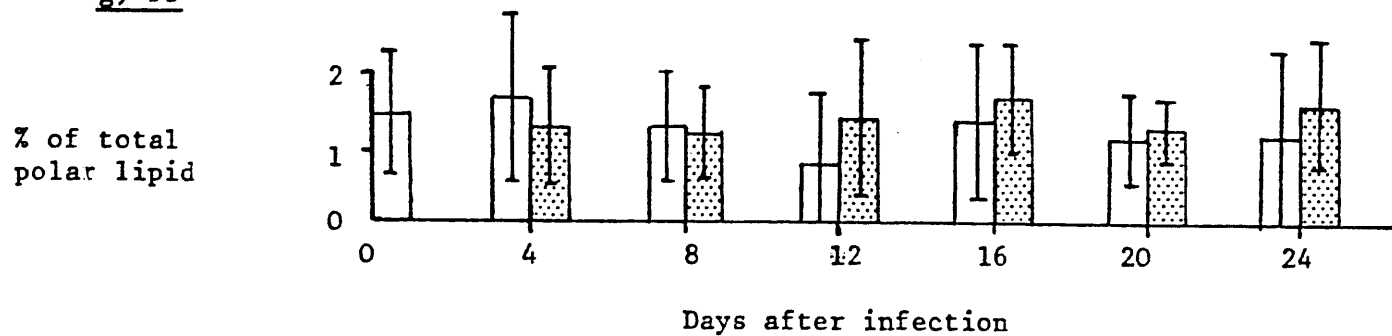


Figure 24 (cont.)

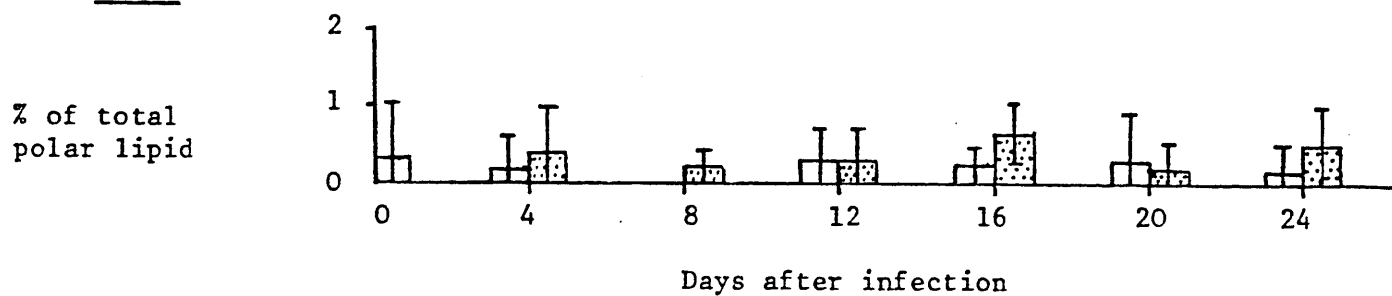
f) PI



g) PS



h) PA



Unshaded Uninfected Plants

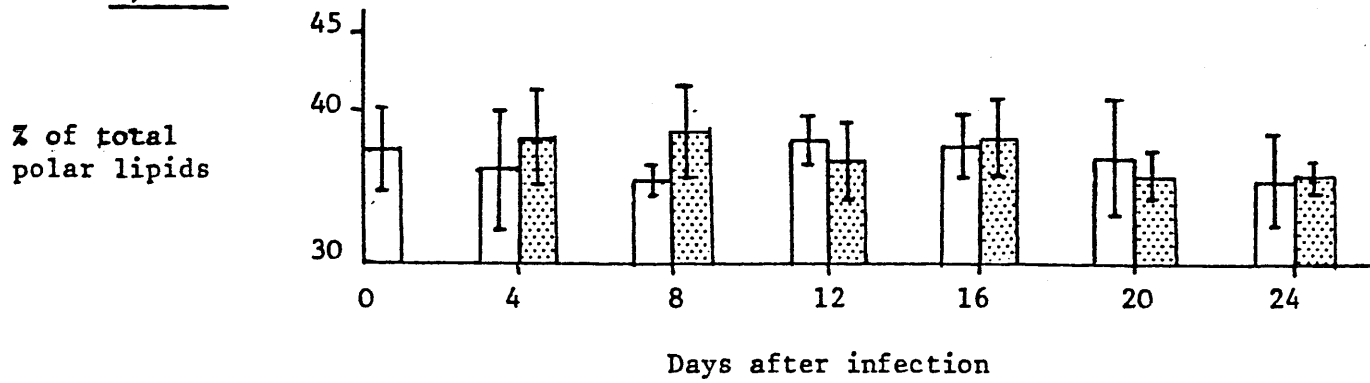
Shaded Infected Plants

I 95% Confidence Limits

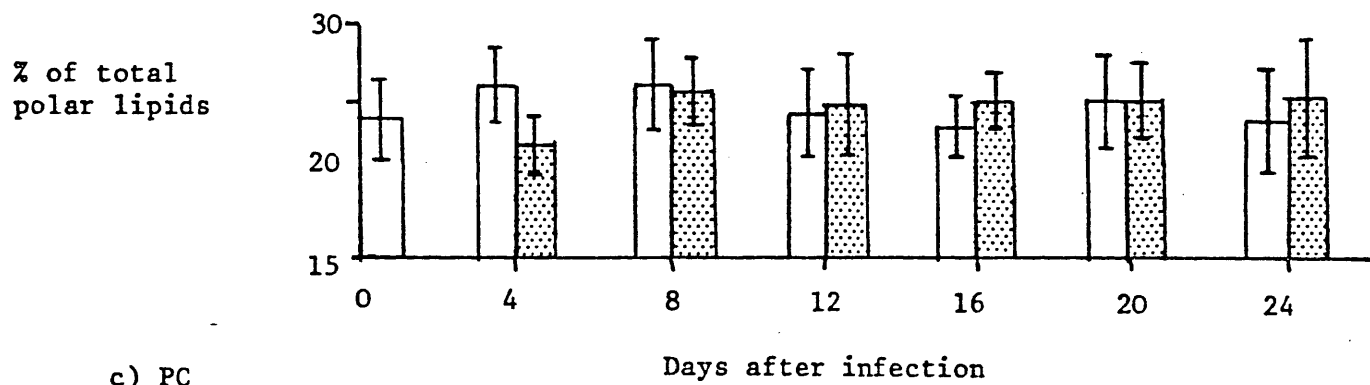
Figure 25: The effect of infection with E. pisi on the individual polar lipid composition in the leaves of the pea cultivar Hurst Green Shaft

Figure 25

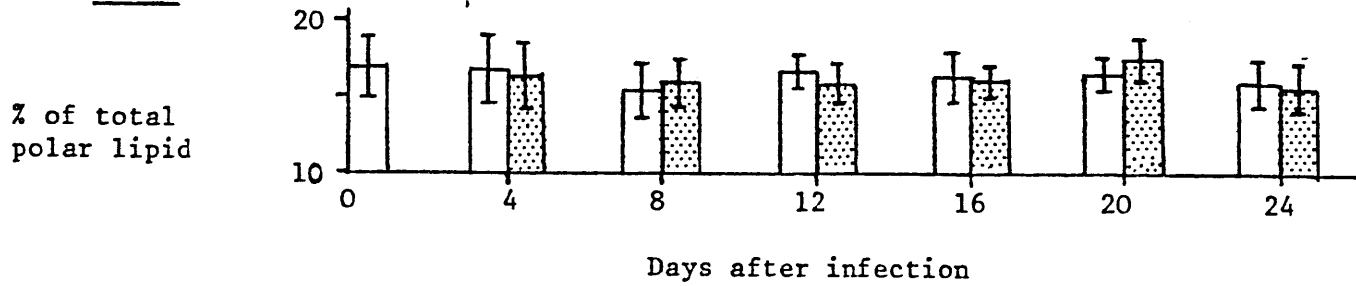
a) MGDG



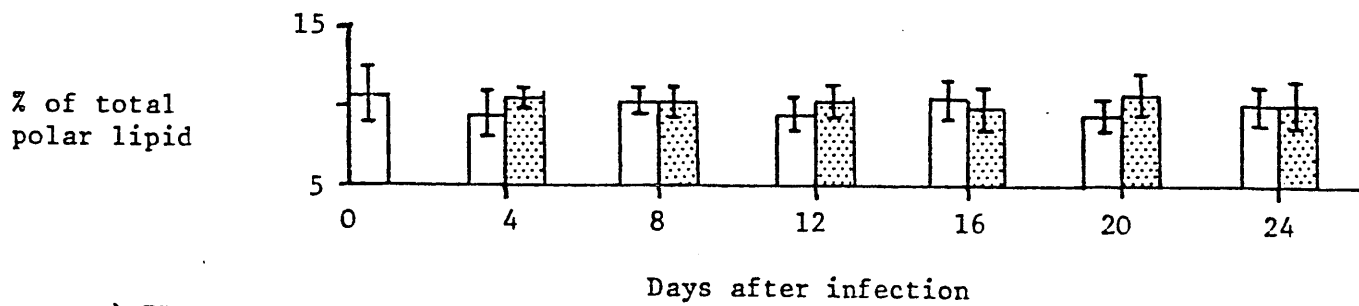
b) DGDG



c) PC



d) PG



e) PE

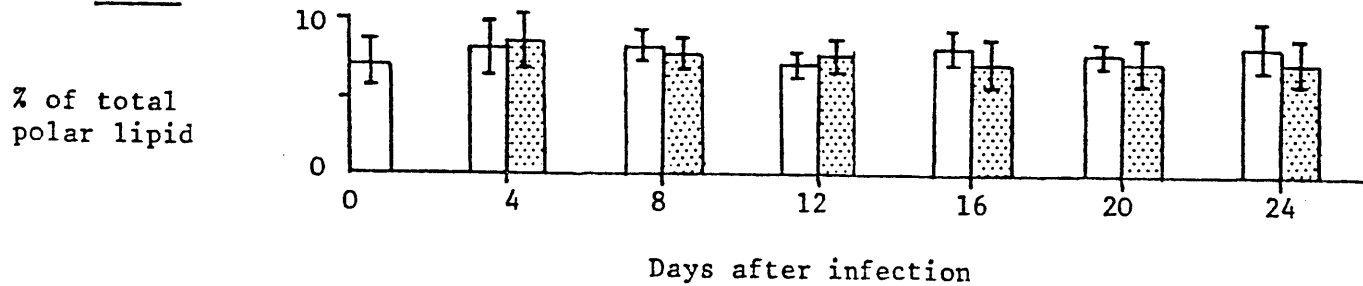
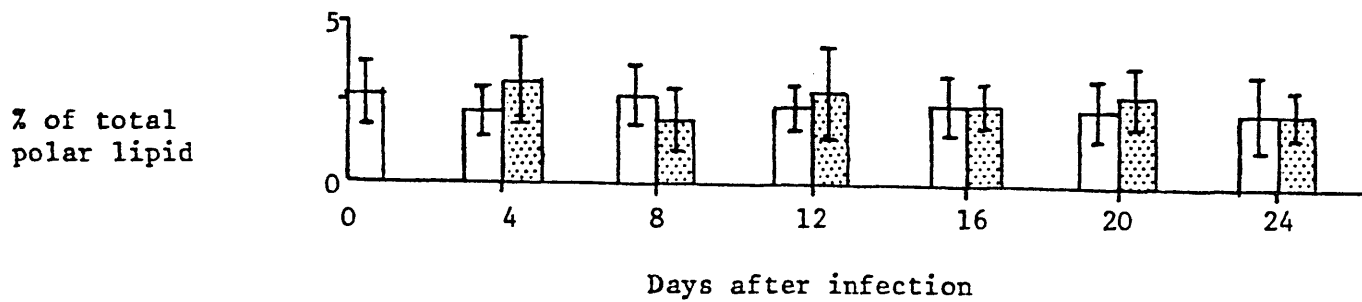
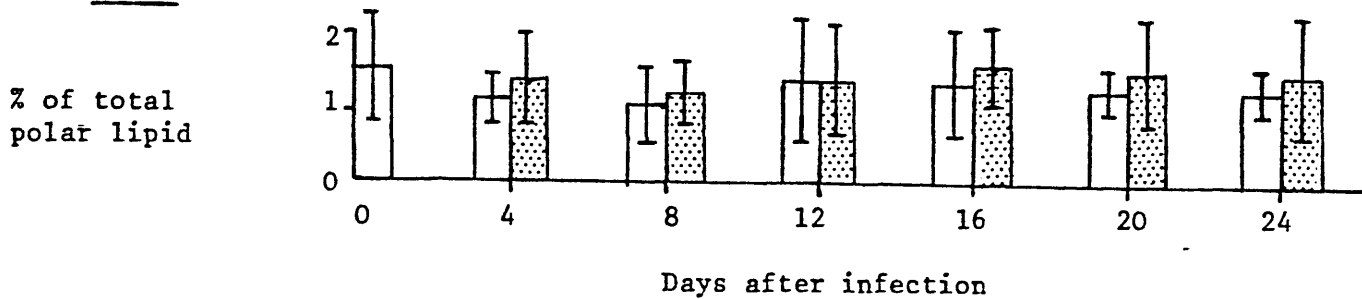


Figure 25 (cont.)

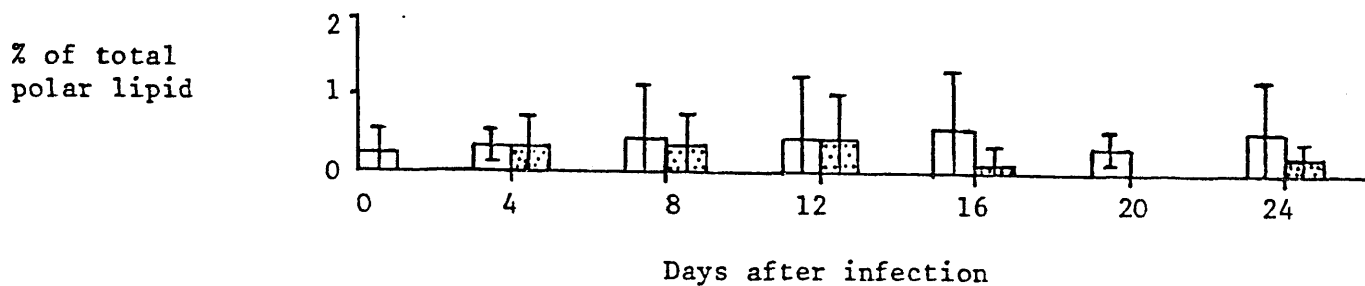
f) PI



g) PS



h) PA



Unshaded Uninfected Plants

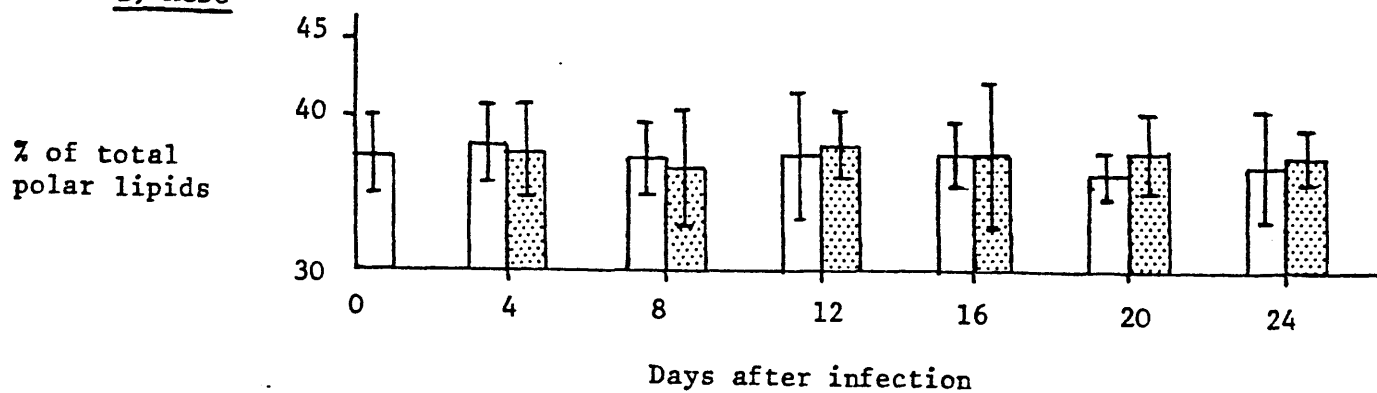
Shaded Infected Plants

I 95% Confidence Limits

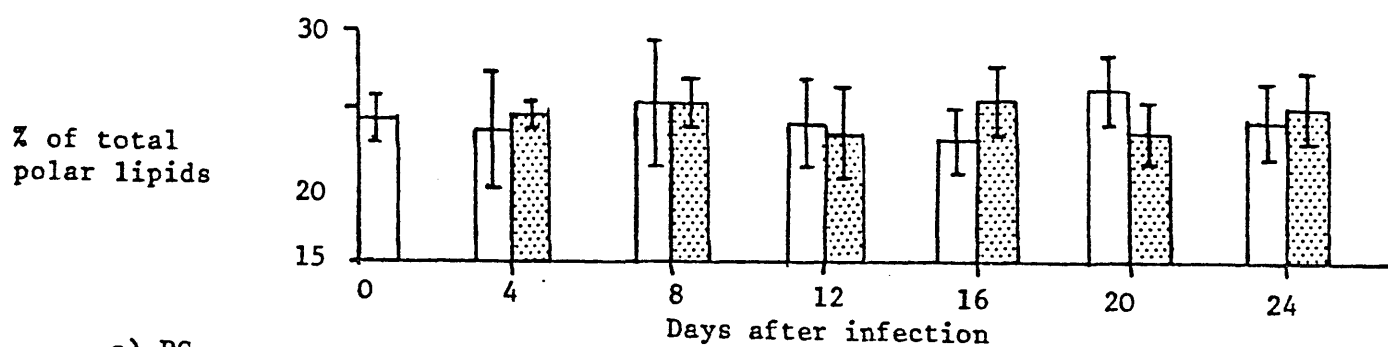
Figure 26: The effect of infection with E. pisi on the individual polar lipid composition in the leaves of the pea cultivar Pilot

Figure 26

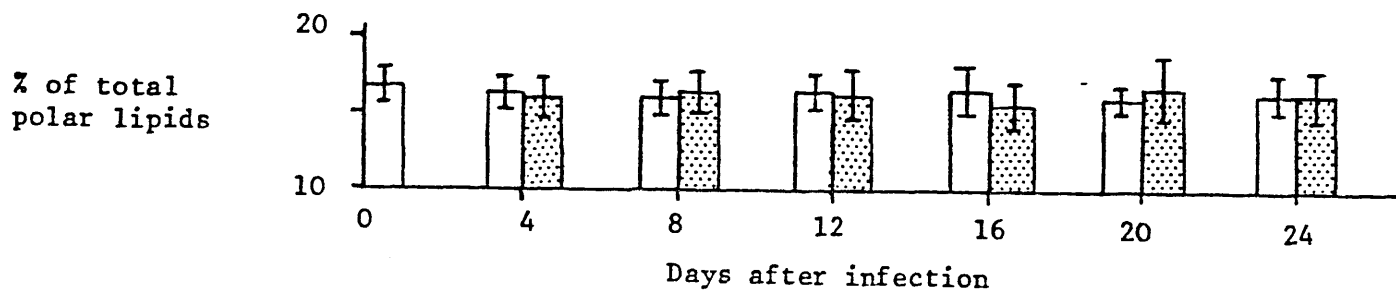
a) MGDG



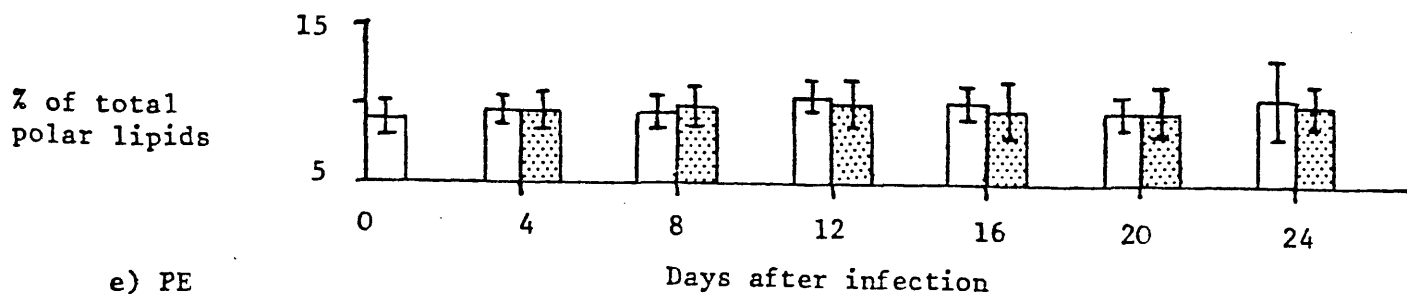
b) DGDG



c) PC



d) PG



e) PE

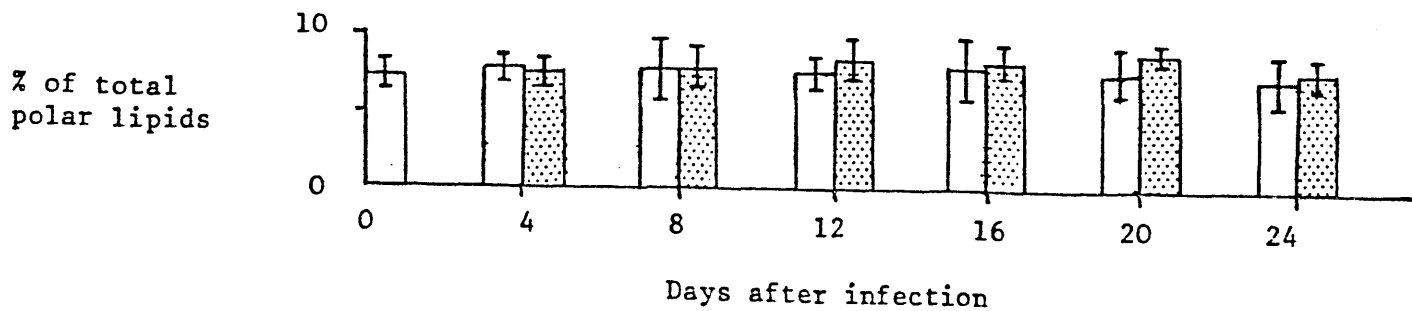
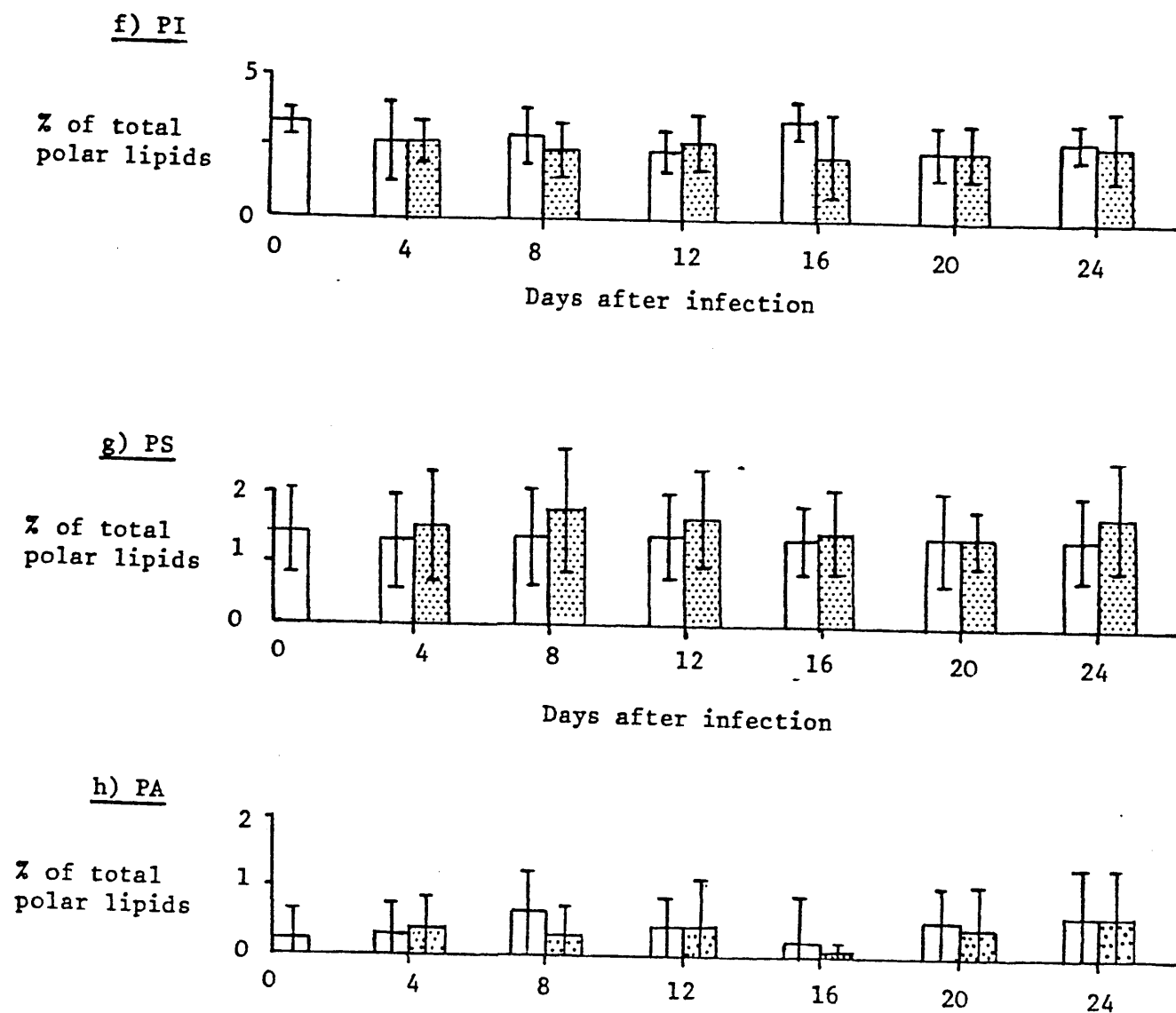


Figure 26 (cont.)



Unshaded Uninfected Plants

Shaded Infected Plants

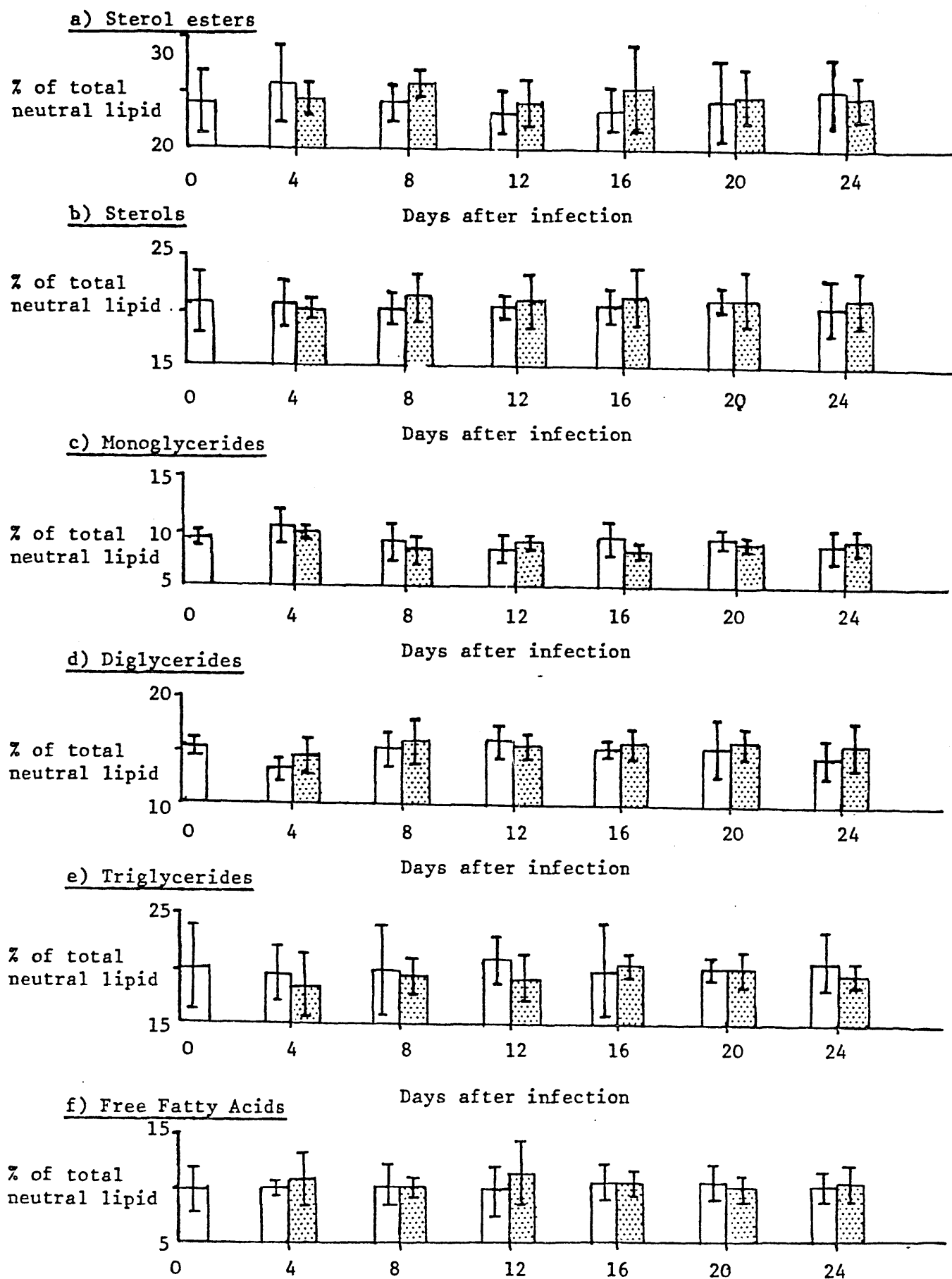
I 95% Confidence Limits

4.2.5. Individual neutral lipids

The percentage composition of the individual neutral lipids in uninfected leaves at the beginning of the experiment in the cultivar Onward was as follows: Sterol esters $24.5 \pm 3.7\%$, Sterols $20.6 \pm 2.6\%$, monoglycerides $9.3 \pm 0.9\%$, diglycerides $15.7 \pm 0.7\%$, triglycerides $20.0 \pm 3.7\%$, and free fatty acids $9/8 \pm 0.1\%$ (Figure 27). The results from the other cultivars (Figures 28, 29 and 30) show that there were no significant differences ($p = 0.05$) in the percentage composition of neutral lipids between cultivars, nor did these values change significantly ($p = 0.05$) during the course of a 24 day period of disease development (Figures 27, 28, 29 and 30).

Figure 27: The effect of infection with E. pisi on the individual neutral lipid composition in the leaves of the pea cultivar Onward

Figure 27

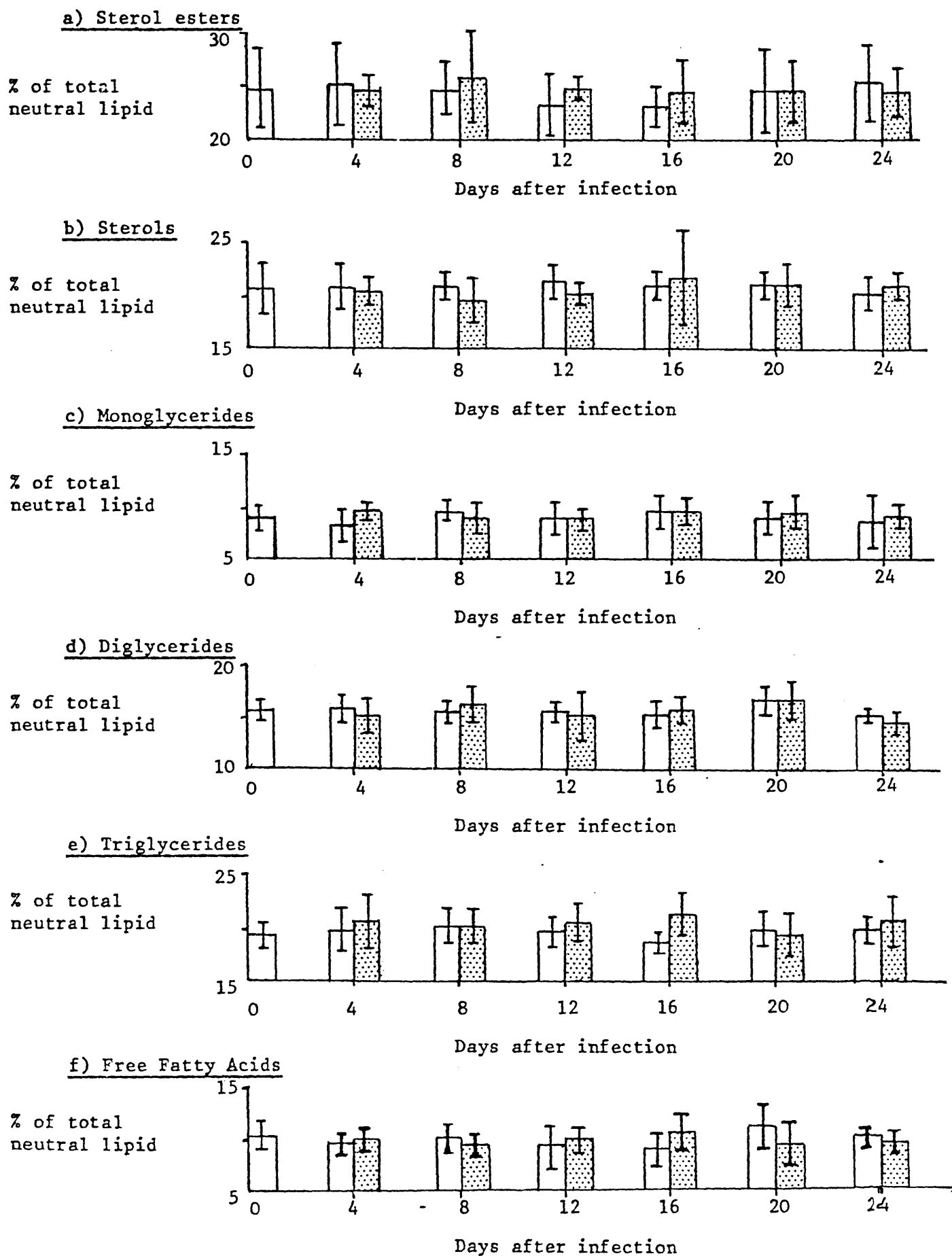


Unshaded - Uninfected Plants; Shaded - Infected Plants

I 95% Confidence Limits

Figure 28: The effect of infection with E. pisi on the individual neutral lipid composition in the leaves of the pea cultivar, Feltham First

Figure 28



I 95% Confidence Limits; Unshaded Uninfected Plants; Shaded Infected P1

Figure 29: The effect of infection with E. pisi on the individual neutral lipid composition in the leaves of the pea cultivar Hurst Green Shaft

Figure 29

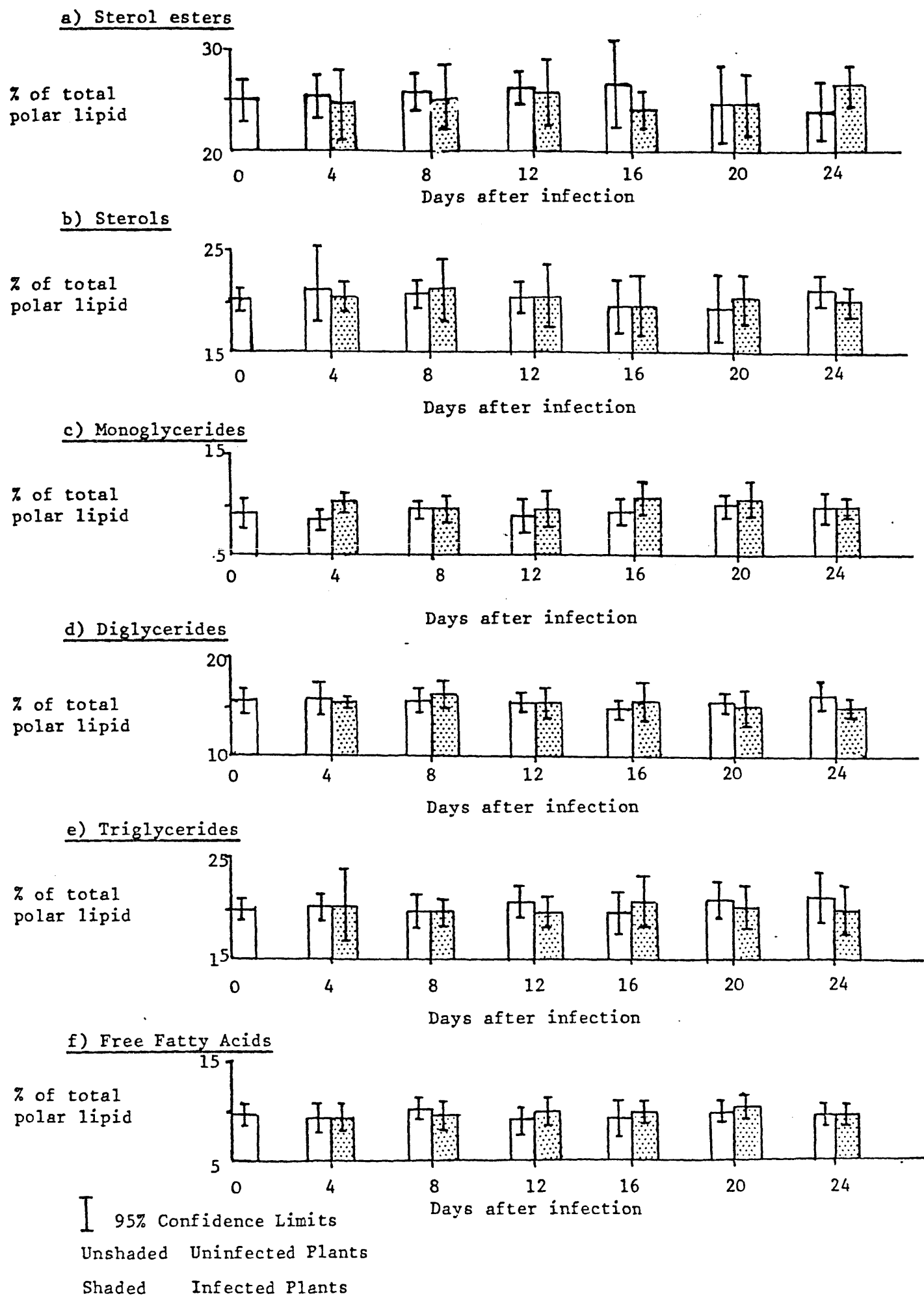
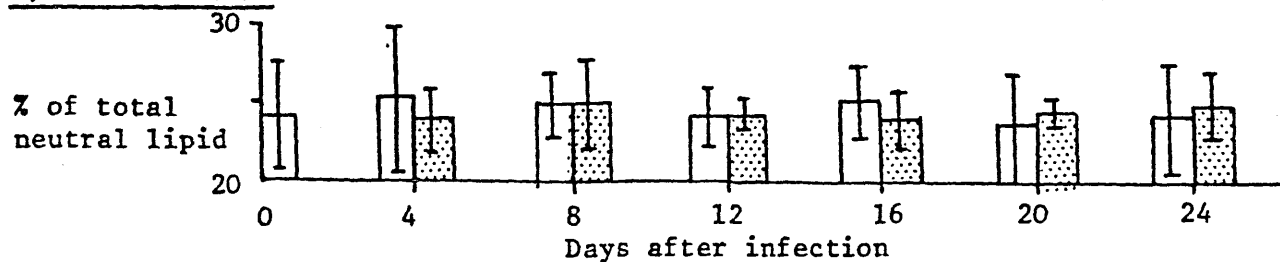


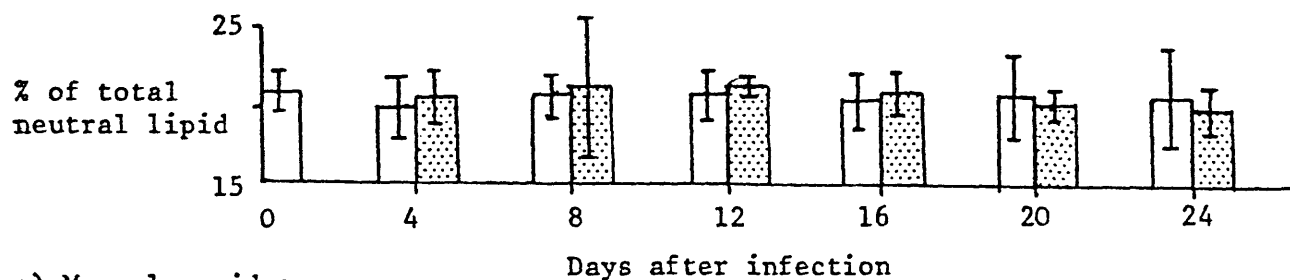
Figure 30: The effect of infection with E. pisi on the individual neutral lipid composition in the leaves of the pea cultivar, Pilot

Figure 30

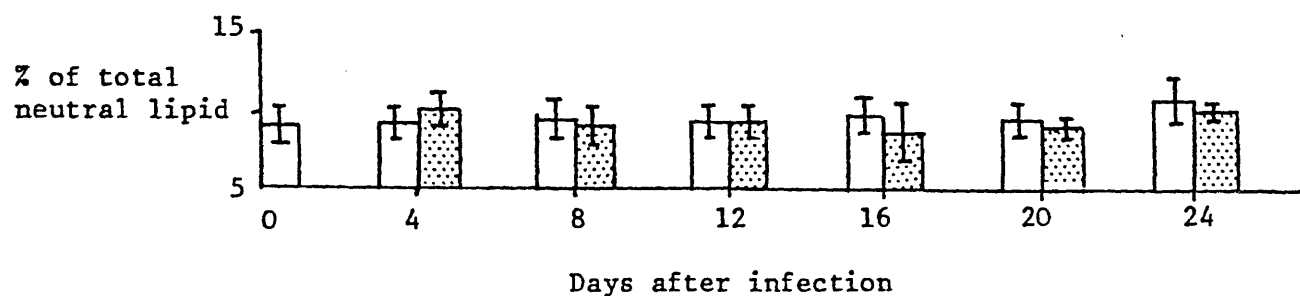
a) Sterol esters



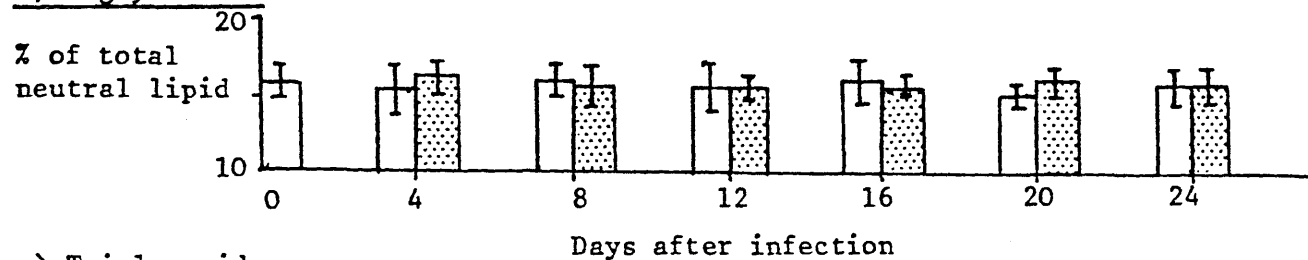
b) Sterols



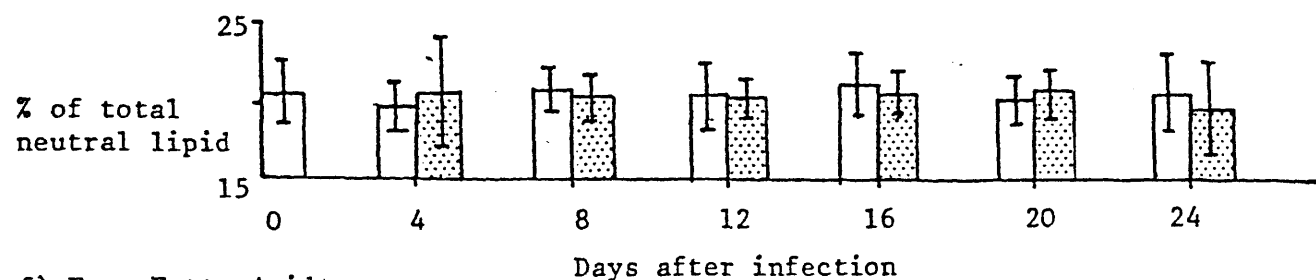
c) Monoglycerides



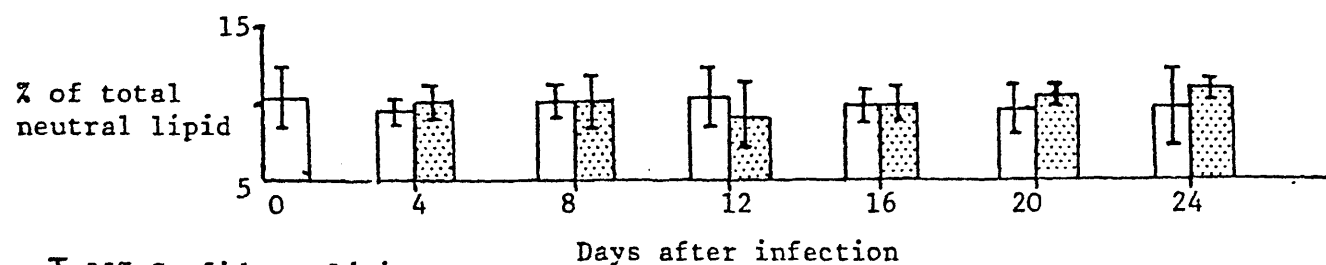
d) Diglycerides



e) Triglycerides



f) Free Fatty Acids



┌ 95% Confidence Limits

Unshaded Uninfected Plants
Shaded Infected Plants

4.2.6 Polar Lipid bound fatty acids

The percentage composition of the individual fatty acids bound to the polar lipids at the beginning of the experiment in the cultivar Onward were as follows: palmitic acid $13.9 \pm 2.0\%$, palmitoleic acid $2.3 \pm 0.3\%$, stearic acid $2.6 \pm 0.4\%$, oleic acid $1.5 \pm 0.9\%$, linoleic acid $14.5 \pm 0.7\%$ and linolenic acid $65.4 \pm 3.2\%$ (Figure 31). The other cultivars did not differ significantly from ($p = 0.05\%$) from Onward in this respect (Figures 32, 33 and 34) and for none of the fatty acids did the values change appreciably during the first 24 days after infection (Figures 31, 32, 33 and 34).

Figure 31: The effect of infection with E. pisi on the total polar lipid fatty acid composition in the leaves of the cultivar Onward

Figure 31

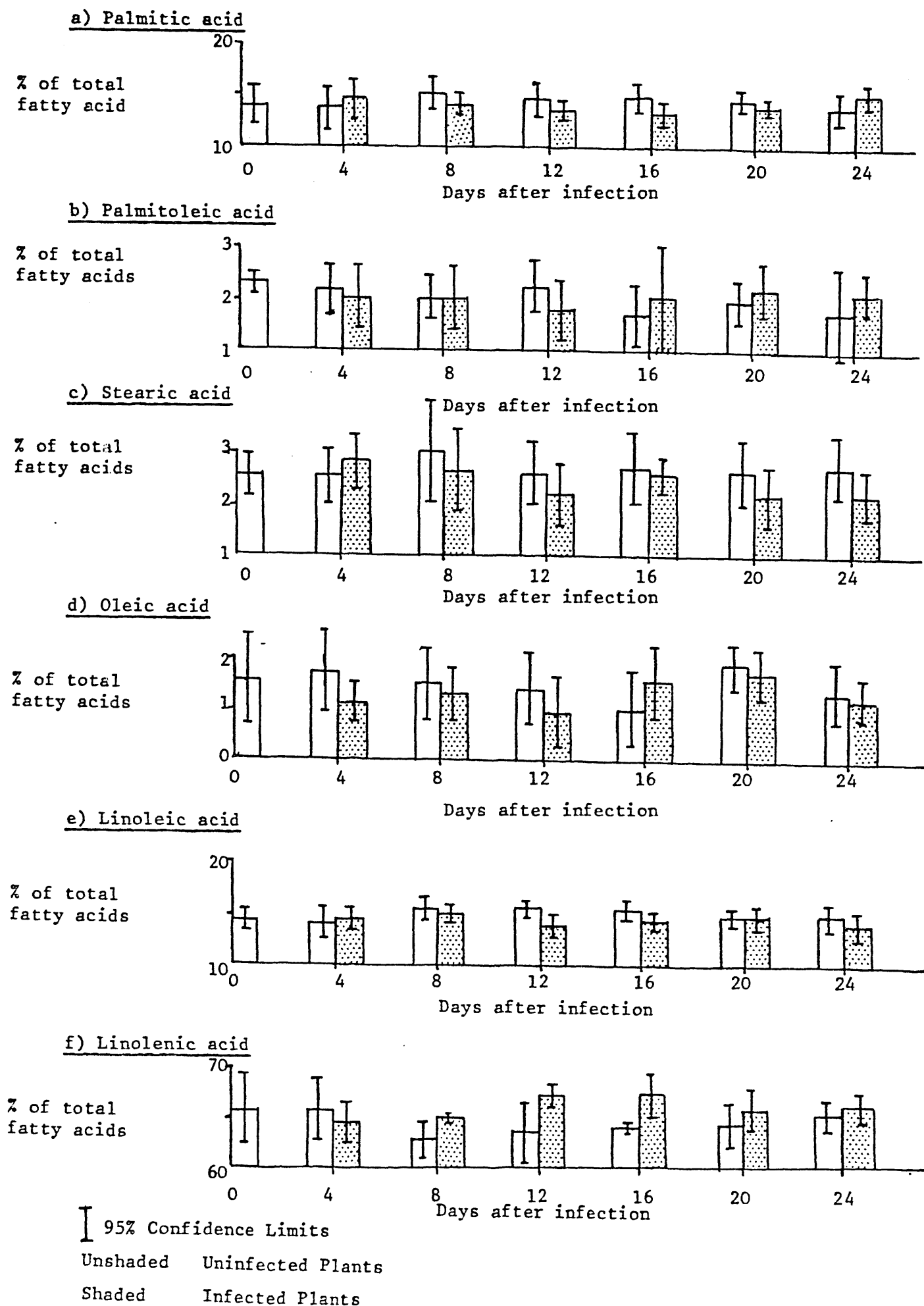


Figure 32: The effect of infection with E. pisi on the total polar lipid fatty acid composition in the leaves of the cultivar Feltham First

Figure 32

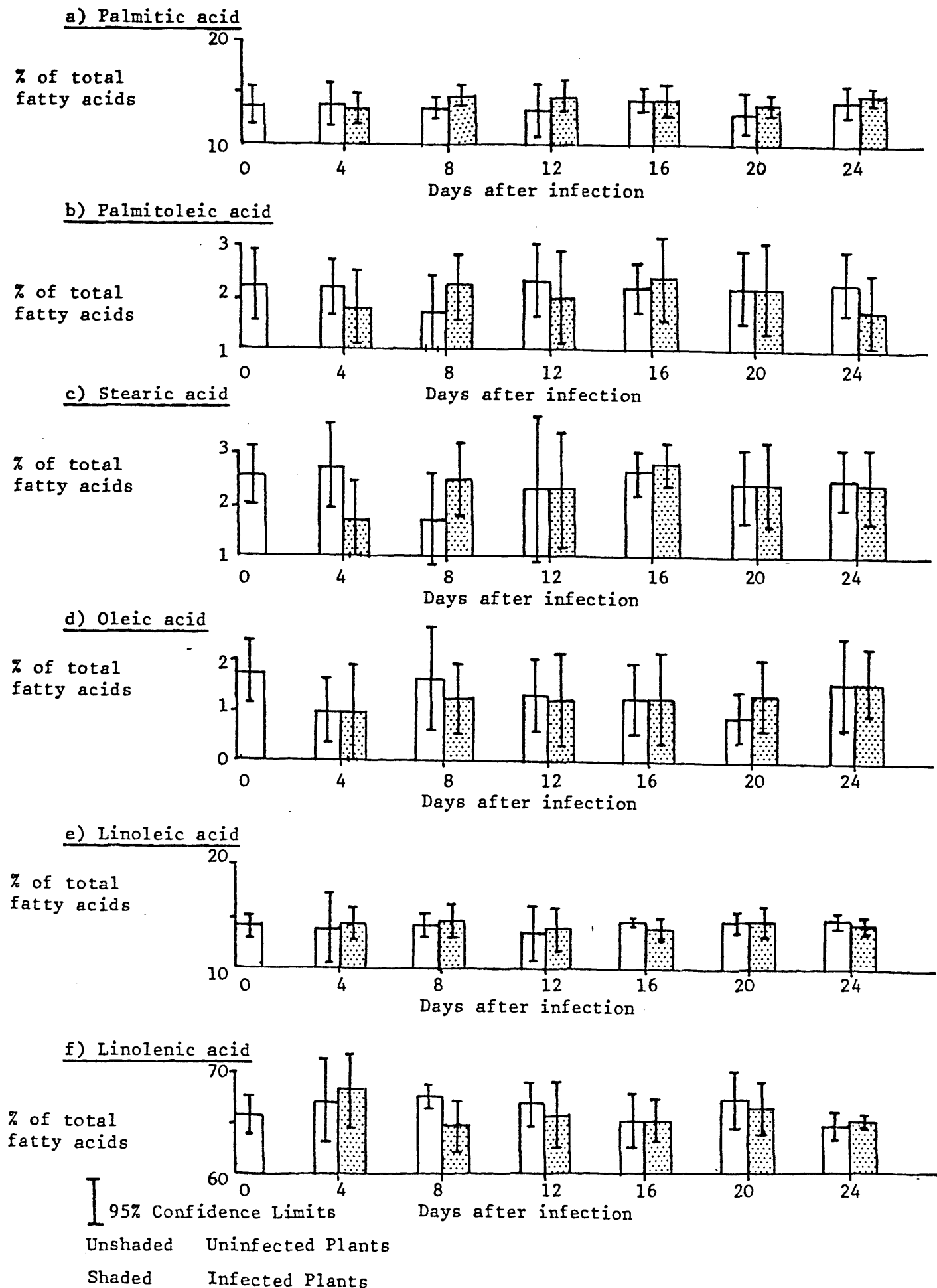


Figure 33: The effect of infection with E. pisi on the total polar lipid fatty acid composition in the leaves of the cultivar Hurst Green Shaft

Figure 33

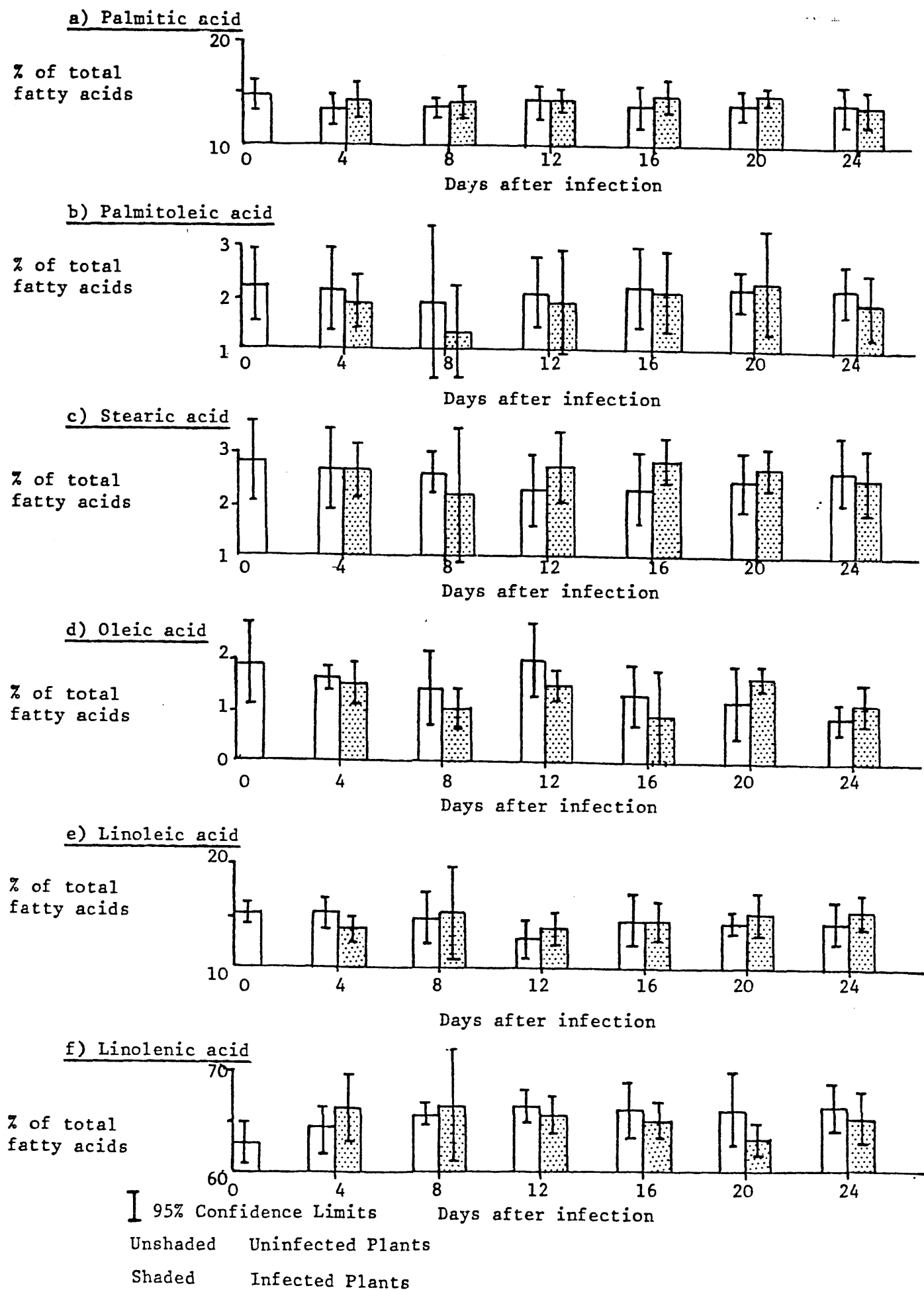
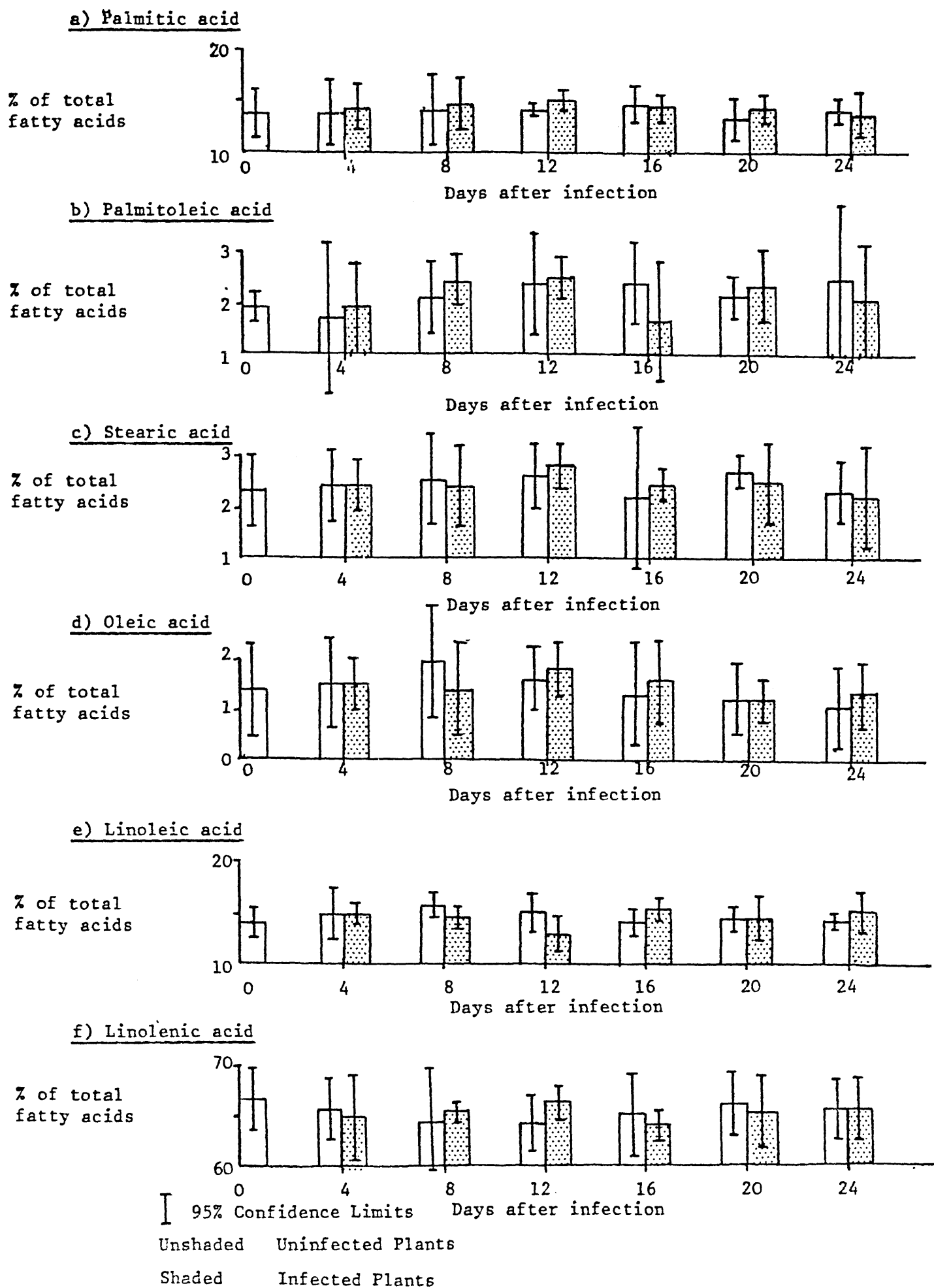


Figure 34: The effect of infection with E. pisi on the total polar lipid fatty acid composition in the leaves of the cultivar Pilot

Figure 34



4.2.7. Neutral Lipid bound fatty acids

The percentage composition of the total fatty acids from the neutral lipid fraction at the beginning of the experiment in the cultivar Onward was as follows (Figure 35):- lauric acid $3.8 \pm 1.6\%$, myristic acid $8.4 \pm 1.2\%$, palmitic acid $24.0 \pm 2.2\%$, st^aeric acid $4.4 \pm 0.9\%$, oleic acid $9.0 \pm 0.3\%$, linoleic acid $28.3 \pm 1.3\%$ and linolenic acid $21.9 \pm 1.2\%$. The results from the other cultivars (Figures 36, 37 and 38) show that there was no significant difference ($p = 0.05$) in the amount of each fatty acid as a percentage of the total neutral lipid fatty acid composition in the four cultivars tested at the beginning of the experiment. Furthermore there were also no significant changes ($p = 0.05$) in these values during the course of the 24 day infection period irrespective of whether the plants were infected or not (Figures 35, 36, 37 and 38).

Figure 35: The effect of infection with E. pisi on the total neutral lipid fatty acid composition in the leaves of the cultivar Onward

Figure 35

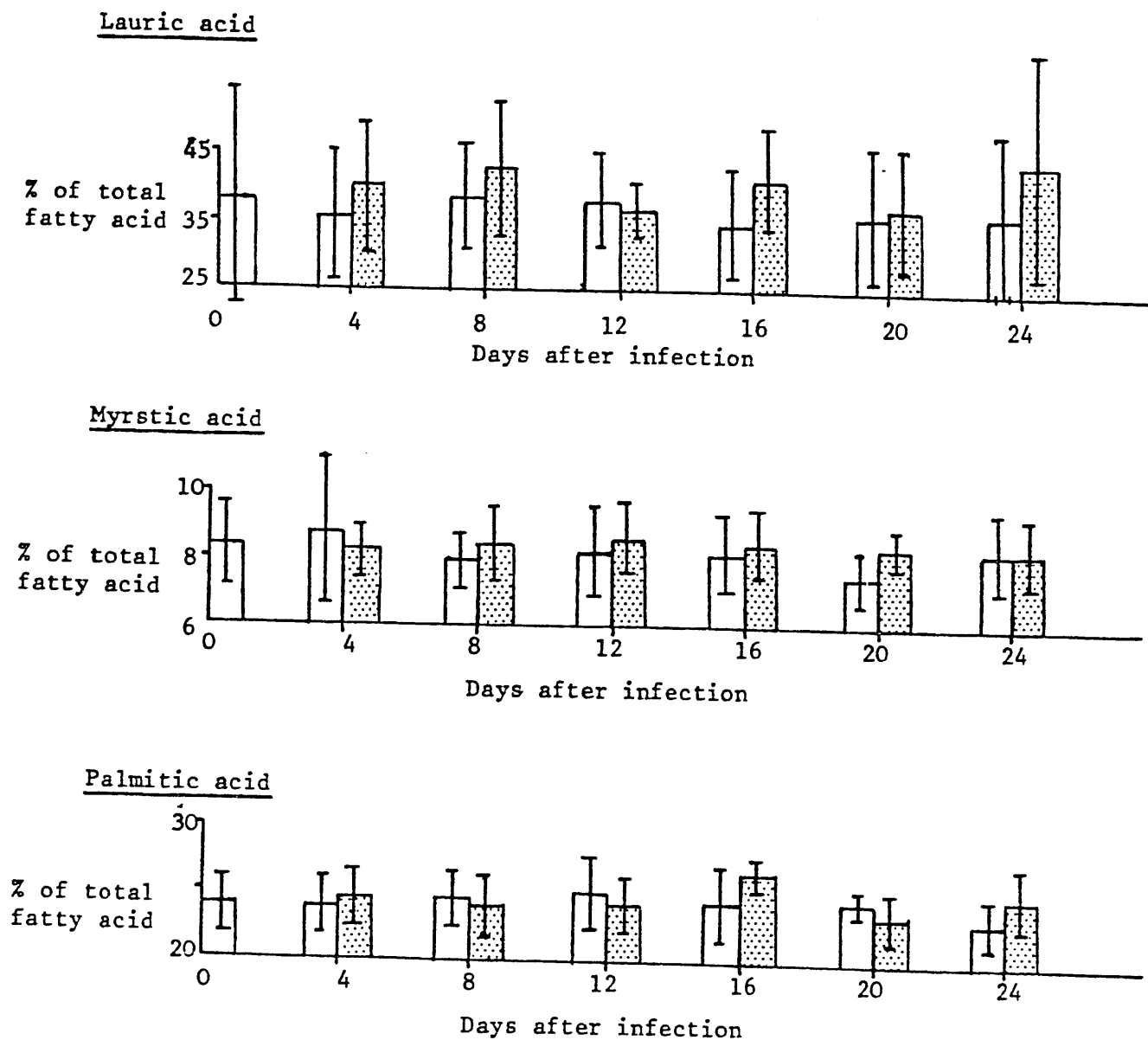
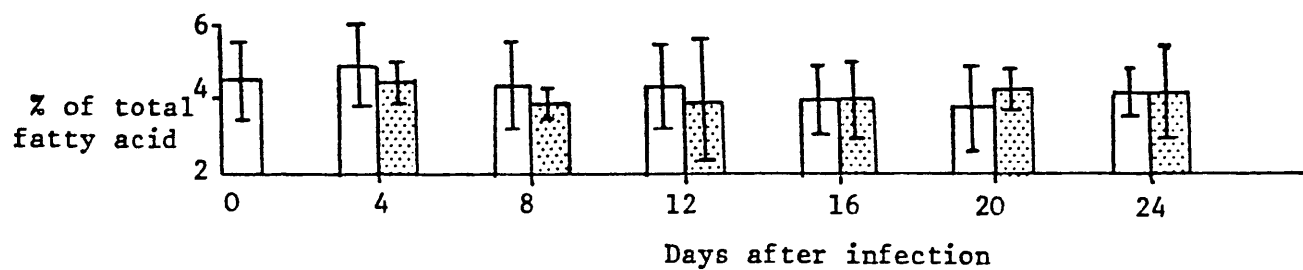
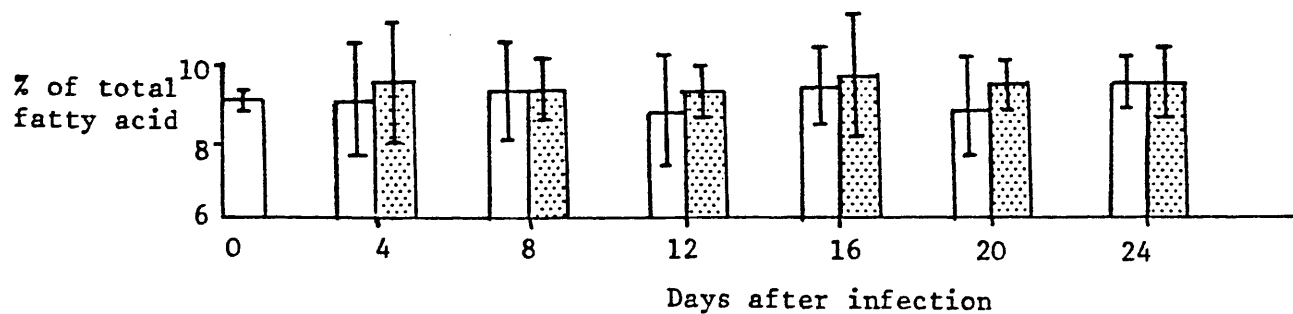


Figure 35 (cont.)

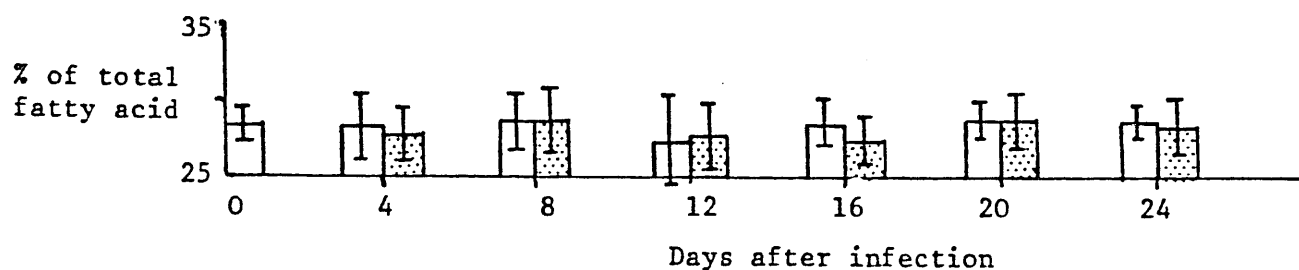
Stearic acid



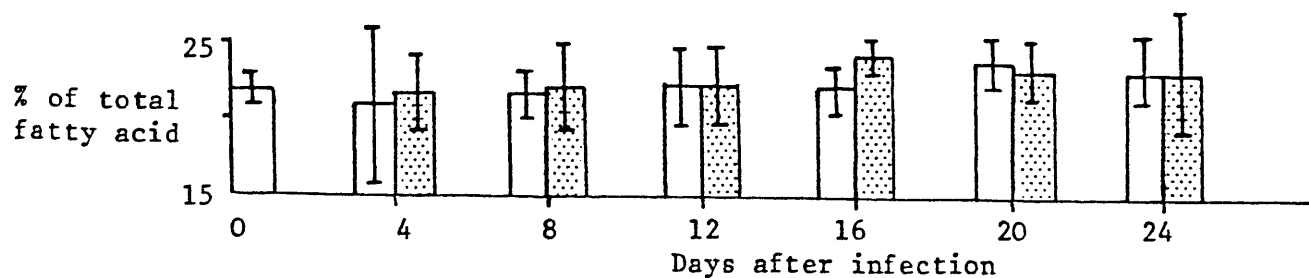
Oleic acid



Linoleic acid



Linolenic acid



Unshaded Uninfected Plants

Shaded Infected Plants

I 95% Confidence Limits

Figure 36: The effects of infection with E. pisi on the total neutral lipid fatty acid composition of the leaves of the pea cultivar Feltham First

Figure 36

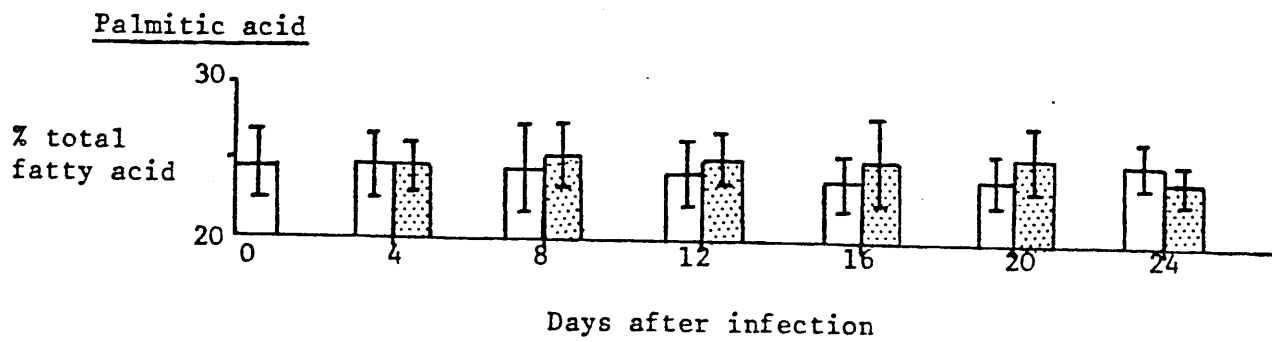
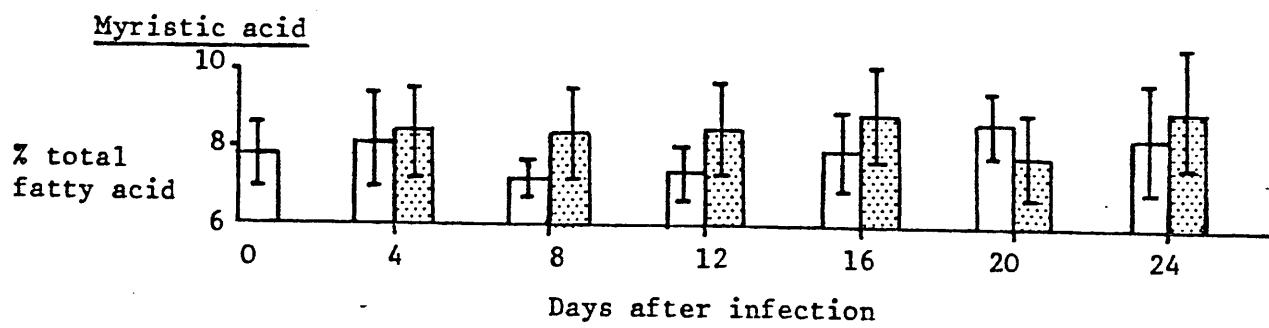
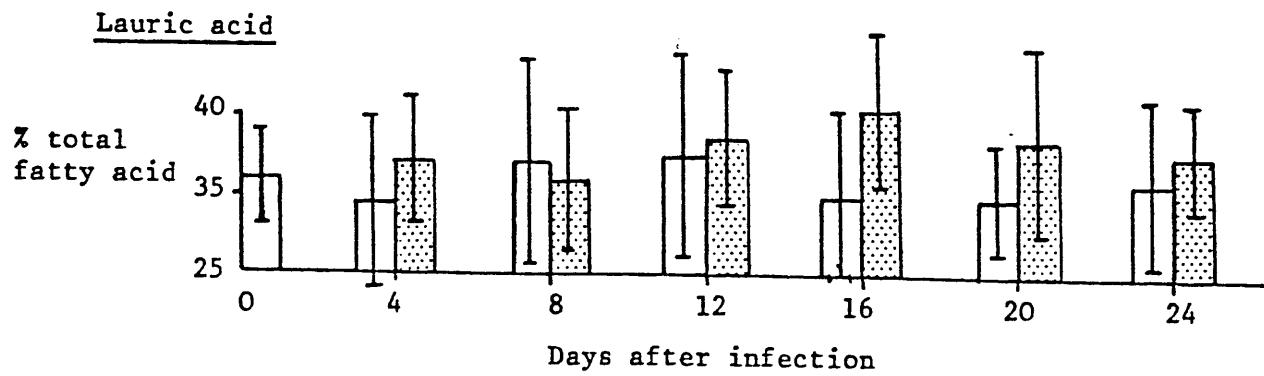
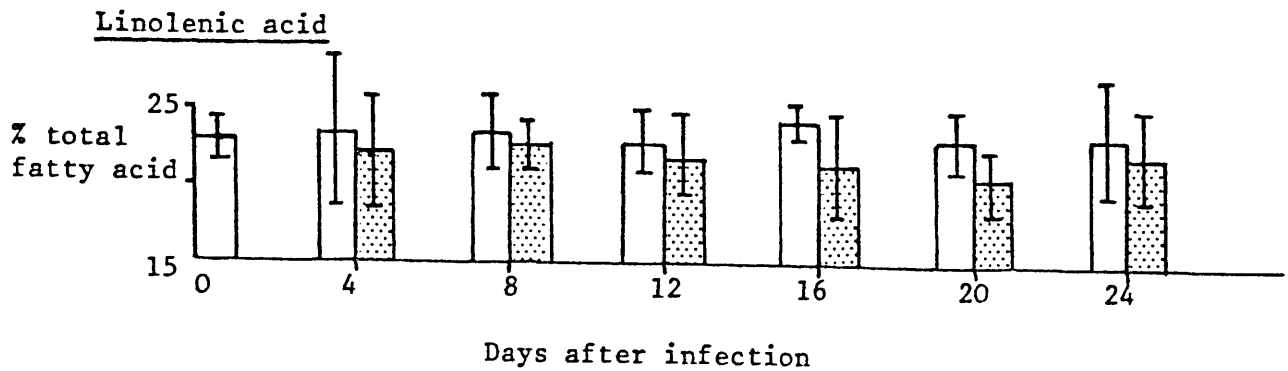
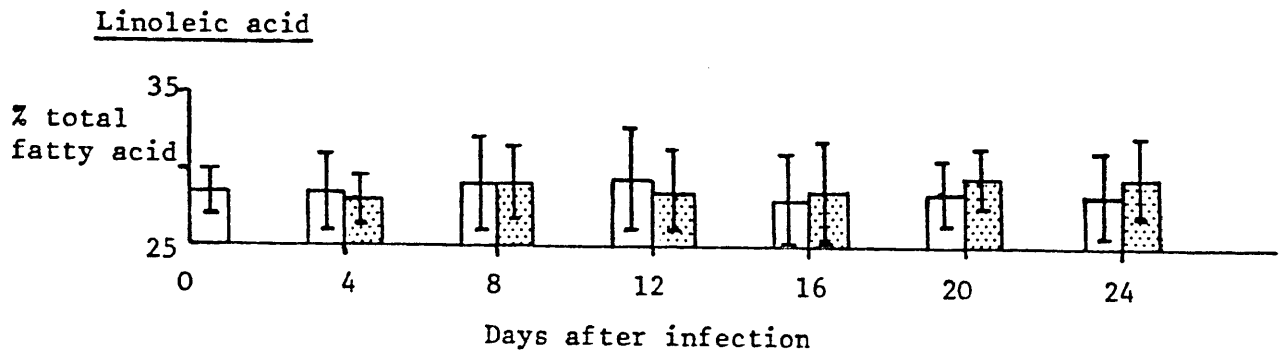
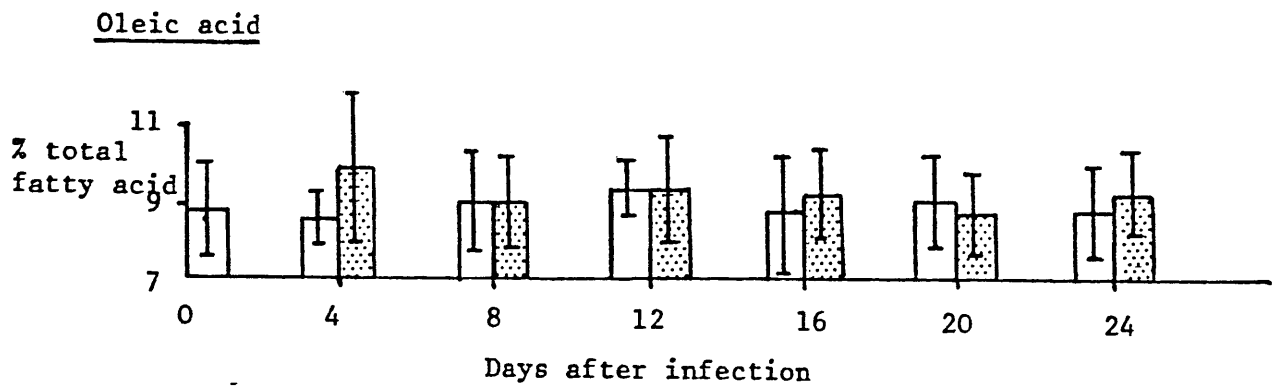
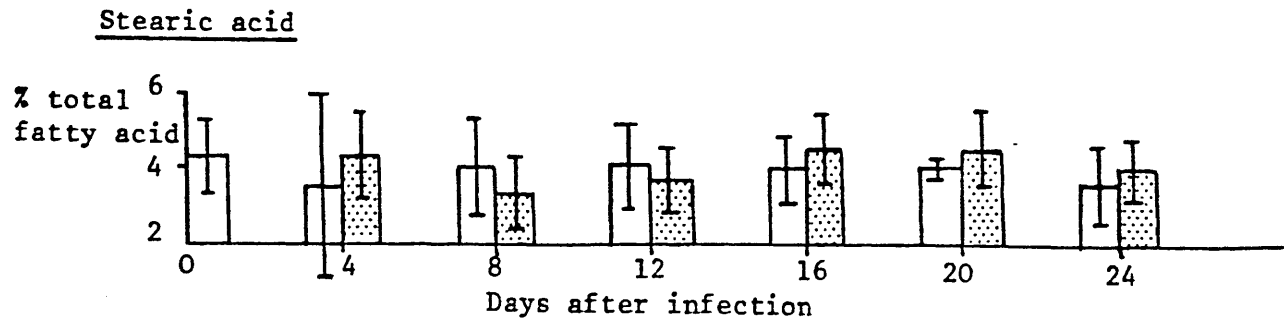


Figure 36 (cont.)



Unshaded Uninfected Plants

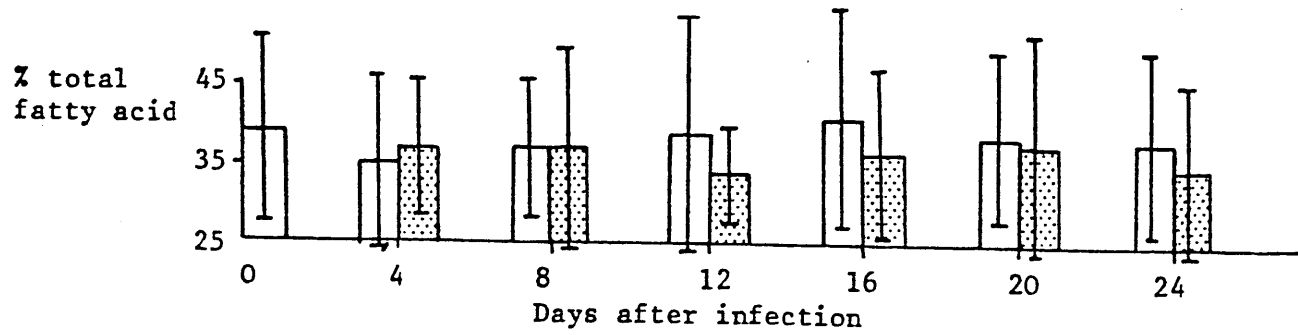
Shaded Infected Plants

I 95% Confidence Limits

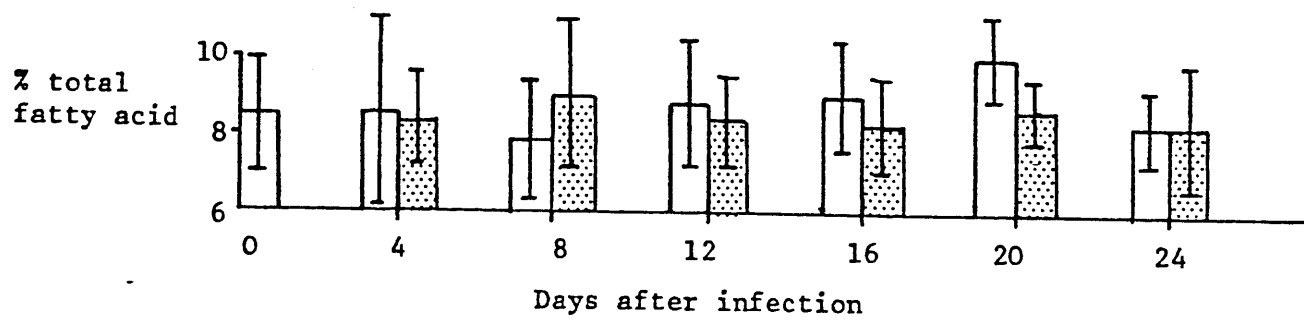
Figure 37: The effect of infection with E. pisi on the total neutral lipid fatty acid composition in the leaves of the pea cultivar Hurst Green Shaft

Figure 37

Lauric acid



Myristic acid



Palmitic acid

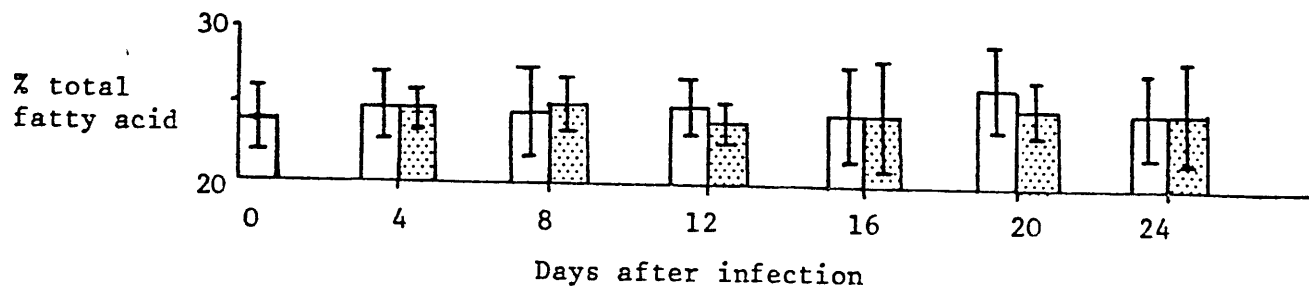
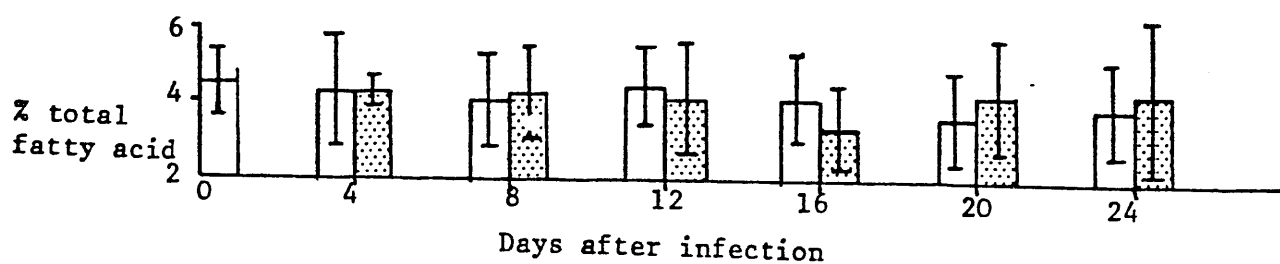
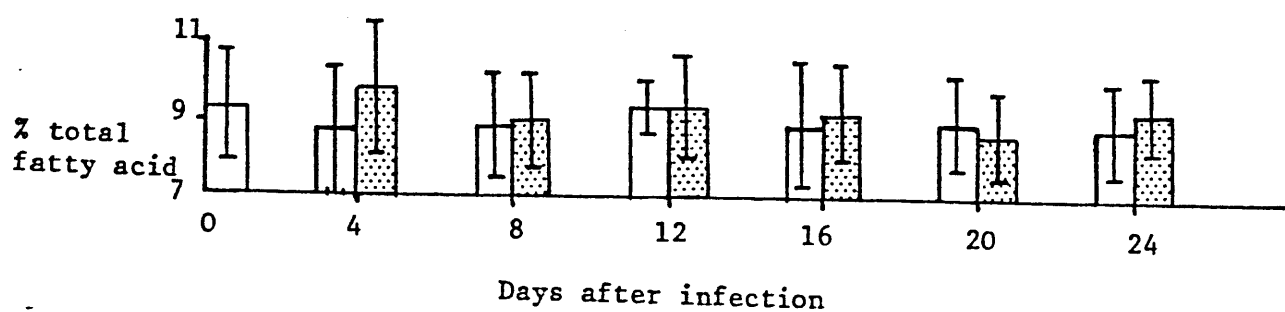


Figure 37 (cont.)

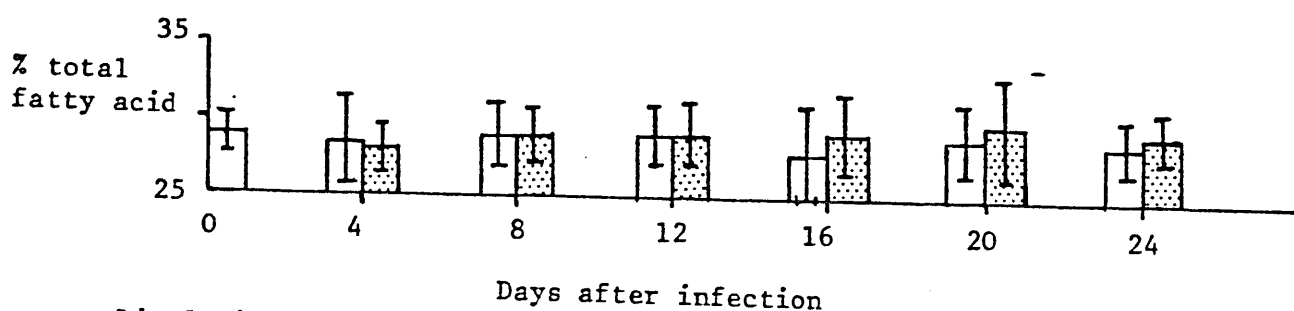
Stearic acid



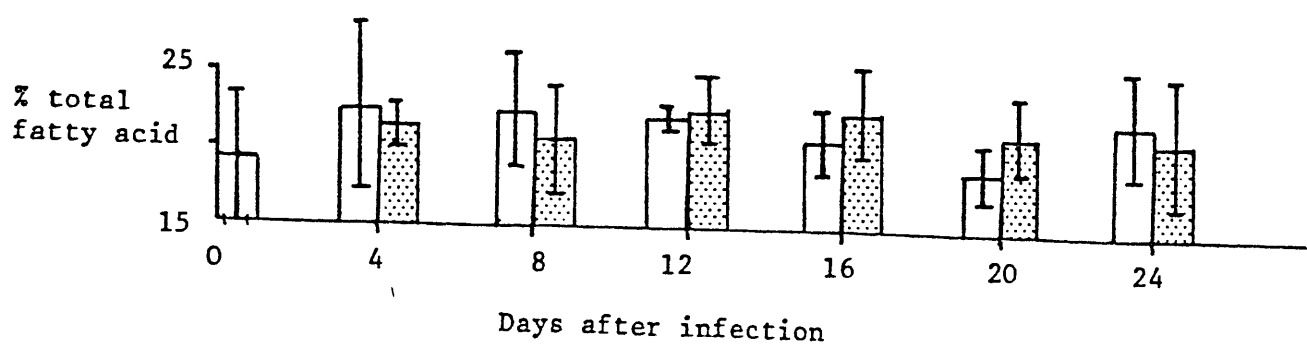
Oleic acid



Linoleic acid



Linolenic acid



Unshaded Uninfected Plants

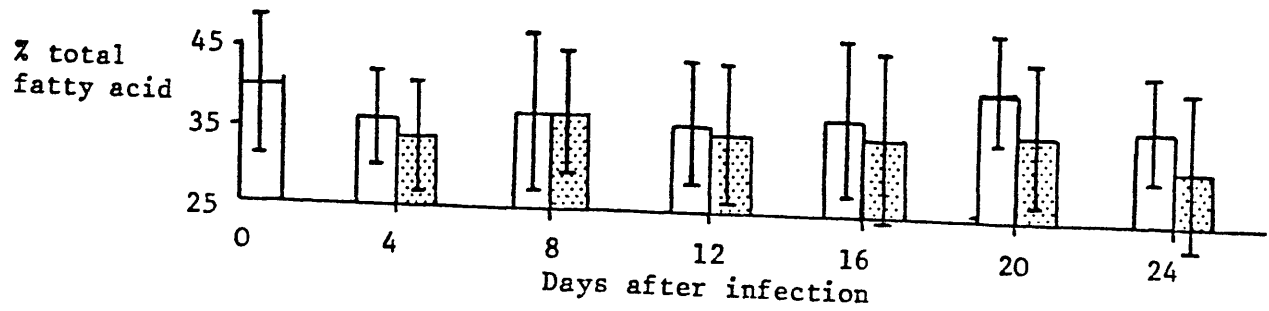
Shaded Infected Plants

I 95% Confidence Limits

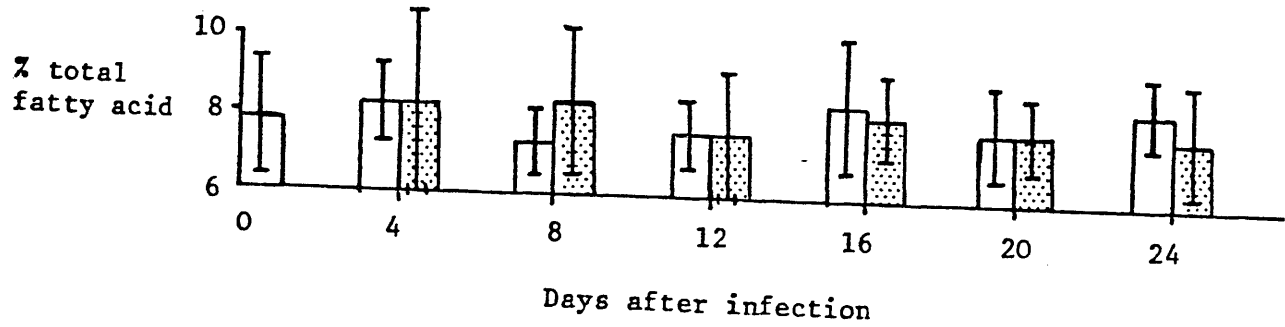
Figure 38: The effect of infection with E. pisi on the total neutral lipid fatty acid composition in the leaves of the pea cultivar Pilot

Figure 38

Lauric acid



Myristic acid



Palmitic acid

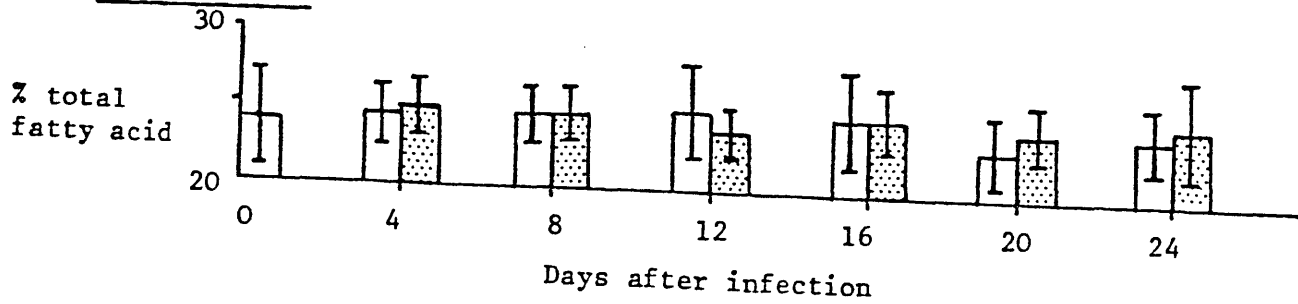
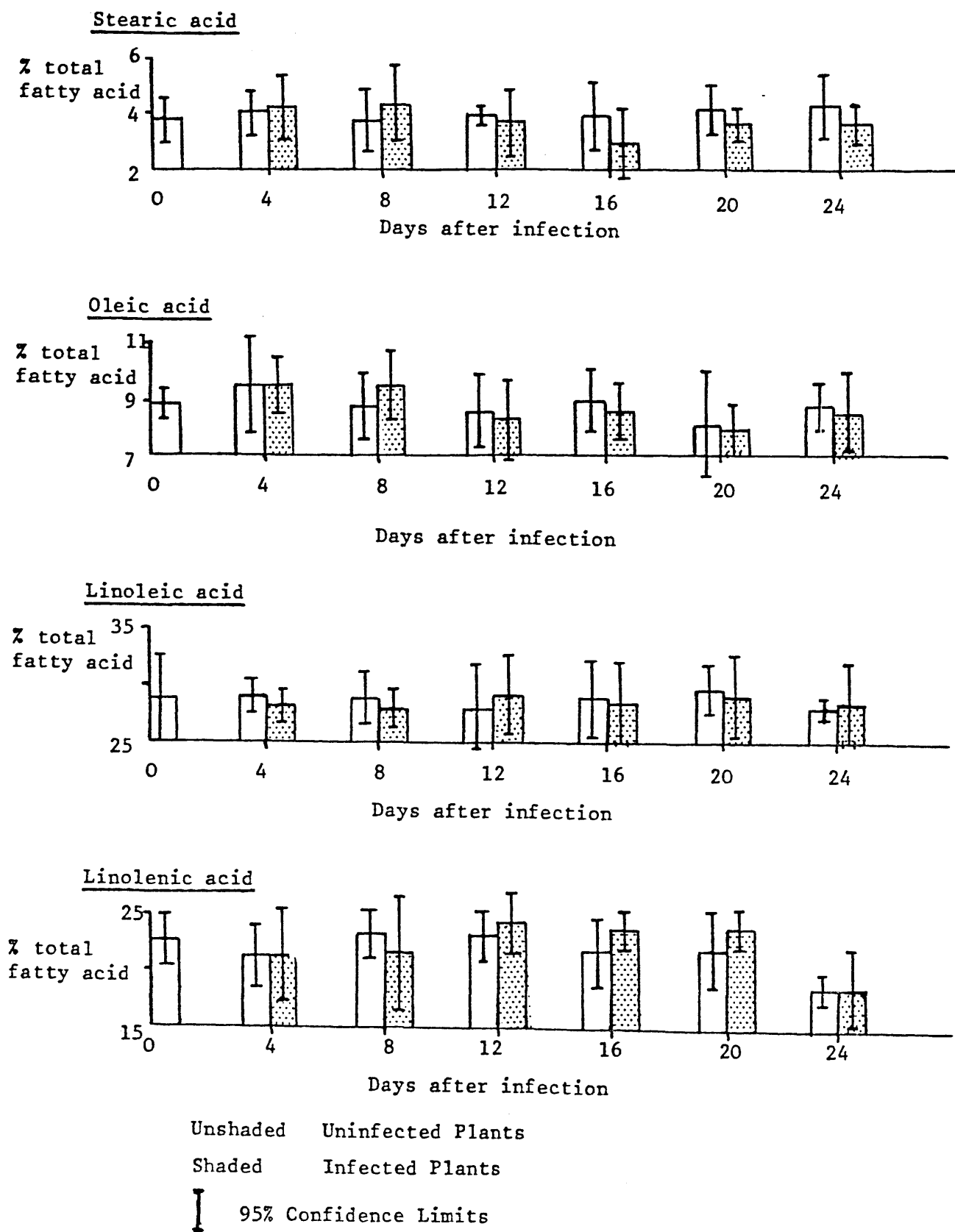


Figure 38 (cont.)



4.2.8. Fatty acids of individual lipid classe

The analysis was extended to include the fatty acids of individual polar lipid classes. Table 17 shows representative data for the cultivar Onward. Fatty acid composition was strongly class dependent. For example palmitic acid typically constituted 22% of the fatty acids in PC, 27% in PE, only 3% in MGDG and 10% in DGDG (Table 17). However there was no indication that plant age per se, infection or time after infection directly affected the results. Data for the other three cultivars were very similar and are recorded in Appendix I.

Table 17: The effect of infection by *E. pisi* on the percentage fatty acid composition of different polar lipids in the leaves of the pea cultivar Onward

a) PC in uninfected plants

Fatty Acid	Days after infection						
	0	4	8	12	16	20	24
Palmitic acid	22.0 ± 2.0	22.7 ± 2.7	22.6 ± 2.6	23.0 ± 2.3	22.5 ± 4.1	22.9 ± 1.6	22.0 ± 1.3
Palmitoleic acid	-	0.1 ± 0.2	-	-	0.1 ± 0.3	-	-
Stearic acid	4.8 ± 0.6	4.6 ± 1.4	4.5 ± 2.6	4.6 ± 0.6	4.4 ± 0.7	4.3 ± 2.1	4.5 ± 0.9
Oleic acid	2.2 ± 1.0	2.5 ± 1.0	2.6 ± 0.7	2.0 ± 1.0	2.5 ± 0.7	2.7 ± 0.7	2.1 ± 0.6
Linoleic acid	40.7 ± 3.7	38.8 ± 4.4	40.2 ± 3.0	39.8 ± 1.6	38.9 ± 3.0	41.6 ± 3.9	40.0 ± 2.8
Linolenic acid	29.6 ± 2.7	31.3 ± 7.4	29.9 ± 3.3	30.5 ± 3.6	31.4 ± 1.2	28.5 ± 1.6	31.3 ± 3.1

b) PC in infected plants

Fatty Acid	Days after infection					
	4	8	12	16	20	24
Palmitic acid	22.1 ± 3.1	23.4 ± 1.1	22.4 ± 3.2	23.3 ± 1.9	22.9 ± 1.8	23.0 ± 1.2
Palmitoleic acid	0.1 ± 0.1	-	-	-	-	-
Stearic acid	4.7 ± 0.9	4.6 ± 0.8	4.3 ± 0.6	4.4 ± 0.9	4.3 ± 1.4	4.6 ± 0.9
Oleic acid	2.6 ± 0.7	2.0 ± 1.1	2.5 ± 0.7	2.4 ± 1.1	2.4 ± 1.1	2.2 ± 0.9
Linoleic acid	40.5 ± 3.3	40.5 ± 3.0	41.8 ± 2.7	40.2 ± 2.7	41.4 ± 3.0	40.5 ± 3.5
Linolenic acid	29.9 ± 1.0	29.5 ± 2.0	26.7 ± 1.4	29.3 ± 3.1	28.9 ± 3.3	29.5 ± 1.6

c) PE in uninfected plants

Fatty Acid	Days after infection						
	0	4	8	12	16	20	24
Palmitic acid	27.0 ± 6.7	26.5 ± 2.6	25.9 ± 2.3	26.5 ± 3.8	25.8 ± 4.2	25.3 ± 2.8	24.8 ± 2.7
Stearic acid	4.2 ± 1.1	4.1 ± 1.1	4.1 ± 2.2	4.5 ± 0.8	4.0 ± 1.2	4.0 ± 0.9	4.6 ± 0.7
Oleic acid	1.8 ± 1.0	2.3 ± 0.8	2.3 ± 1.0	2.4 ± 0.7	1.9 ± 1.2	2.6 ± 0.7	1.7 ± 0.8
Linoleic acid	39.2 ± 4.1	40.7 ± 2.1	40.6 ± 2.7	39.9 ± 2.7	39.3 ± 4.9	39.8 ± 3.1	40.2 ± 4.2
Linolenic acid	27.7 ± 5.4	26.3 ± 3.4	27.0 ± 4.3	26.6 ± 4.1	28.9 ± 2.2	28.2 ± 2.2	28.7 ± 2.8

d) PE in infected plants

Fatty Acid	Days after infection					
	4	8	12	16	20	24
Palmitic acid	25.8 ± 2.2	26.5 ± 3.6	27.3 ± 2.3	26.5 ± 2.6	26.3 ± 3.2	25.9 ± 3.4
Stearic acid	4.8 ± 0.7	4.0 ± 1.2	4.4 ± 1.9	5.0 ± 0.2	4.3 ± 1.0	4.6 ± 0.9
Oleic acid	2.3 ± 0.7	1.6 ± 1.5	2.5 ± 0.7	2.0 ± 1.1	1.9 ± 1.2	2.4 ± 0.6
Linoleic acid	38.2 ± 3.6	40.0 ± 4.6	38.7 ± 5.6	38.5 ± 3.1	39.1 ± 4.5	40.7 ± 4.6
Linolenic acid	28.9 ± 1.4	27.9 ± 3.2	27.0 ± 3.5	27.9 ± 2.3	28.4 ± 1.8	26.4 ± 5.5

e) PG in uninfected plants

Fatty Acid	Days after infection						
	0	4	8	12	16	20	24
Palmitic acid	25.6 ± 2.3	26.1 ± 1.3	22.5 ± 2.1	24.9 ± 3.0	24.9 ± 3.2	24.8 ± 2.9	25.0 ± 2.0
Palmitoleic acid	20.4 ± 2.4	21.0 ± 2.3	21.2 ± 2.4	21.0 ± 0.6	21.0 ± 2.1	21.9 ± 2.2	21.1 ± 1.7
Stearic acid	2.4 ± 0.8	3.4 ± 1.5	2.2 ± 0.7	2.4 ± 1.1	2.4 ± 0.9	2.6 ± 0.9	2.8 ± 0.8
Oleic acid	2.7 ± 1.3	2.4 ± 0.8	2.1 ± 0.7	3.2 ± 1.7	3.2 ± 1.5	3.3 ± 0.9	2.3 ± 0.7
Linoleic acid	11.2 ± 1.3	11.5 ± 1.5	11.6 ± 2.6	11.7 ± 0.7	12.1 ± 2.1	11.2 ± 1.8	11.9 ± 2.1
Linolenic acid	35.2 ± 4.9	35.5 ± 5.9	40.3 ± 3.0	36.8 ± 3.8	36.4 ± 3.2	36.0 ± 3.3	36.8 ± 4.8

f) PG in infected plants

Fatty Acid	Days after infection					
	4	8	12	16	20	24
Palmitic acid	24.6 ± 2.4	24.3 ± 2.7	25.5 ± 2.4	23.5 ± 2.1	25.2 ± 3.6	24.4 ± 2.9
Palmitoleic acid	20.9 ± 1.4	21.0 ± 1.4	21.6 ± 1.2	21.9 ± 1.5	20.6 ± 1.4	20.7 ± 2.5
Stearic acid	2.6 ± 1.0	2.4 ± 0.7	2.3 ± 1.2	2.7 ± 0.8	2.4 ± 0.8	2.4 ± 1.0
Oleic acid	2.2 ± 0.8	2.7 ± 0.8	2.5 ± 0.5	2.2 ± 0.8	2.0 ± 1.0	2.5 ± 0.6
Linoleic acid	12.3 ± 1.7	12.6 ± 1.4	13.0 ± 1.2	12.6 ± 1.4	12.6 ± 1.9	13.0 ± 1.6
Linolenic acid	37.2 ± 2.7	36.6 ± 4.6	35.2 ± 3.9	37.0 ± 5.0	37.0 ± 2.8	36.7 ± 5.0

g) PI, PS and PA in uninfected plants*

Fatty Acid	Days after infection						
	0	4	8	12	16	20	24
Palmitic acid	26.6 ± 7.4	27.0 ± 1.8	25.7 ± 1.9	26.7 ± 4.4	27.5 ± 3.6	24.6 ± 1.5	27.4 ± 3.0
Stearic acid	4.4 ± 1.2	3.7 ± 2.7	4.3 ± 1.3	3.5 ± 1.7	3.6 ± 2.5	4.3 ± 1.0	4.2 ± 1.0
Oleic acid	1.8 ± 1.0	2.4 ± 0.7	2.2 ± 0.6	1.6 ± 0.8	2.1 ± 1.3	2.0 ± 0.3	2.4 ± 0.9
Linoleic acid	39.6 ± 4.2	41.8 ± 3.0	39.6 ± 6.3	39.8 ± 5.0	38.8 ± 4.1	39.3 ± 5.1	40.4 ± 4.3
Linolenic acid	27.6 ± 5.2	25.1 ± 4.4	28.1 ± 4.5	28.3 ± 2.9	28.2 ± 4.0	29.8 ± 3.3	25.5 ± 2.9

h) PI, PS and PA in infected plants*

Fatty Acid	Days after infection					
	4	8	12	16	20	24
Palmitic acid	25.9 ± 1.4	28.1 ± 3.9	27.7 ± 2.7	25.4 ± 2.0	26.8 ± 3.2	25.8 ± 1.9
Stearic acid	4.1 ± 1.2	3.9 ± 0.7	3.6 ± 0.7	3.6 ± 1.6	3.6 ± 1.9	3.9 ± 1.6
Oleic acid	2.2 ± 0.6	1.8 ± 1.3	2.2 ± 0.6	2.1 ± 1.1	2.1 ± 0.4	1.7 ± 1.0
Linoleic acid	39.6 ± 6.4	40.5 ± 3.3	39.1 ± 6.5	41.0 ± 4.1	38.7 ± 3.2	40.2 ± 4.3
Linolenic acid	26.3 ± 6.0	25.6 ± 2.7	28.0 ± 4.3	27.8 ± 3.2	26.7 ± 3.1	28.3 ± 1.7

i) MGDG in uninfected plants

Fatty Acid	Days after infection						
	0	4	8	12	16	20	24
Palmitic acid	3.8 ± 3.9	2.5 ± 0.7	3.3 ± 1.5	3.6 ± 1.5	4.1 ± 1.1	3.6 ± 0.7	2.9 ± 1.3
Stearic acid	0.8 ± 0.4	0.9 ± 0.6	1.2 ± 0.7	1.1 ± 0.6	0.8 ± 0.5	1.4 ± 0.8	1.1 ± 0.8
Oleic acid	1.2 ± 1.1	1.3 ± 0.7	1.3 ± 0.6	1.0 ± 0.8	1.6 ± 0.9	1.3 ± 0.3	1.4 ± 0.6
Linoleic acid	3.8 ± 1.0	4.2 ± 0.8	4.1 ± 1.0	4.5 ± 0.8	4.0 ± 1.3	3.7 ± 1.7	4.2 ± 1.0
Linolenic acid	90.4 ± 5.3	90.6 ± 2.4	89.0 ± 3.4	89.4 ± 1.6	89.2 ± 2.5	89.9 ± 1.9	90.4 ± 2.0

j) MGDG in infected plants

Fatty Acid	Days after infection					
	4	8	12	16	20	24
Palmitic acid	3.0 ± 1.3	2.9 ± 1.5	3.6 ± 2.1	3.6 ± 1.2	3.7 ± 1.7	3.8 ± 1.4
Stearic acid	1.2 ± 0.6	1.0 ± 0.6	1.2 ± 1.0	1.3 ± 0.7	1.1 ± 0.5	1.0 ± 0.6
Oleic acid	1.5 ± 0.7	1.4 ± 0.7	1.6 ± 0.4	1.3 ± 0.5	1.3 ± 0.6	1.2 ± 0.7
Linoleic acid	4.0 ± 1.0	4.0 ± 1.0	4.6 ± 0.7	4.1 ± 0.2	4.1 ± 0.9	4.4 ± 0.9
Linolenic acid	90.2 ± 2.4	91.0 ± 1.4	90.0 ± 1.5	89.6 ± 2.9	89.9 ± 2.0	89.6 ± 1.7

k) DGDG in uninfected plants

Fatty Acid	Days after infection						
	0	4	8	12	16	20	24
Palmitic acid	9.8 ± 1.6	10.2 ± 1.7	10.7 ± 0.9	10.6 ± 1.7	9.8 ± 1.5	10.9 ± 1.4	11.1 ± 1.3
Stearic acid	2.9 ± 0.6	2.9 ± 1.0	2.3 ± 0.7	2.3 ± 1.2	2.7 ± 1.0	2.6 ± 1.0	2.5 ± 0.6
Oleic acid	1.0 ± 0.3	1.2 ± 0.9	1.2 ± 0.5	1.0 ± 0.7	1.3 ± 0.6	1.1 ± 0.2	1.5 ± 0.6
Linoleic acid	3.1 ± 0.6	2.7 ± 0.8	2.9 ± 1.0	2.7 ± 0.9	3.1 ± 1.0	2.9 ± 0.3	3.2 ± 0.3
Linolenic acid	83.1 ± 2.6	83.2 ± 2.8	82.9 ± 1.9	83.2 ± 0.4	83.0 ± 1.4	82.5 ± 2.1	82.0 ± 0.7

l) DGDG in infected plants

Fatty Acid	Days after infection					
	4	8	12	16	20	24
Palmitic acid	9.5 ± 1.0	9.6 ± 1.3	9.6 ± 1.3	9.7 ± 1.8	9.6 ± 2.6	8.9 ± 0.4
Stearic acid	2.8 ± 0.4	2.6 ± 0.9	2.7 ± 1.4	2.6 ± 1.0	2.8 ± 1.2	2.5 ± 0.8
Oleic acid	0.9 ± 0.5	0.8 ± 0.7	0.9 ± 0.4	1.1 ± 0.7	1.0 ± 0.9	0.7 ± 0.5
Linoleic acid	3.0 ± 0.7	3.0 ± 1.1	3.3 ± 1.3	3.2 ± 0.6	3.6 ± 1.5	2.6 ± 0.9
Linolenic acid	83.8 ± 0.7	83.7 ± 3.0	83.4 ± 2.2	83.3 ± 1.6	82.7 ± 2.3	85.4 ± 1.3

*Data for these three lipid classes aggregated since together they constitute only 5% of the polar lipids

4.3. Lipid metabolism in infected and uninfected pea epidermal cells

4.3.1. Experiments to improve the viability of stripped epidermal cells

When strips of epidermal cells were taken from the leaves of uninfected pea plants from several different cultivars the following values, using the method described in Section 3.1.6, for percentage epidermal cell viability were obtained, Onward $14.5 \pm 11.5\%$, Feltham First $16.9 \pm 12.5\%$, Hurst Green Shaft $14.3 \pm 10.3\%$, Pilot $16.0 \pm 12.2\%$, Perfection $12.3 \pm 11.6\%$, Meteor $14.1 \pm 11.3\%$ and Dwarf Defiance $12.2 \pm 10.6\%$. When leaves which had been infected 21 days previously were tested cell viability was lower, viz: Onward $4.7 \pm 80\%$, Feltham First $7.8 \pm 5.9\%$, Hurst Green Shaft $5.6 \pm 7.5\%$, Pilot $6.1 \pm 4.9\%$, Perfection $7.7 \pm 6.2\%$, Meteor $7.4 \pm 8.1\%$ and Dwarf Defiance $5.9 \pm 6.5\%$. All these results represent the means of 10 replicates and are recorded together with the standard error.

The results were disquieting because although much of the loss of viability was probably associated with the mechanical stripping process itself, the possibility remained that even in intact tissue many epidermal cells might be senescent and that would be reflected in the lipid composition. Consequently comparisons were made with strips taken from stem tissue which is also susceptible to powdery mildew.

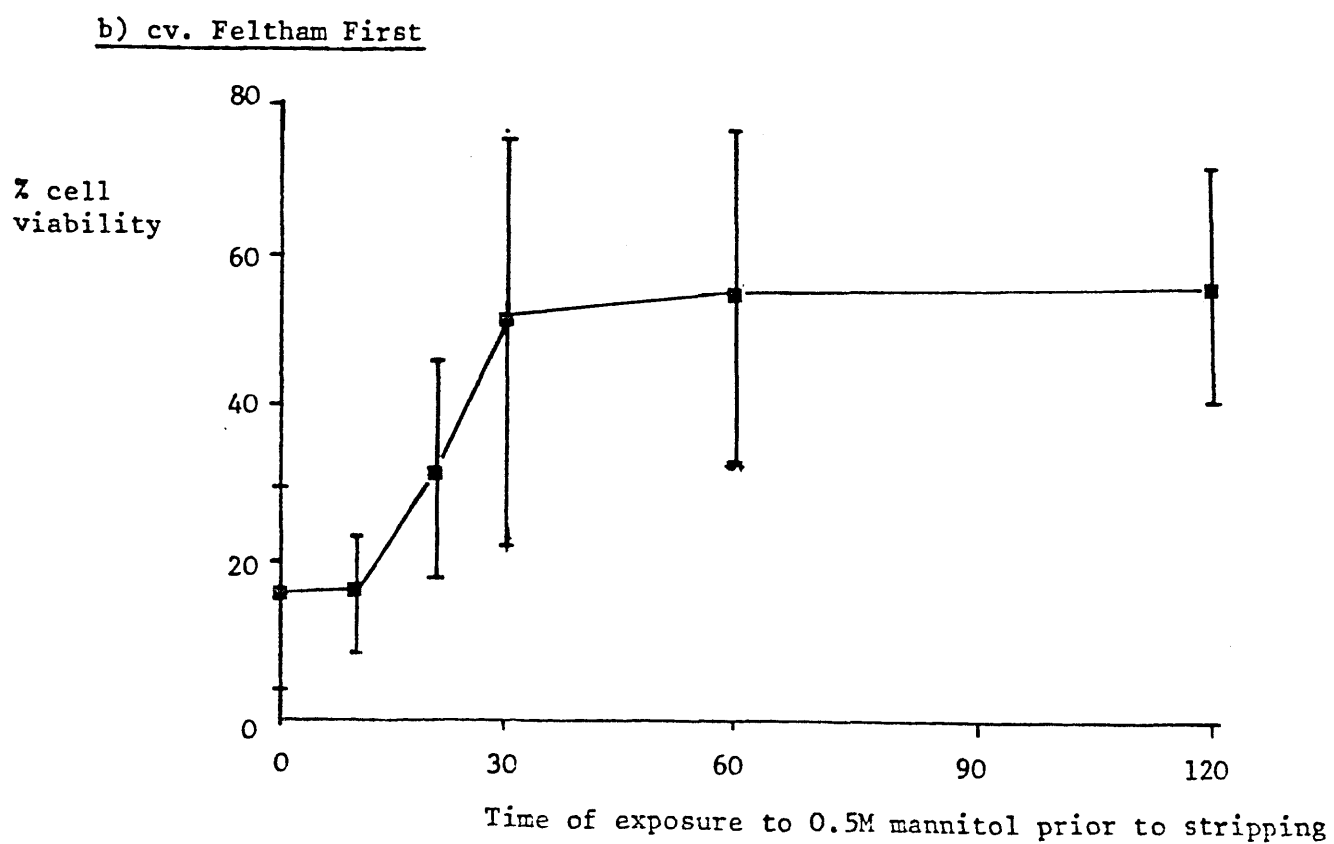
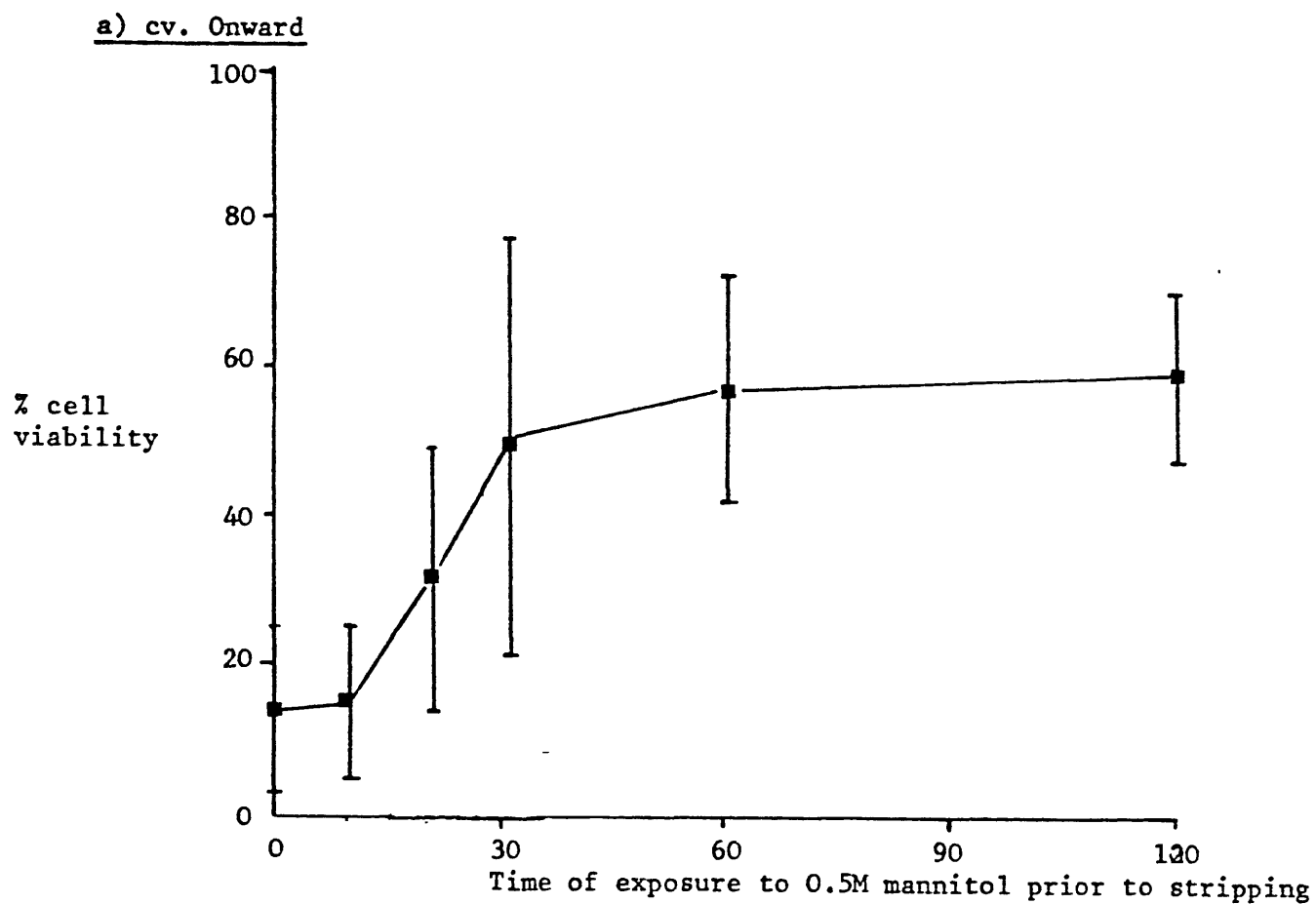
When uninfected stem tissue was used the percentage of viable cells for each cultivar was Onward $29.1 \pm 18.7\%$, Feltham First $31.8 \pm 19.4\%$, Hurst Green Shaft $35.2 \pm 15.6\%$, Pilot $28.6 \pm 22.2\%$, Perfection $21.8 \pm 15.3\%$, Meteor $40.9 \pm 23.9\%$ and Dwarf Defiance $29.7 \pm 12.6\%$. For epidermal cells taken from plant stems that had been infected 21 days previously the results were:- Onward $11.6 \pm 7.0\%$, Feltham First $14.0 \pm 7.4\%$, Hurst Green Shaft $12.9 \pm 7.5\%$, Pilot $11.2 \pm 6.3\%$, Perfection $13.2 \pm 7.9\%$, Meteor $12.9 \pm 7.6\%$, and Dwarf Defiance $13.2 \pm 6.8\%$. Although an improvement over leaves the overall viability was still much lower than desired.

It was decided to test the effect of plasmolysis in an attempt to minimize the shock effects associated with peeling off the epidermal cells. Various osmotica were used of which mannitol at 0.5M proved one of the most suitable. The results presented in Figure 39 and Table 18 show

the effect of pretreatment for various intervals on resulting viability of the epidermal strips. When uninfected leaf tissue was used there was a steady increase in the cell viability as the time of exposure to mannitol was increased, the majority of the effect occurring in the first 30 minutes. When the leaves had been exposed to mannitol for between 1 and 2 hours 55-60% of the cells from all cultivars tested were viable (Figure 39) by the criteria employed.

Figure 39: The effect of exposure to 0.5M mannitol on the viability of epidermal cells in strips taken from leaves of the cultivar Onward

Figure 39



When the leaf tissue that had been infected with *E. pisi* for 21 days was treated in the same way there was only a very small increase in cell viability to 9-11% for all the cultivars tested (Table 18).

Table 18: The effect of exposure to 0.5M mannitol on the viability of epidermal cells from leaves, infected for 21 days, by *E. pisi*, of different cultivars.

Cultivar	Time of exposure of 0.5M mannitol (mins)					
	0	10	20	30	60	120
Onward	4.7 ± 8.0	7.3 ± 6.9	8.6 ± 7.9	9.3 ± 5.0	11.0 ± 4.8	11.0 ± 5.9
Feltham First	7.8 ± 5.9	7.9 ± 5.8	9.4 ± 4.6	9.3 ± 6.0	9.9 ± 5.8	10.1 ± 6.1
Hurst Green Shaft	5.6 ± 7.5	7.2 ± 7.3	9.2 ± 5.0	9.8 ± 5.2	10.4 ± 6.4	11.2 ± 4.9
Pilot	6.1 ± 4.9	5.7 ± 6.6	7.5 ± 5.0	8.1 ± 7.5	8.9 ± 5.5	9.1 ± 5.5
Perfection	7.7 ± 6.2	6.9 ± 6.2	8.5 ± 5.4	11.0 ± 6.1	10.7 ± 4.6	10.5 ± 5.6
Meteor	7.4 ± 8.1	6.7 ± 5.4	6.8 ± 6.0	9.9 ± 6.1	9.4 ± 6.1	10.4 ± 6.4
Dwarf Defiance	5.9 ± 6.5	6.2 ± 7.8	8.2 ± 5.8	9.3 ± 3.9	10.5 ± 5.9	10.5 ± 4.2

For stem tissue the results were more encouraging. After phasmolysis for one hour the percentage cell viability in epidermal strips of various cultivars was:- Onward 90.9 ± 8.6%, Feltham First 91.6 ± 12.4%, Hurst Green Shaft 88.1 ± 14.4%, Pilot 36.3 ± 19.0%, Perfection 88.3 ± 14.4%, Meteor 90.3 ± 8.2% and Dwarf Defiance 88.8 ± 11.1%. The improvement in viability with time is shown for cultivar Onward as a representative example in Figure 40.

After infection the stem epidermal cells were of reduced viability, but still much improved over equivalent leaf tissue. After two hours plasmolysis cell viabilities were:- Onward 72.3 ± 28.0%, Feltham First 74.2 ± 17.4%, Hurst Green Shaft 65.8 ± 19.8%, Pilot 27.5 ± 11.8%, Perfection 63.4 ± 18.8%, Meteor 70.2 ± 24.8% and Dwarf Defiance 73.4 ± 20.0%. With a single exception all cultivars behaved similarly and the results obtained using Feltham First are shown (Figure 41) as a representative example. The cultivar Pilot showed little response to plasmolysis. The reason for this was not investigated and this cultivar was not used further.

Figure 40: The effect of exposure to 0.5M mannitol on the viability of epidermal cells in strips from stems of cultivar Onward

Figure 40

i) Onward

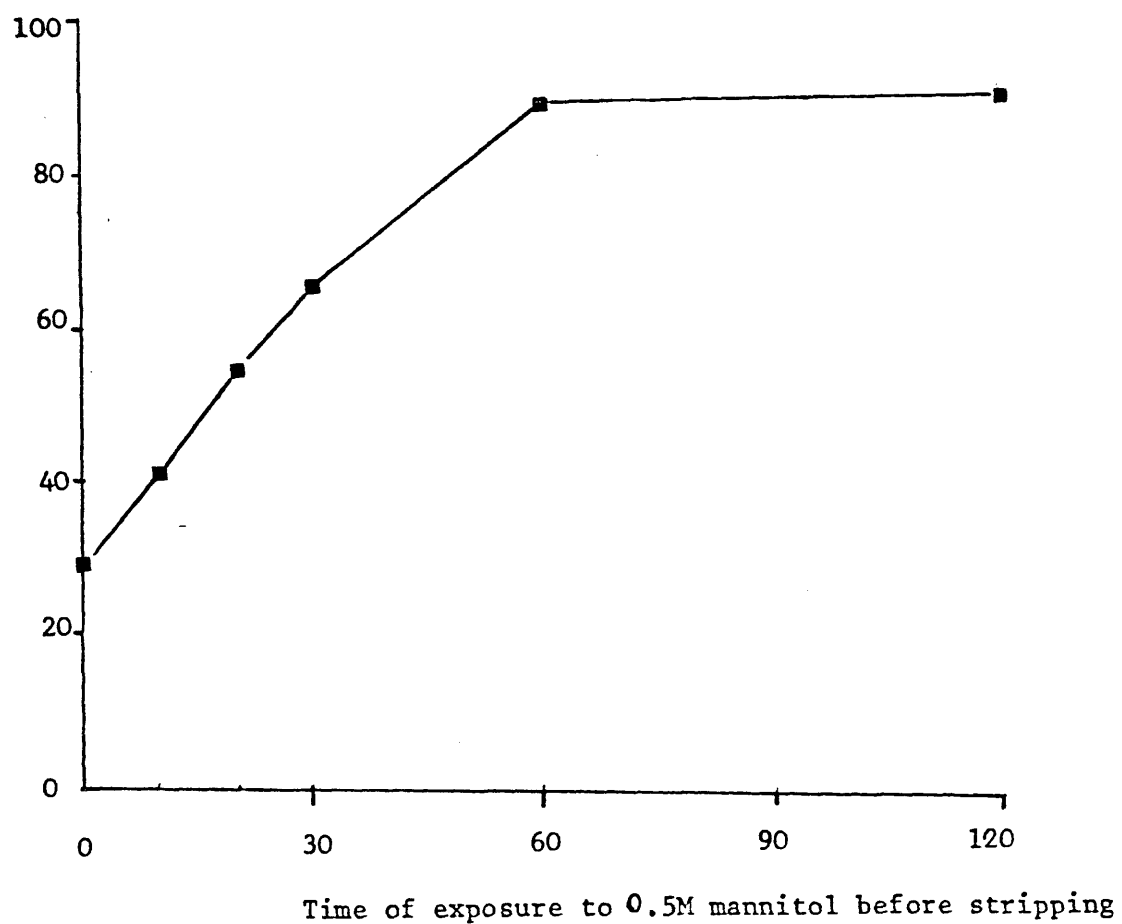
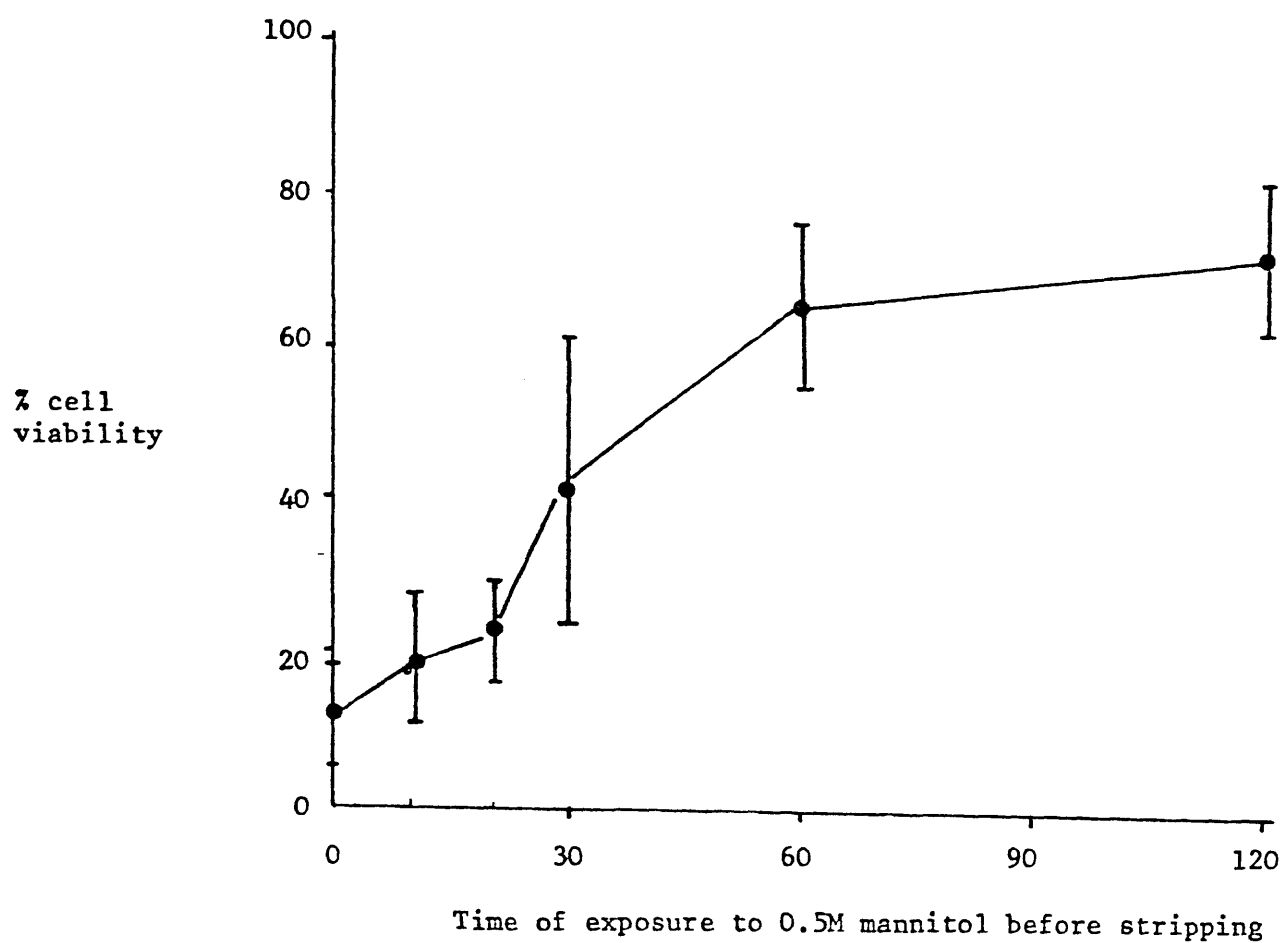


Figure 41: The effect of exposure of 0.5M mannitol on the viability of epidermal cells from stems of cultivar Feltham First infected for 21 days by E. pisi

Figure 41

ii) Feltham First



Mean of 10 replicates

 95% Confidence Limits

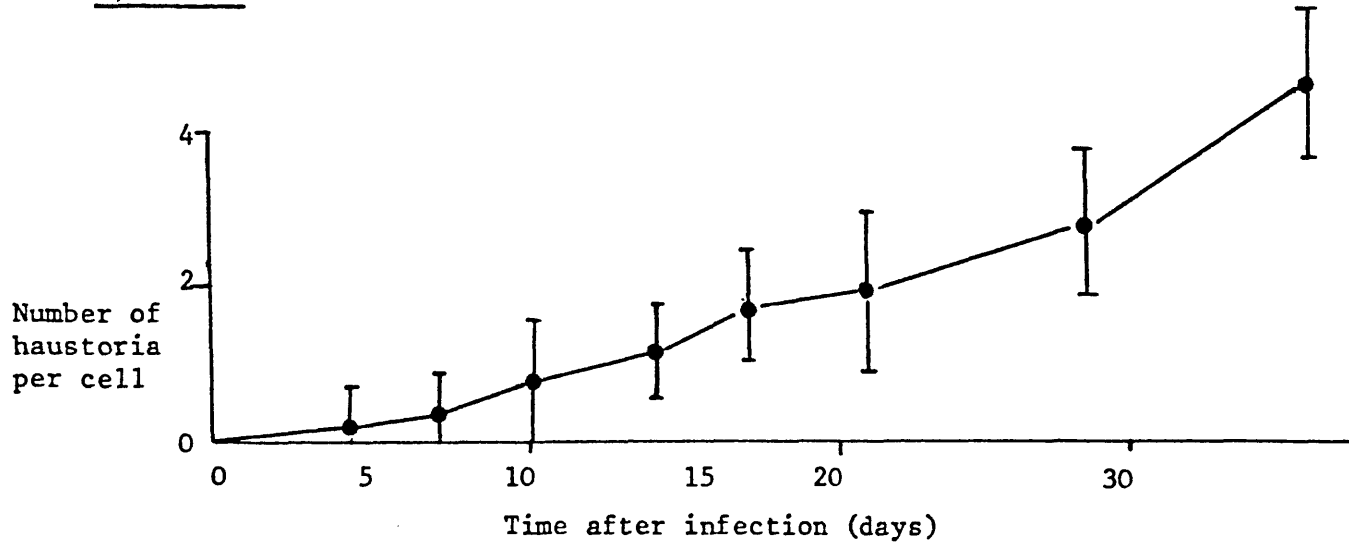
4.3.2. Experiments to assess the extent of infection by E. pisi at different time periods

The reason for this series of experiments was to assess the validity of time after infection as a suitable criterion of the extent of infection within cells. Infection was assessed as the average number of haustoria in each epidermal cell. The assessments were made at 9 different times during an infection period of 35 days. At each sampling point the mean of 10 replicate observations was calculated and the results presented in Figure 42. For each cultivar the results reveal an approximately linear increase in haustorial number with time. For all cultivars the slopes were approximately constant and equal and at no time were there significant ($p = 0.05$) differences between cultivars.

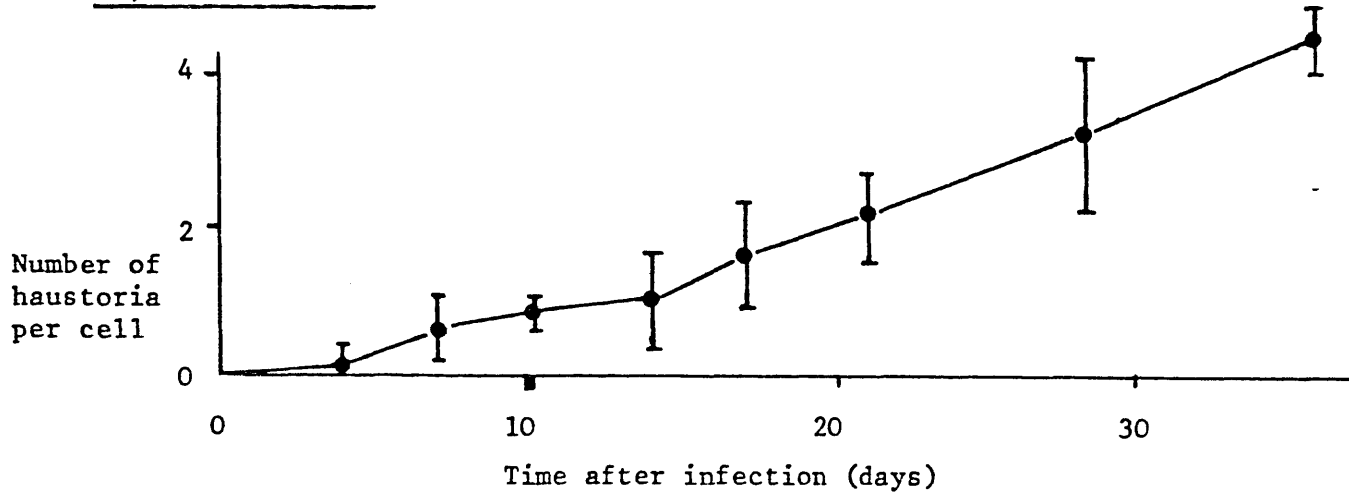
Figure 42: The number of haustoria in each epidermal cell at different times after infection

Figure 42

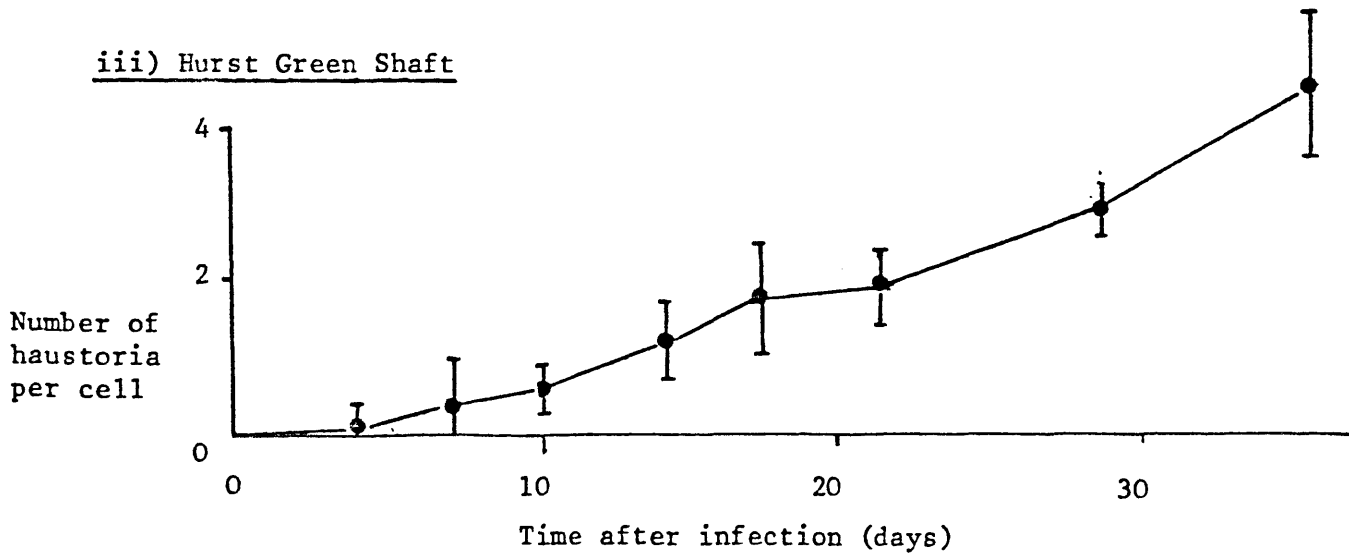
i) Onward



ii) Feltham First



iii) Hurst Green Shaft

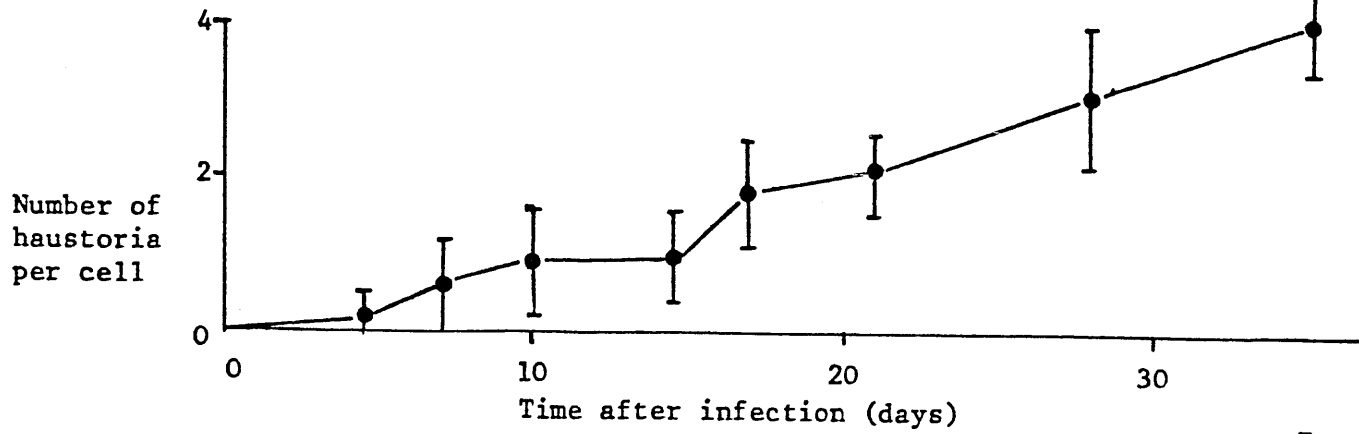


Results were the mean of 10 replicates

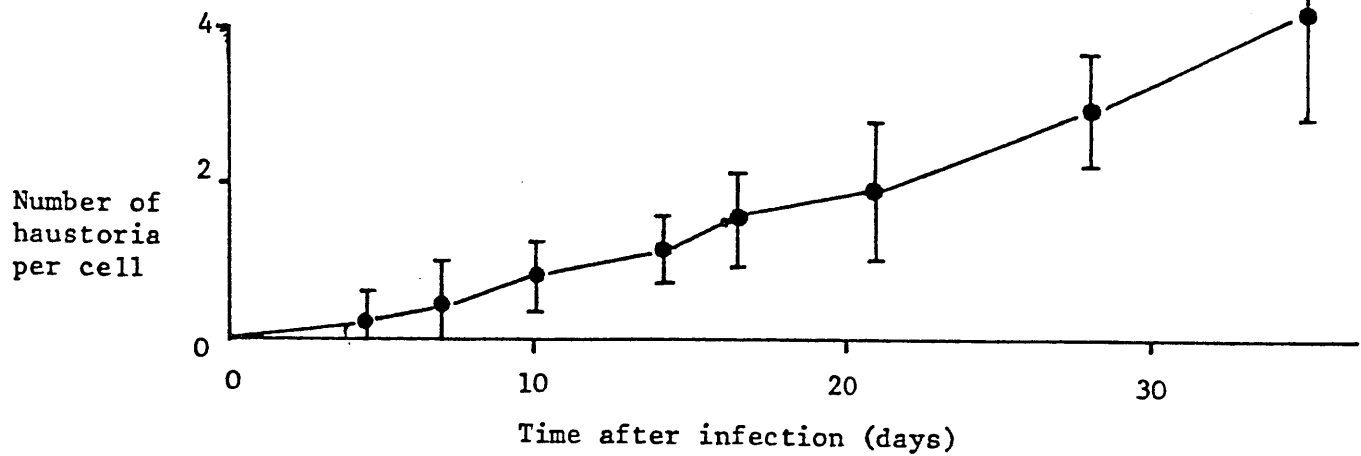
I 95% Confidence Limits

Figure 42 (cont.)⁴

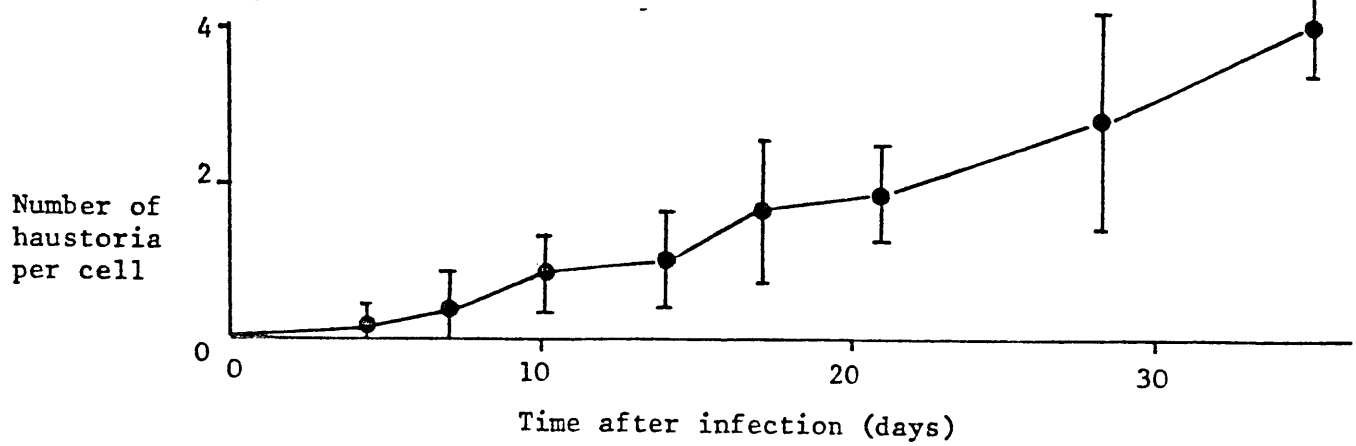
iv) Pilot



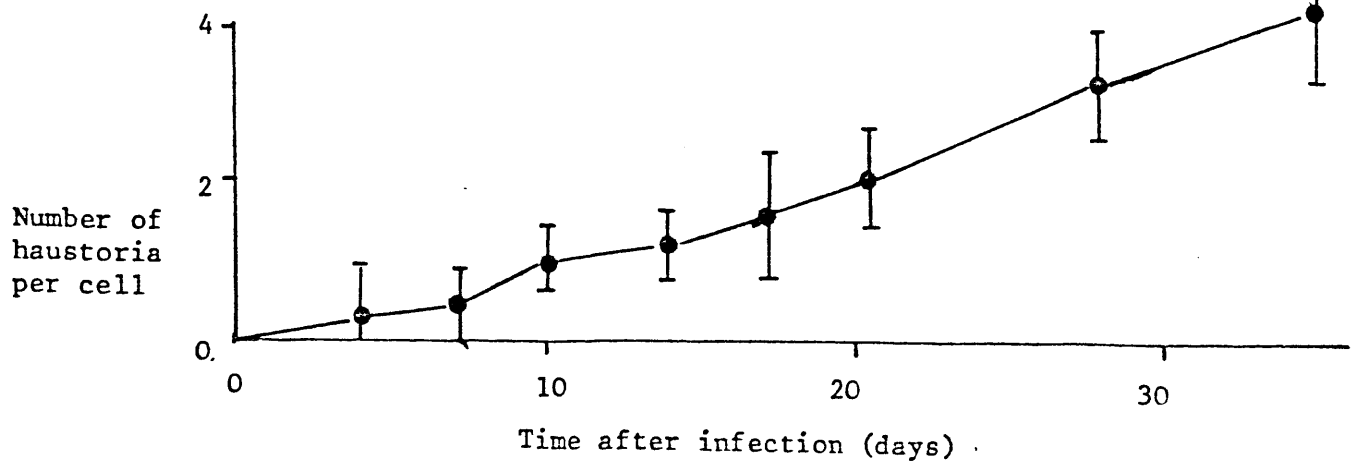
v) Perfection



vi) Meteor



vii) Dwarf Defiance



4.3.3. Cellular contamination of "epidermal" strips

These experiments were undertaken as it was important to know both how much non epidermal cell contamination was present in the strips and whether these values varied with the amount of infection. The extent of contamination is expressed as the % of the non epidermal cells in the strips and the amount of contamination was assessed up to the 35th day after infection. The results from healthy and infected stem epidermis are recorded in Table 19.

There were no significant cultivar-related differences ($p = 0.05$) in the degree of non epidermal cell contamination. Although there was a trend for an increase in the non-epidermal cell contamination with time after infection (Figure 43) there was no significant difference ($p = 0.05$) between the values for each cultivar at any one time during the 35 day infection period. Clearly stems are a purer source of epidermis than leaves.

Table 19: Contamination of epidermal strips by non epidermal cells in different cultivars

a) Leaves

Cultivars	% Non Epidermal cells	
	Uninfected Plants	Plants infected for 35 days
Onward	32.5 ± 16.6	52.3 ± 7.9
Feltham First	28.6 ± 18.0	54.0 ± 11.5
Hurst Green Shaft	23.6 ± 16.2	49.1 ± 8.9
Pilot	25.1 ± 16.1	50.2 ± 14.7
Perfection	34.7 ± 20.8	46.0 ± 14.4
Meteor	32.3 ± 18.6	52.1 ± 10.6
Dwarf Defiance	36.1 ± 20.1	51.3 ± 14.5

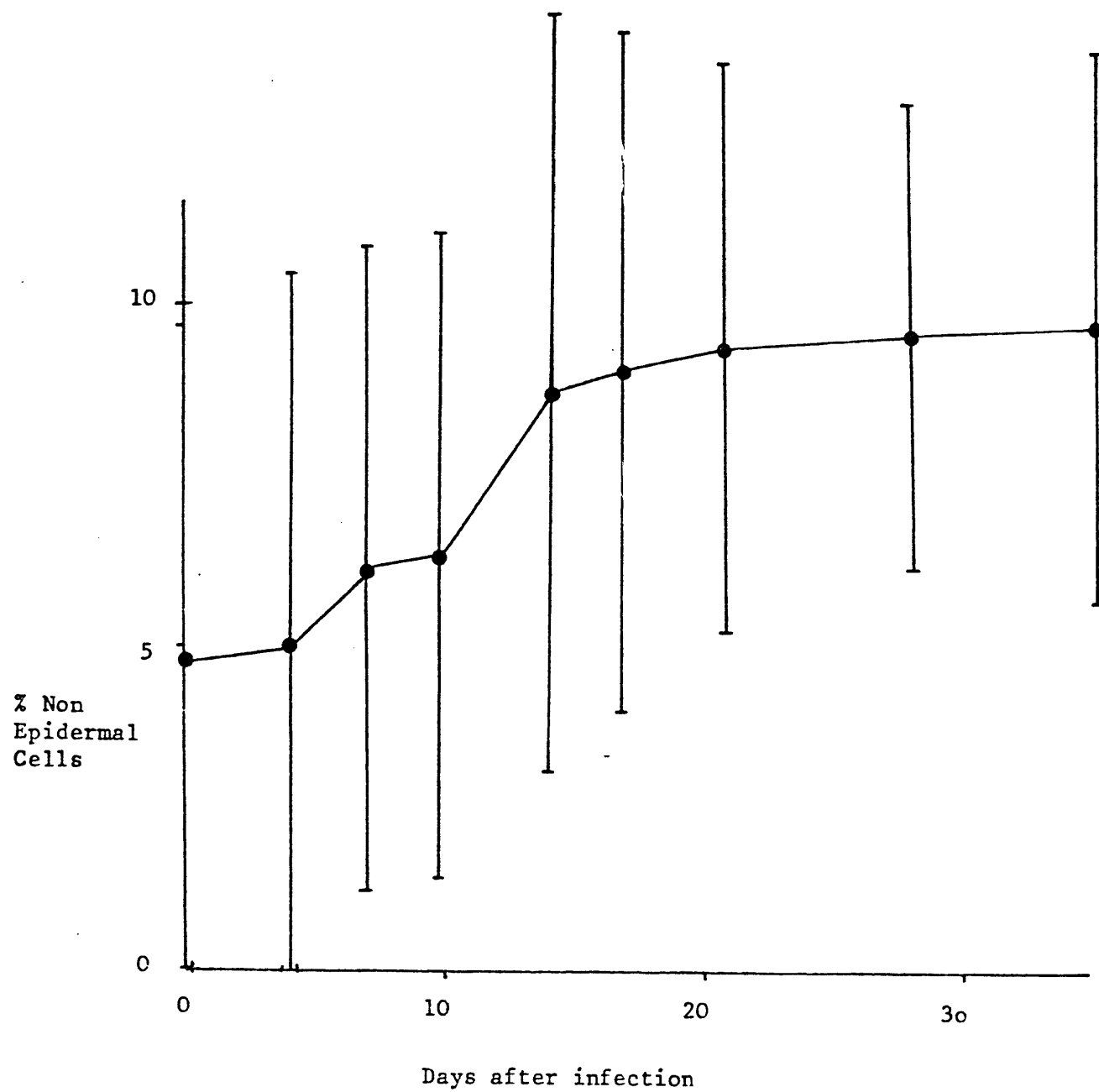
b) Stems

Cultivar	% Non Epidermal Cells	
	Uninfected Plants	Plants Infected for 35 days
Onward	5.9 ± 6.1	9.3 ± 4.3
Feltham First	5.2 ± 7.3	10.1 ± 4.9
Hurst Green Shaft	4.2 ± 5.2	8.9 ± 5.6
Pilot	5.1 ± 5.3	10.4 ± 3.8
Perfection	2.7 ± 4.7	9.7 ± 2.9
Meteor	3.8 ± 3.4	9.9 ± 4.8
Dwarf Defiance	6.5 ± 6.7	10.7 ± 4.1

The results are the means of 10 replicates quoted with 95% confidence limits

Figure 43: The percentage of non epidermal cells in strips from stems of pea plants at different times after infection by E. pisi

Figure 43



I 95% Confidence Limits

4.3.4. The influence of infection by *E. pisi* on the lipid composition in epidermal cells of pea, cultivar Onward

In all these experiments strips of stem epidermis from stems that had been pre-plasmolysed in 0.5M mannitol were used.

a) Total Lipids

The mean total lipid, total polar lipid and total neutral lipids were determined at various times during the course of a 42 day infection period to determine any changes in lipid amounts and also to monitor any alteration in the proportion of polar and neutral lipids. The amounts of total, polar and neutral lipids in uninfected plants did not change significantly ($P = 0.05\%$) throughout the 42 day period (Figure 44). Uninfected plants contained $63.2 \pm 4.8 \mu\text{g lipid g}^{-1}$ dry weight at the start of the experiment falling to $60.8 \pm 3.1 \mu\text{g lipid g}^{-1}$ dry weight after 42 days. Figure 44 shows that in both healthy and infected epidermis polar lipid predominated over neutral lipids by a ratio of approximately four to one and that this relationship remained essentially constant with time.

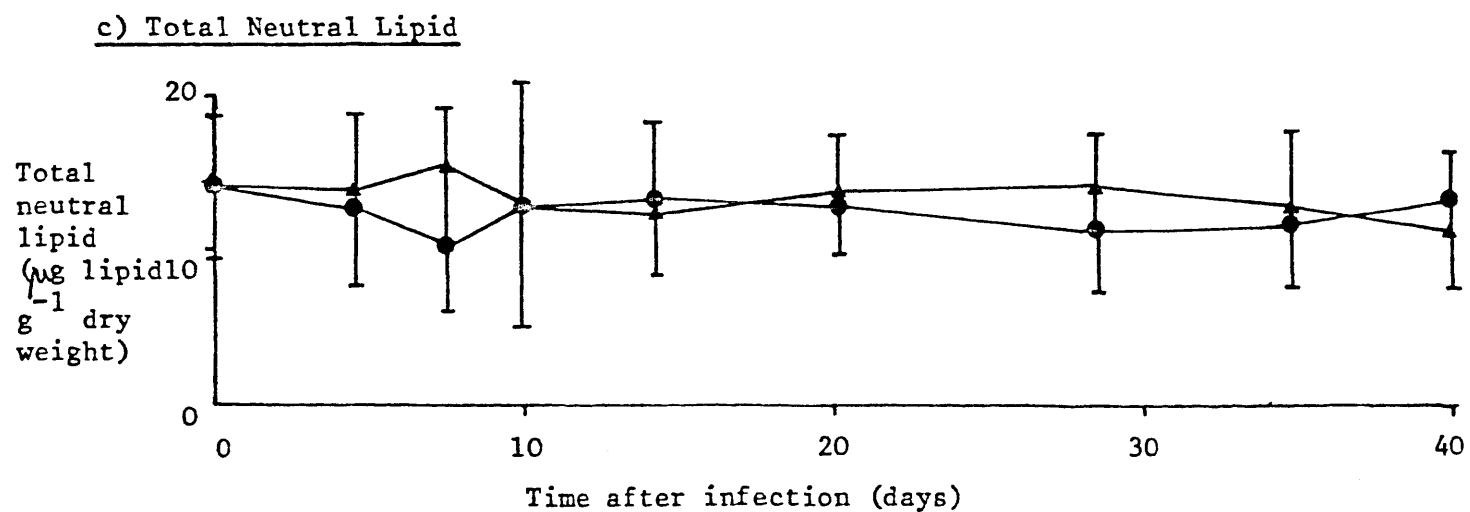
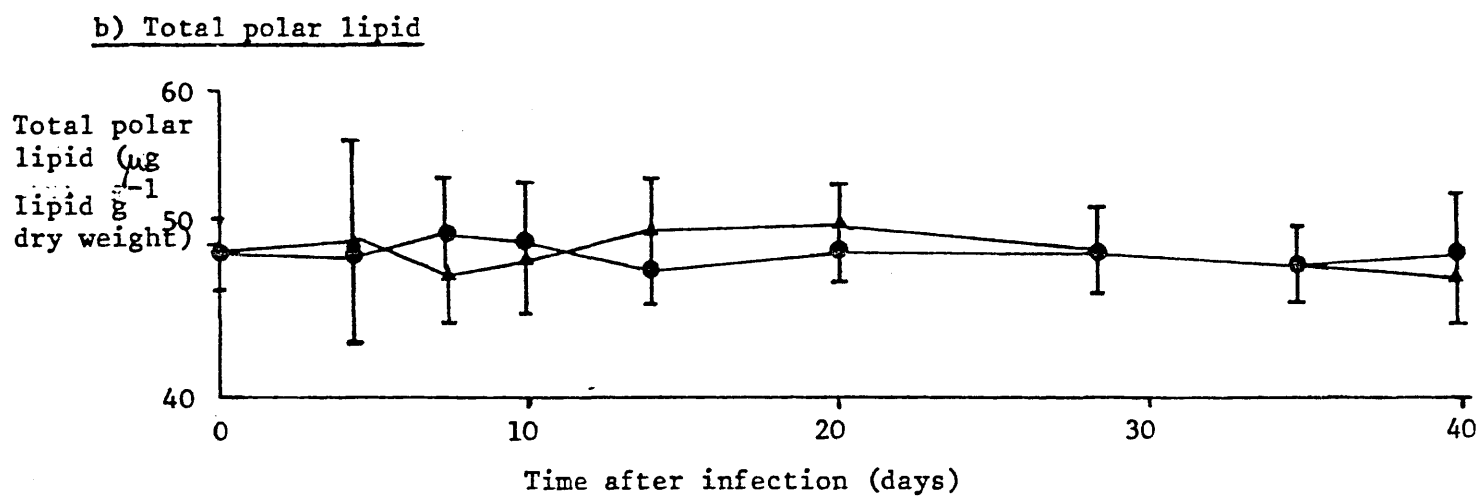
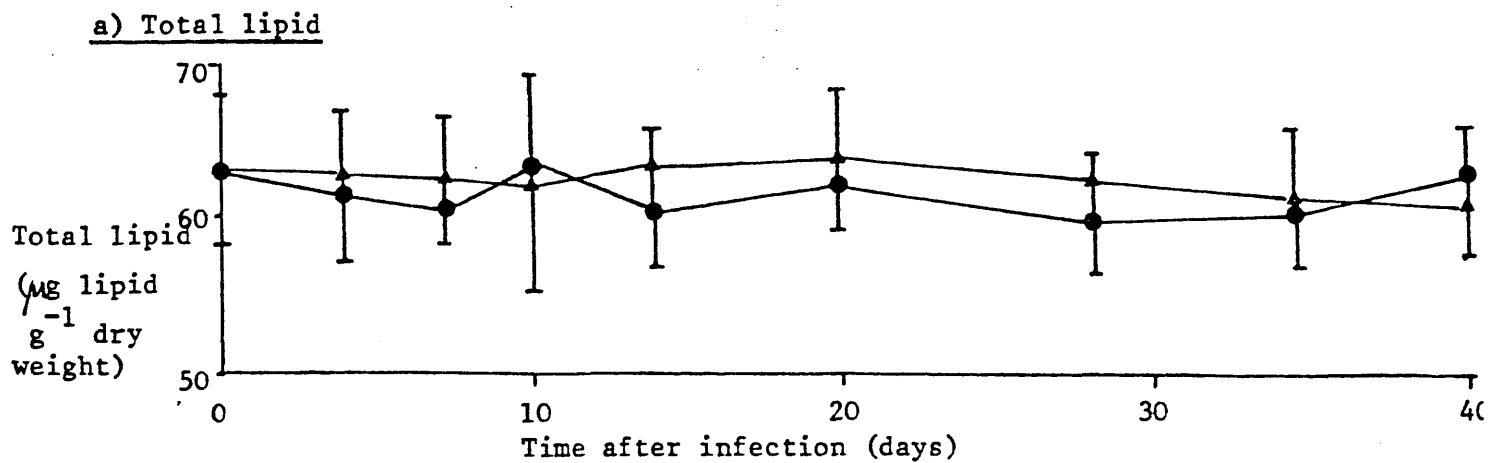
b) Individual Polar Lipids

The individual polar lipids were assessed during the same 42 day time period and at the same intervals. The percentage composition of each of the individual polar lipids at the beginning of the experiment in epidermal cells from uninfected plants were as follows:- PC $46.0 \pm 4.9\%$, PE $25.5 \pm 5.3\%$, PG $6.2 \pm 2.3\%$, PI $14.7 \pm 2.3\%$, PS $3.7 \pm 2.3\%$, PA $0.7 \pm 0.7\%$, MGDG $2.0 \pm 1.1\%$ and DGDG $1.0 \pm 1.1\%$. After 42 days the percentage composition of these lipids in uninfected epidermal cells had not changed significantly (Figure 45).

At all stages during the development of infection, epidermal strips from infected plants had a very similar polar lipid composition. There were however, significant decreases ($p = 0.05$) in the percentage composition of MGDG and DGDG during this period and there was also a trend towards a reduction in the amount of PG although this was not statistically significant ($p = 0.05$) (Figure 45).

Figure 44: The effect of infection by E. pisi on the lipid composition of epidermal strips from the pea cultivar Onward

Figure 44



- Uninfected Plants
- ▲ Infected Plants
- ┃ 95% Confidence Limits

Figure 45: The effect of infection by E. pisi on the individual polar lipid composition in epidermal strips from the cultivar Onward

Figure 45

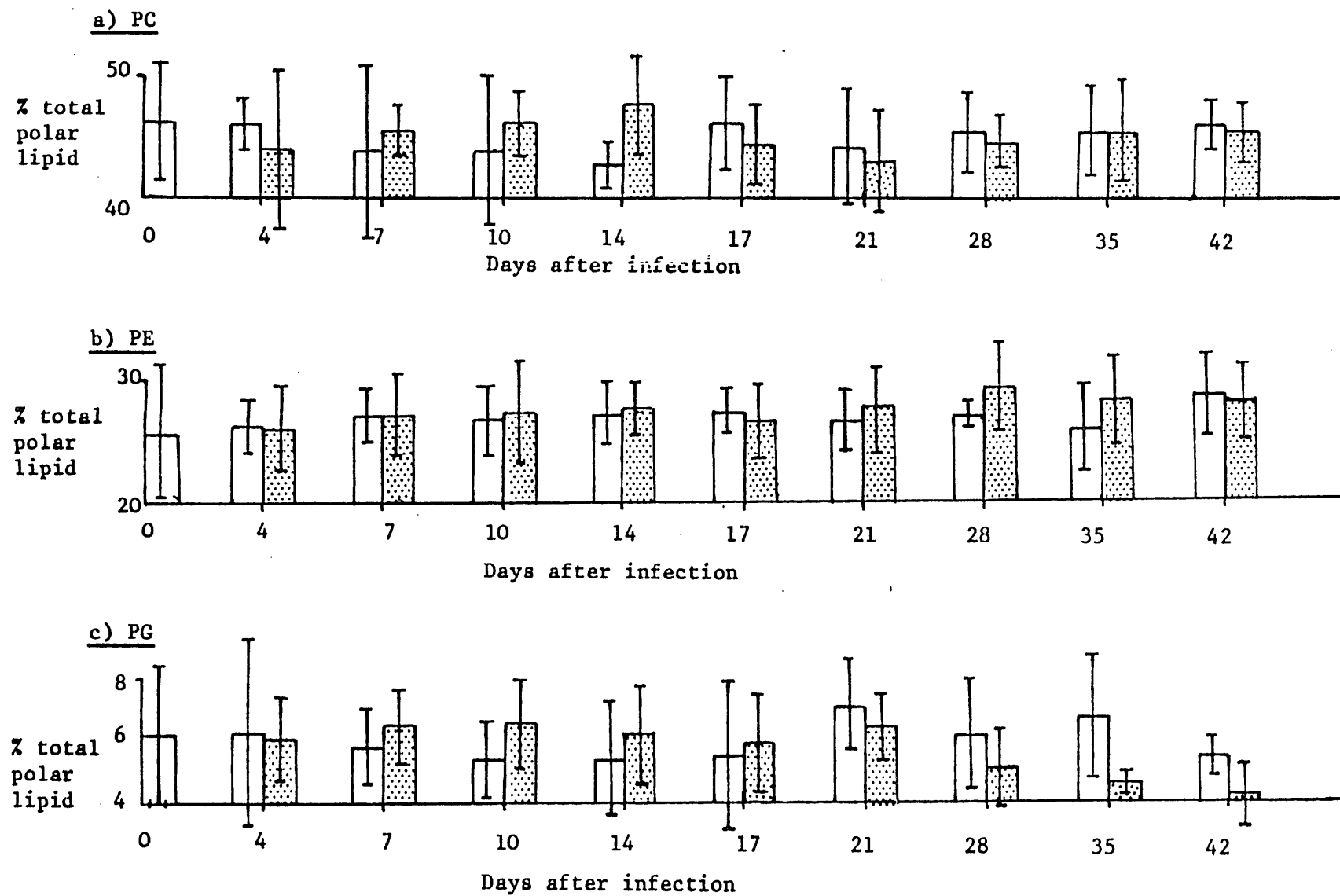
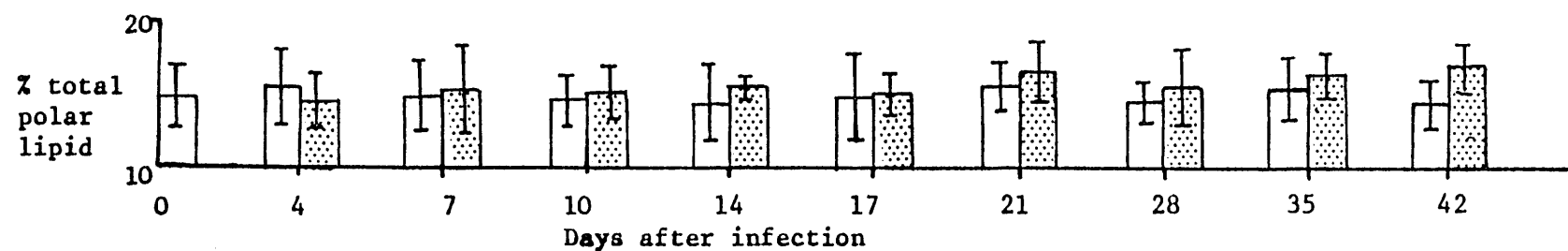
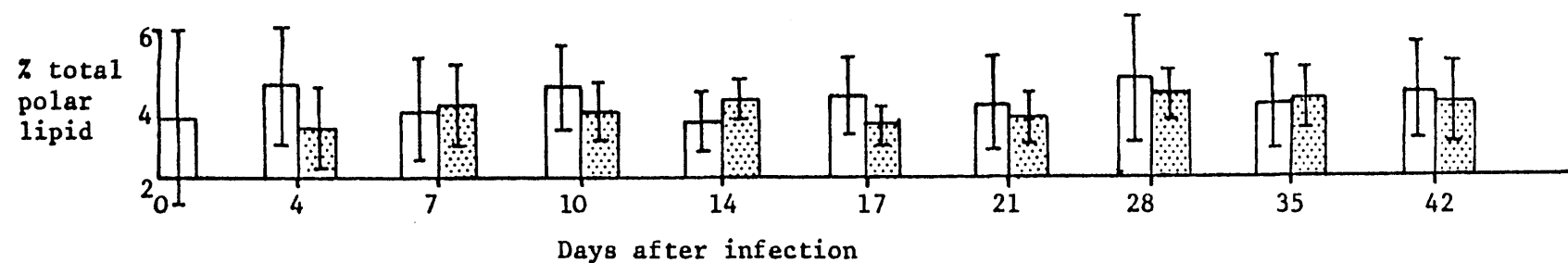


Figure 45 (cont.)

d) PI



e) PS



f) PA

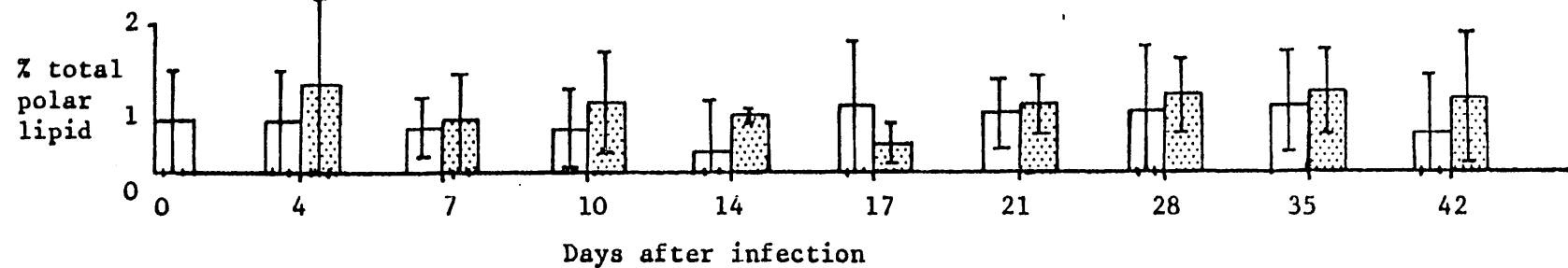
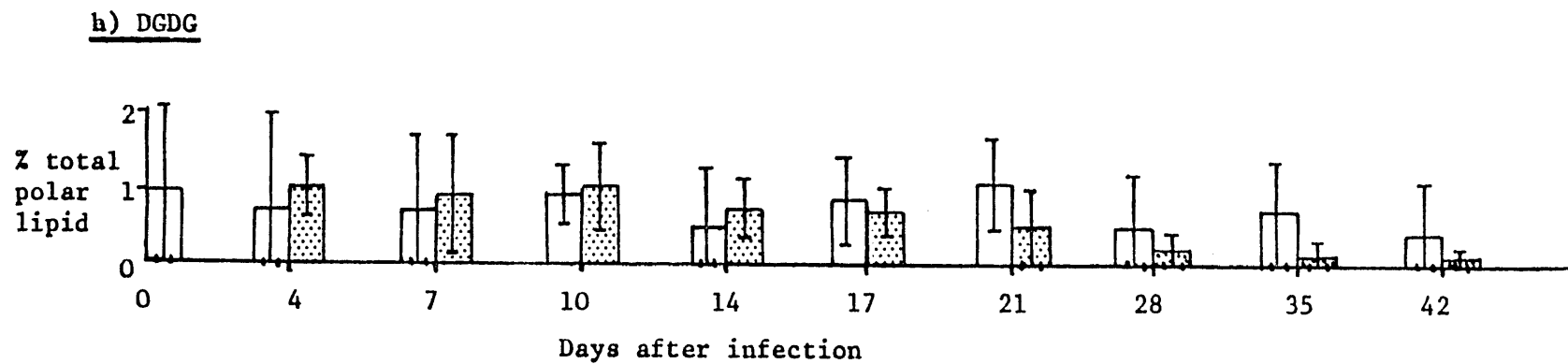
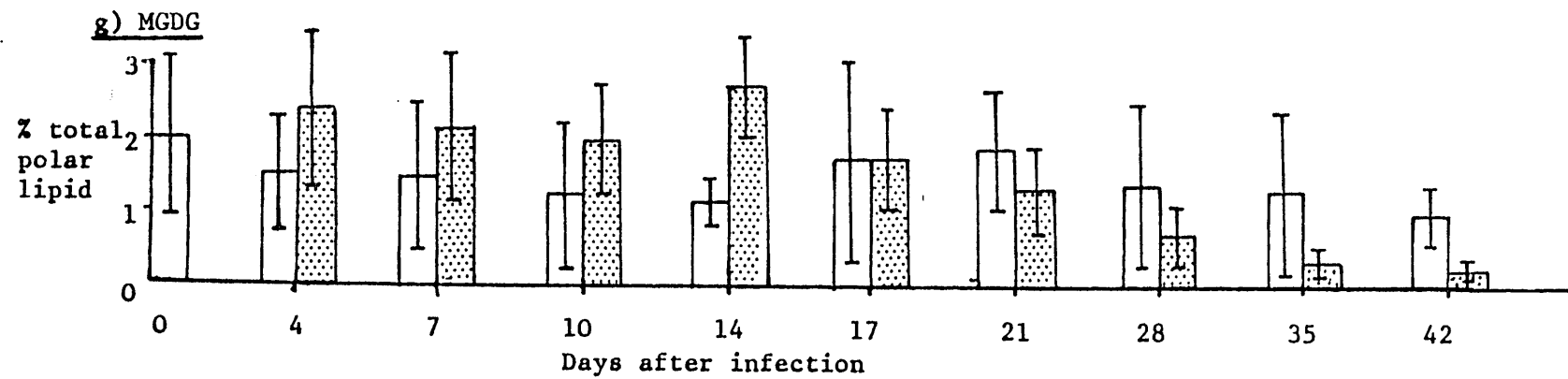


Figure 45 (cont.)



Unshaded Uninfected Plants

Shaded Infected Plants

┌ 95% Confidence Limits

c) Total polar lipid bound fatty acids

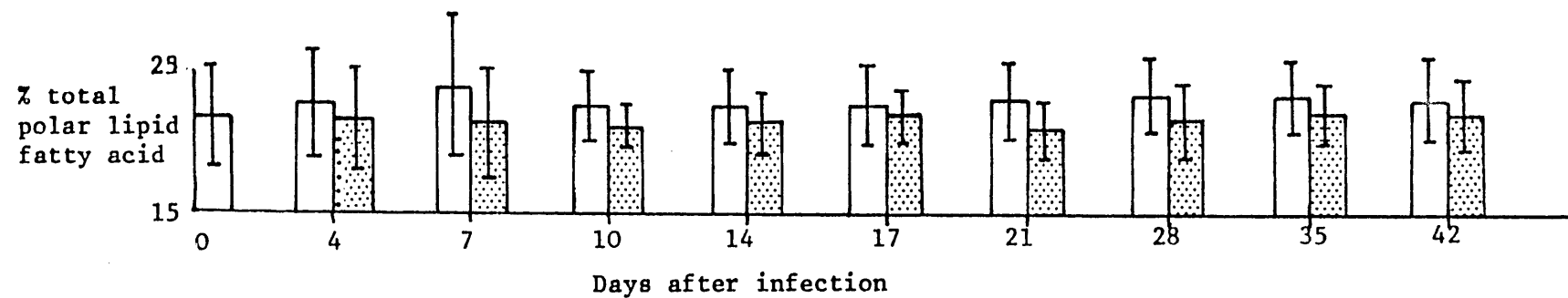
When the total polar lipid bound fatty acids of epidermal cells were assessed over the 42 day period the percentage composition of each fatty acid at the outset was as follows:- palmitic acid $21.8 \pm 3.4\%$, palmitoleic acid $0.5 \pm 0.5\%$, stearic acid $6.9 \pm 1.4\%$, oleic acid $4.2 \pm 1.1\%$, linoleic acid $40.5 \pm 3.3\%$ and linolenic acid $28.1 \pm 2.6\%$. After 42 days these values had not changed significantly ($p = 0.05$) in healthy plants, but in strips from infected plants there was a dramatic decrease in linoleic acid and a concomitant increase in linolenic acid (Figure 46). These changes were first apparent about day 10, if a small and statistically insignificant decline in palmitic acid is discounted, are the only marked changes detectable upon infection.

To test the effect of these changes upon the degree of saturation a fatty acid index was calculated for each stage of the experiment. This index was calculated as follows: $(3 \times \text{fatty acid with 3 double bonds} + 2 \times \text{fatty acids with 2 double bonds} + \text{fatty acids with 1 double bond})$ divided by total saturated fatty acids. Figure 47 shows the effect of infection on the fatty acid index for the total polar lipid-bound fatty acids. The index values for healthy and infected plants appear to diverge from the first sampling period onwards and differ significantly by day 14.

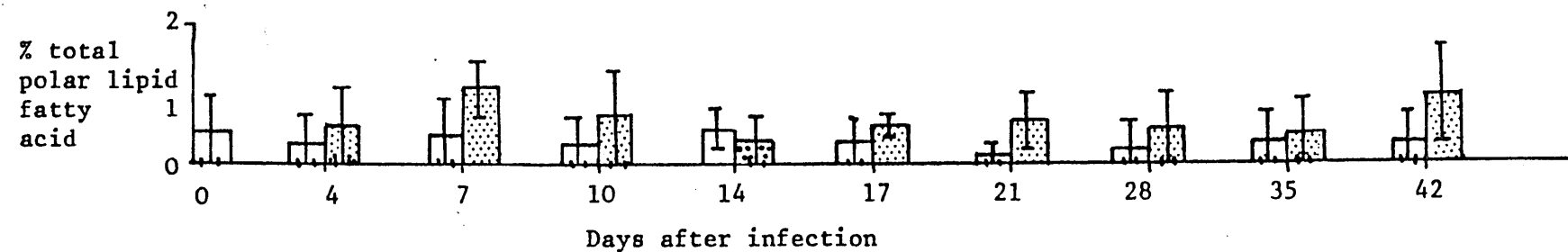
Figure 46: The effect of infection of E. pisi on the total polar lipid fatty acid composition in epidermal strips from the cultivar Onward

Figure 46

a) Palmitic acid



b) Palmitoleic acid



c) Stearic acid

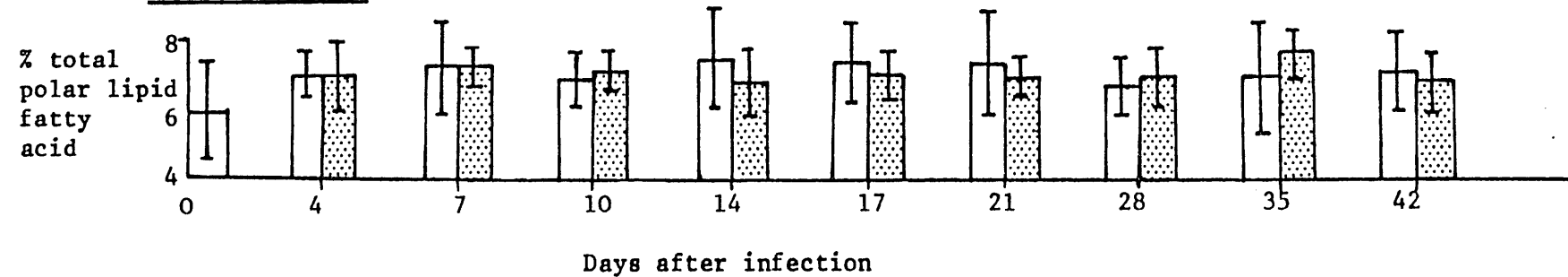
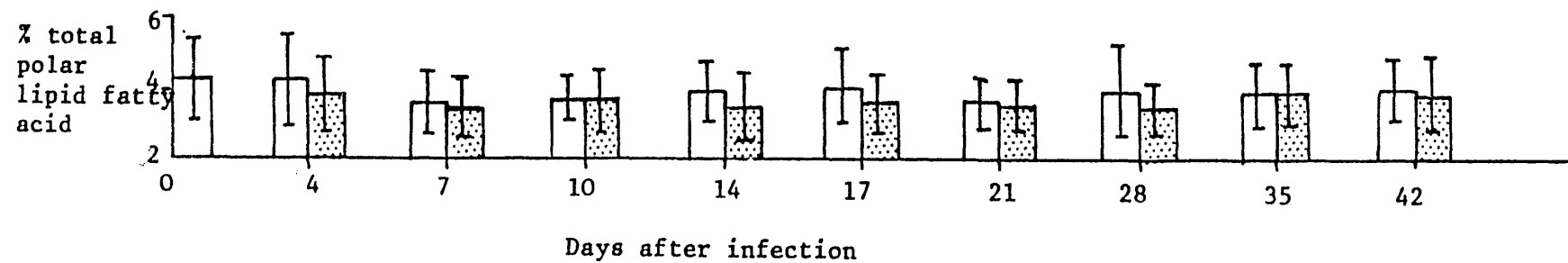


Figure 46 (cont.)

d) Oleic acid



e) Linoleic acid

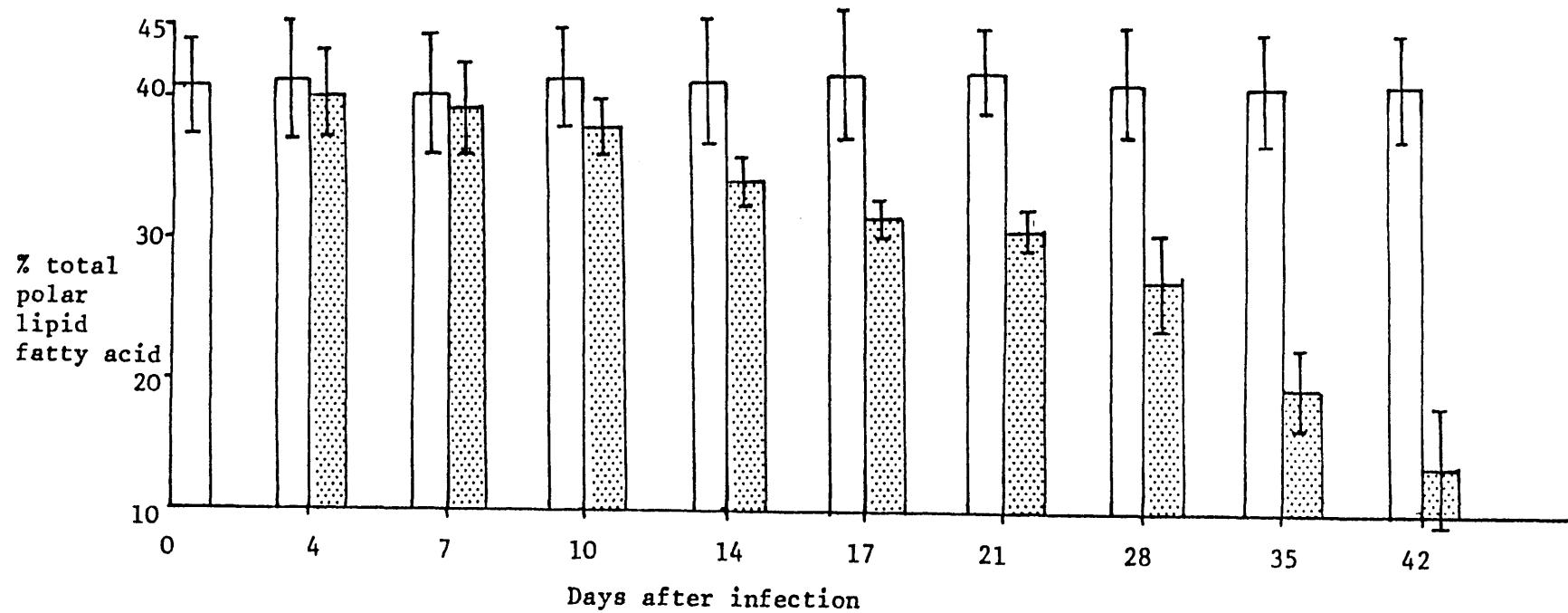
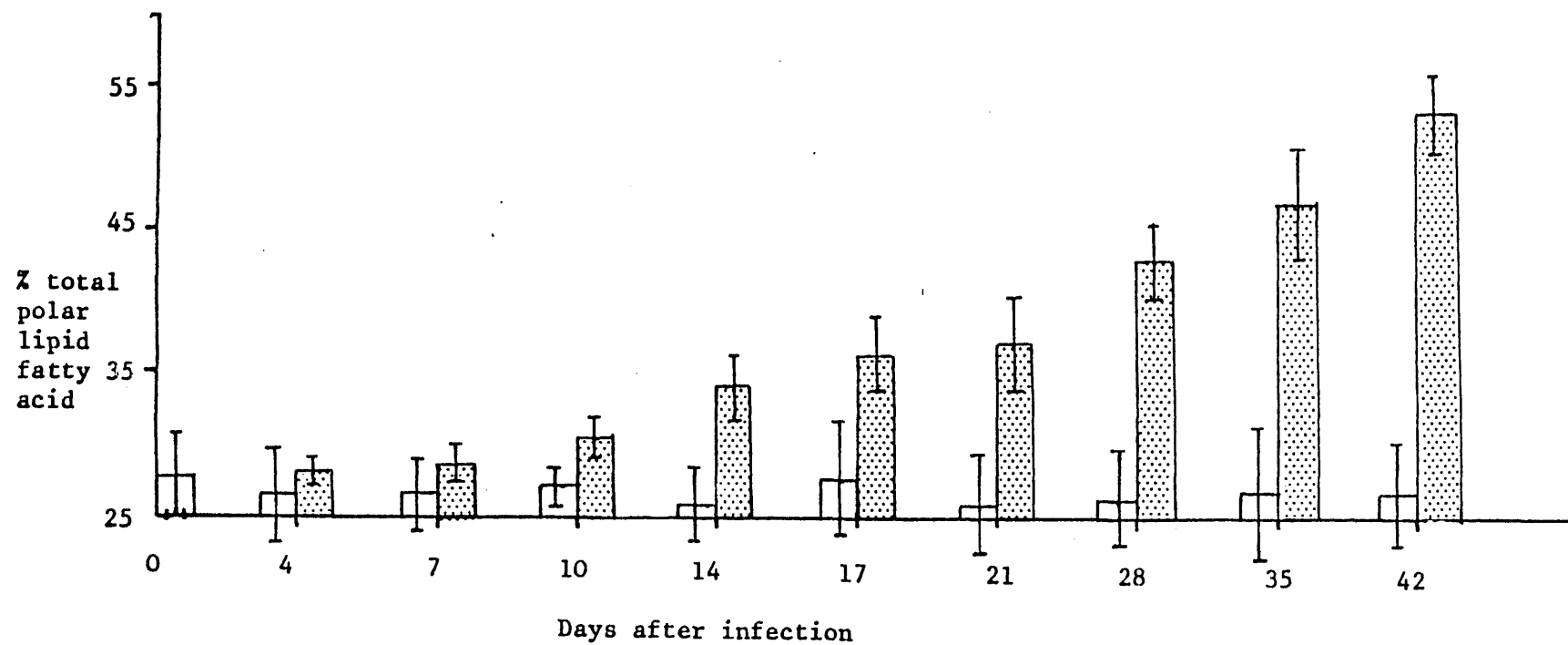


Figure 46 (cont.)

f) Linolenic acid



Unshaded Uninfected Plants

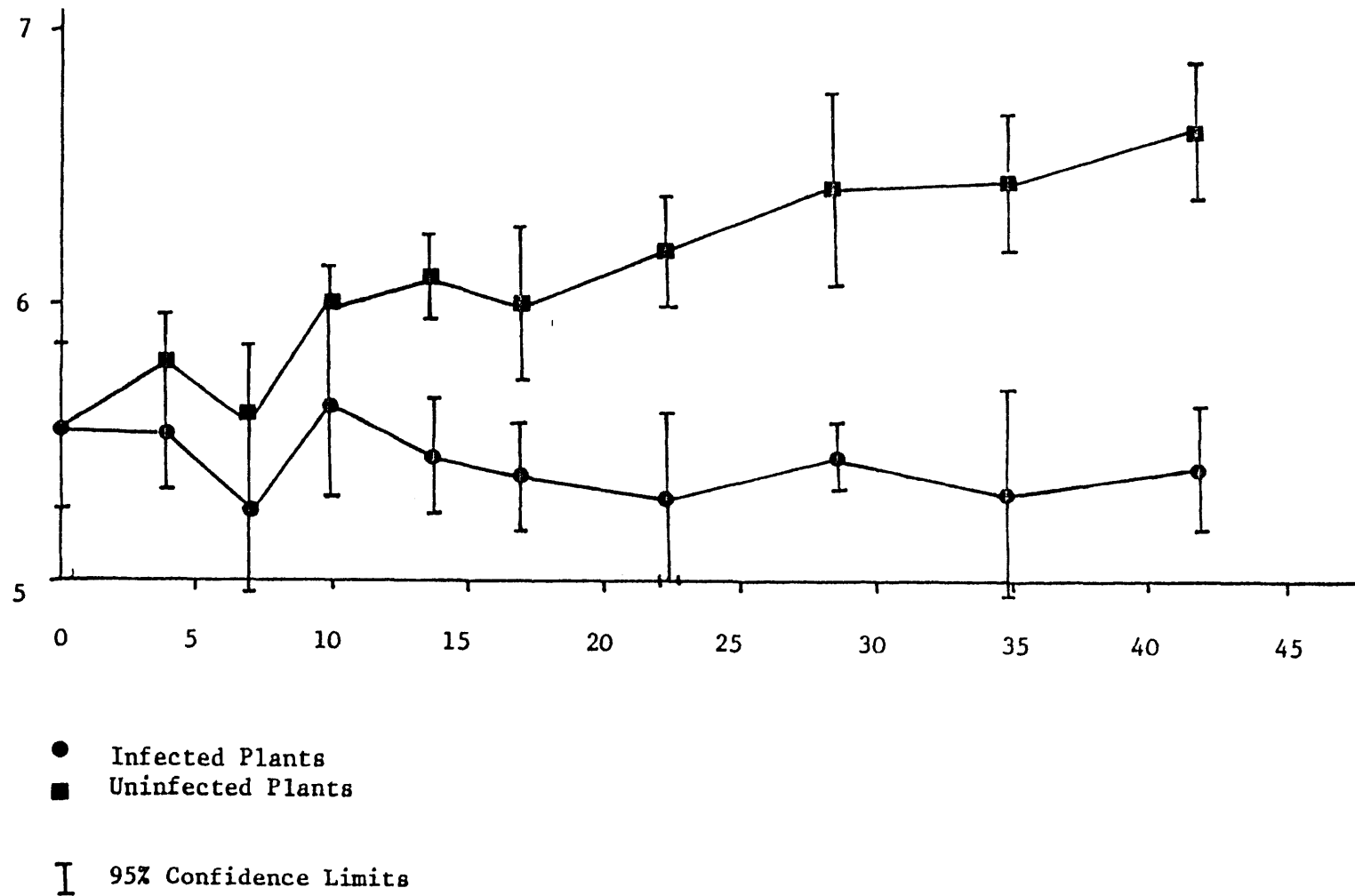
Shaded Infected Plants

I 95% Confidence Limits

Figure 47: The effect of infection of E. pisi on the fatty acid index of polar lipid-bound fatty acids in epidermal strips from stems of the cultivar Onward

Figure 47

Fatty acid index



d) Fatty acids of individual polar lipids

Individual polar lipids were analysed to see if the changes observed in the previous section were general within the class or restricted to particular constituent lipids. The percentage fatty acid composition of each polar lipid at the outset of the experiment is shown in Table 20. In healthy plants these figures do not change significantly ($p = 0.05$) over the 42 day period (Figure 48, 49, 50, 51, 52, 53, 54 and 55). In infected plants the most obvious change was a marked decrease in the content of linoleic acid and a corresponding increase in linolenic acid. This change was observed for each of the phospholipids although for PG the change was smaller and occurred later, but was not seen for either of the galactolipids. As observed for the class as a whole for most of the individual components there was a noticeable increase in the degree of fatty acid unsaturation (Figures 56, 57, 58, 59, 60, 61, 62 and 63). Divergence of the data for healthy and infected plants was generally apparent from an early stage, but statistical significance at the 5% level was not achieved until days 10 (PA), 14 (PE and PS), 17 (PC and PI) or exceptionally day 35 for PG. Relative changes in the proportions of linoleic and linolenic acids accounts for the overwhelming majority of the index changes. Only very minor changes not statistically significant at the 5% level of probability were noted for the other fatty acids. For example in PC, PE and PI there was a tendency for palmitic acid levels to be lower in infected than non-infected epidermis, but for the remaining phospholipids a similar trend was not evident.

Clearly the PG differs markedly from the other phospholipids, it is only polar lipid to have a significant palmitoleic acid content and it contains significantly ($p = 0.05$) less stearic acid and linoleic acids and more linolenic acid than the other phospholipids (Table 20).

The two galactolipids clearly constitute a distinct sub-group within the polar lipid class. Not only is the fatty acid composition totally different from the phospholipids, but also there are virtually no changes resulting from infection. The linolenic acid content of both MGDG and DGDG is so high even in healthy plants (index values ≈ 60 and 20 respectively) that a further increase in the degree of unsaturation could not be easily

achieved.

Table 20: Fatty acids of individual polar lipids of pea stem epidermis

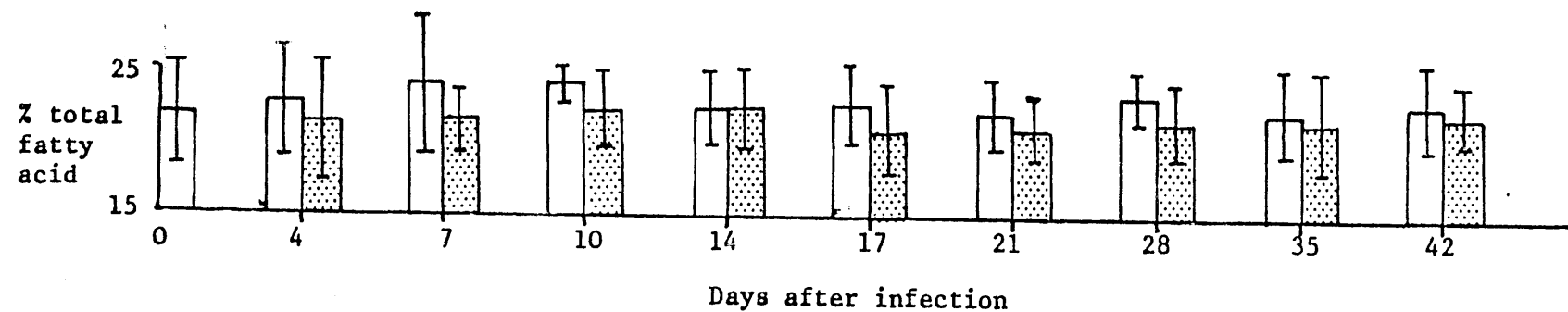
	PC	PE	PG	PI	PS	PA	MGDG	DGDG
Palmitic Acid	22.1 ± 3.3	22.8 ± 2.2	25.0 ± 1.8	22.9 ± 4.2	22.6 ± 3.4	22.5 ± 3.2	3.3 ± 1.7	9.9 ± 1.7
Palmitoleic Acid	0	0.3 ± 0.3	18.7 ± 4.2	0.3 ± 0.4	0	0.1 ± 0.1	0.1 ± 0.1	0
Stearic Acid	6.3 ± 1.0	7.0 ± 0.6	2.3 ± 0.8	7.4 ± 1.5	5.0 ± 1.3	5.6 ± 0.7	0.6 ± 0.5	2.8 ± 0.9
Oleic Acid	3.4 ± 0.7	3.6 ± 0.6	4.2 ± 1.3	2.9 ± 0.8	2.6 ± 0.6	2.7 ± 0.8	1.4 ± 0.5	0.9 ± 0.8
Linoleic Acid	40.4 ± 4.8	39.3 ± 1.6	11.1 ± 1.9	39.8 ± 4.4	40.7 ± 4.6	42.2 ± 4.7	3.8 ± 0.4	3.0 ± 0.8
Linolenic Acid	27.7 ± 2.7	26.9 ± 1.8	37.8 ± 4.6	26.9 ± 1.1	24.0 ± 2.6	26.8 ± 2.0	90.9 ± 2.0	83.3 ± 2.0

All results were the means of 4 replicates quoted with 95% confidence limits

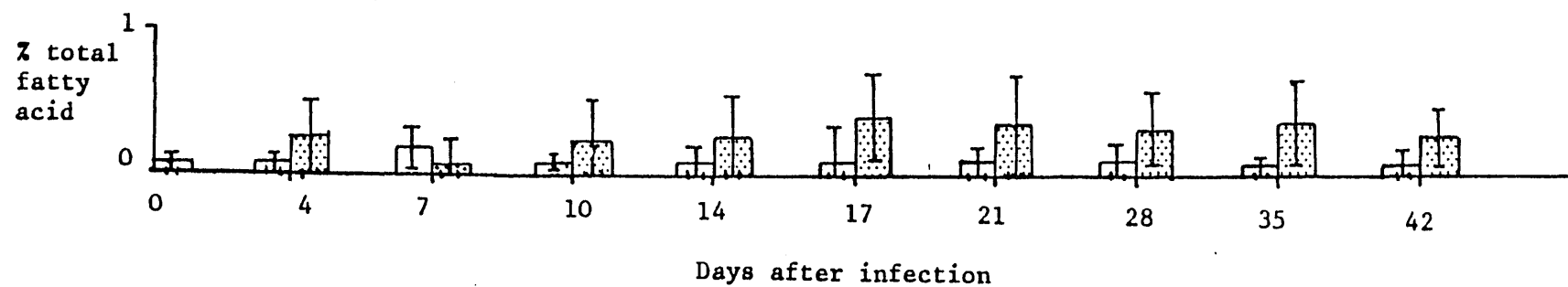
Figure 48: The effect of infection by E. pisi on the fatty acid composition of PC in epidermal strips from the cultivar Onward

Figure 48

a) Palmitic acid



b) Palmitoleic acid



c) Stearic acid

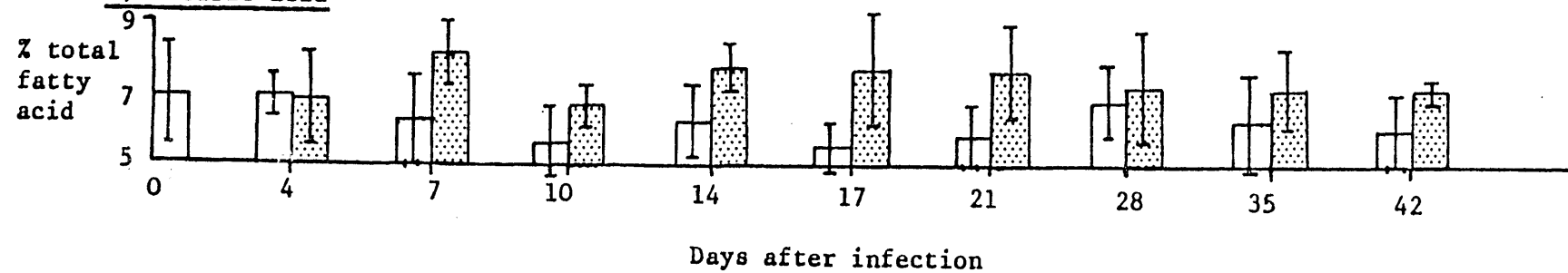
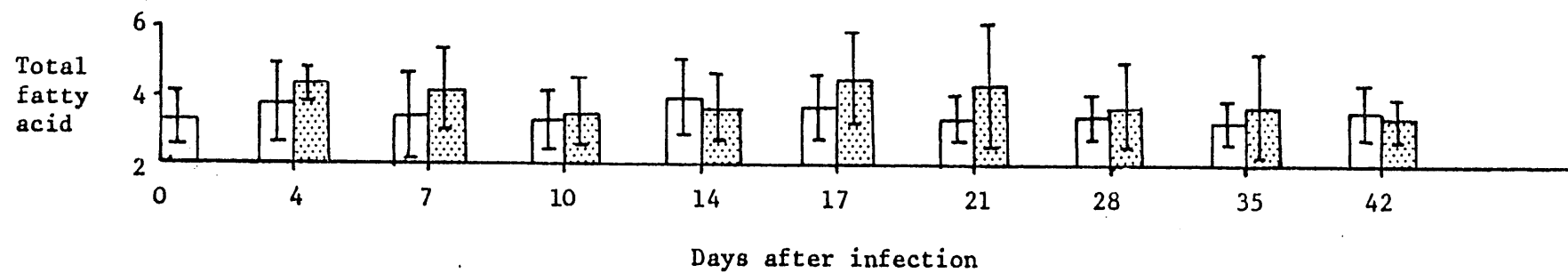


Figure 48 (cont.)

d) Oleic acid



e) Linoleic acid

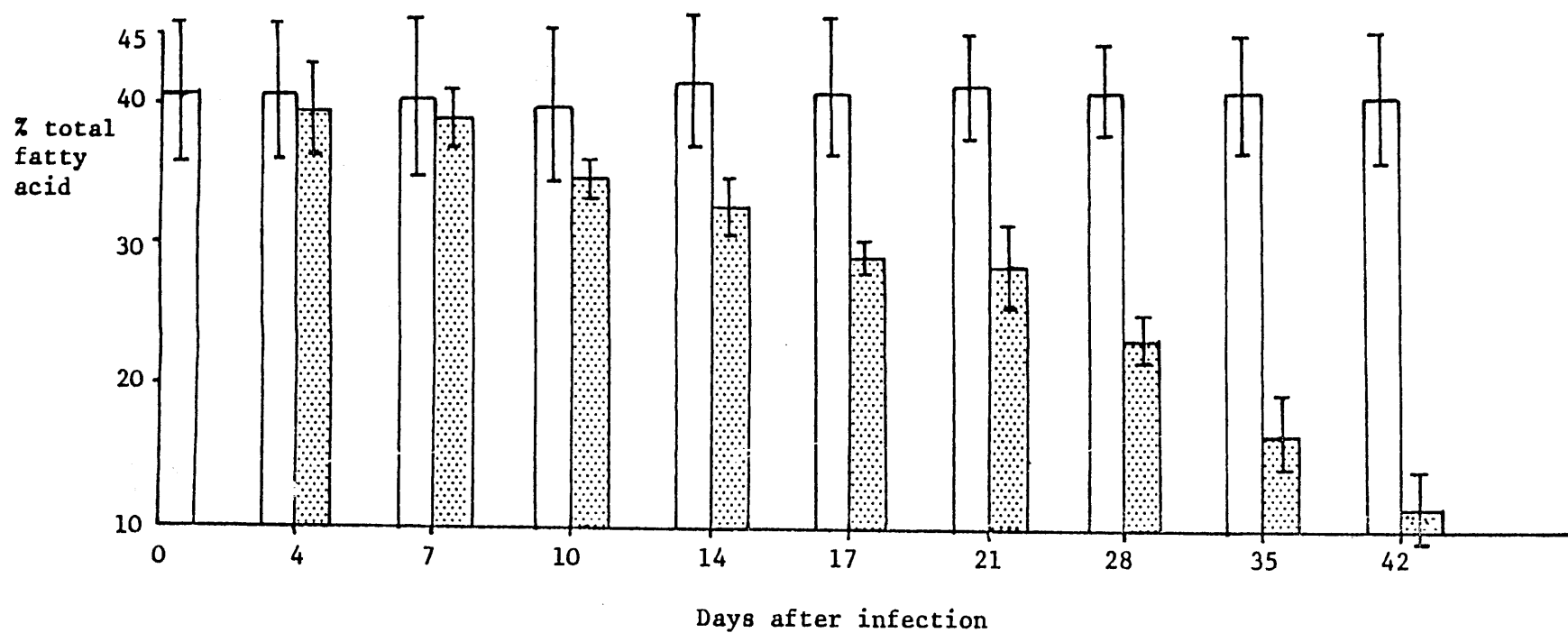
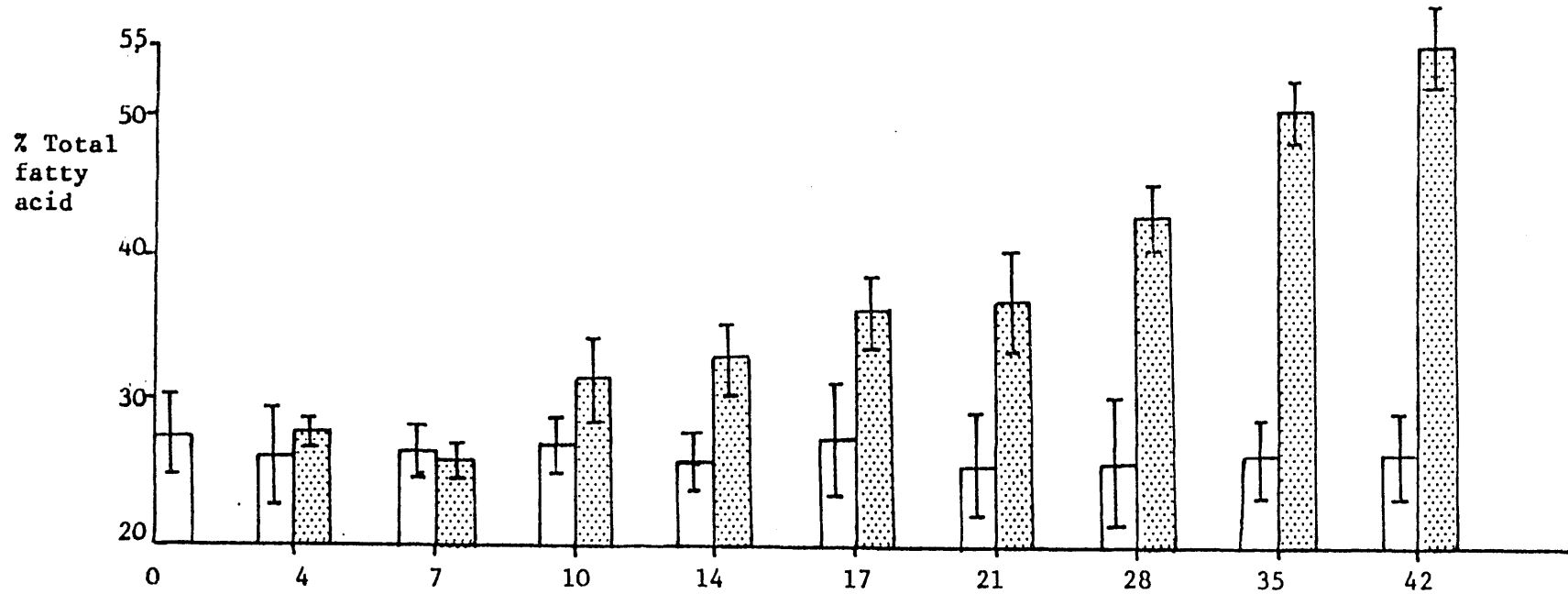


Figure 48 (cont.)

f) Linolenic acid



Unshaded Uninfected Plants

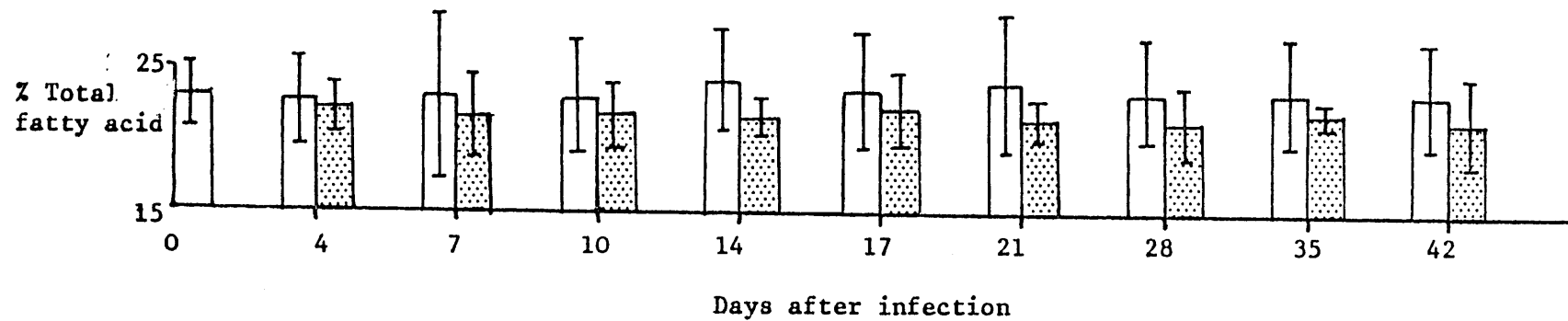
Shaded Infected Plants

I 95% Confidence Limits

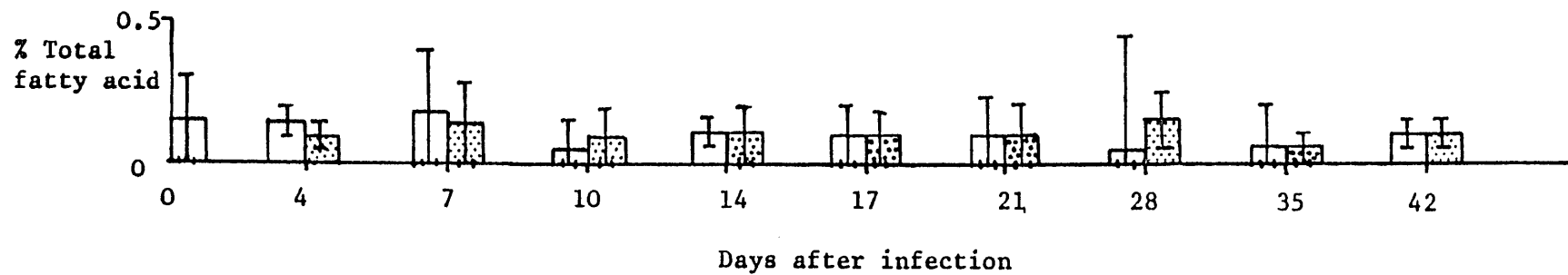
Figure 49: The effect of infection by E. pisi on the fatty acid composition of PE in epidermal strips from the cultivar Onward

Figure 49

a) Palmitic acid



b) Pamitoleic acid



c) Stearic acid

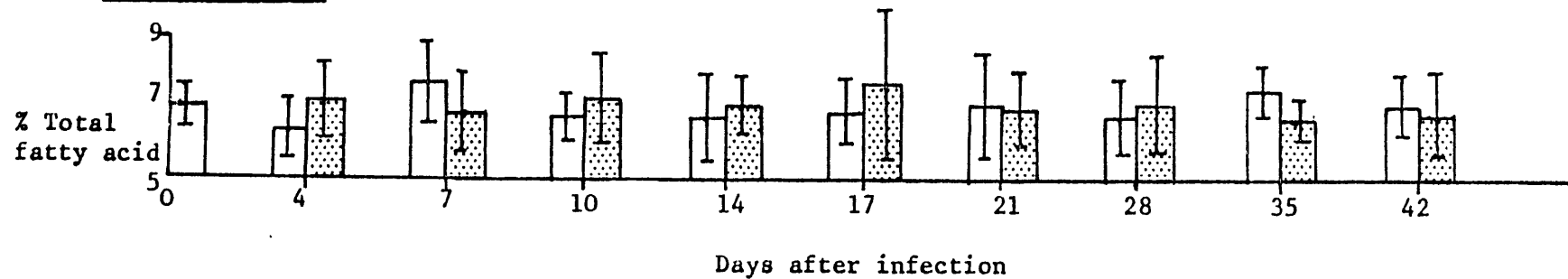


Figure 49 (cont.)

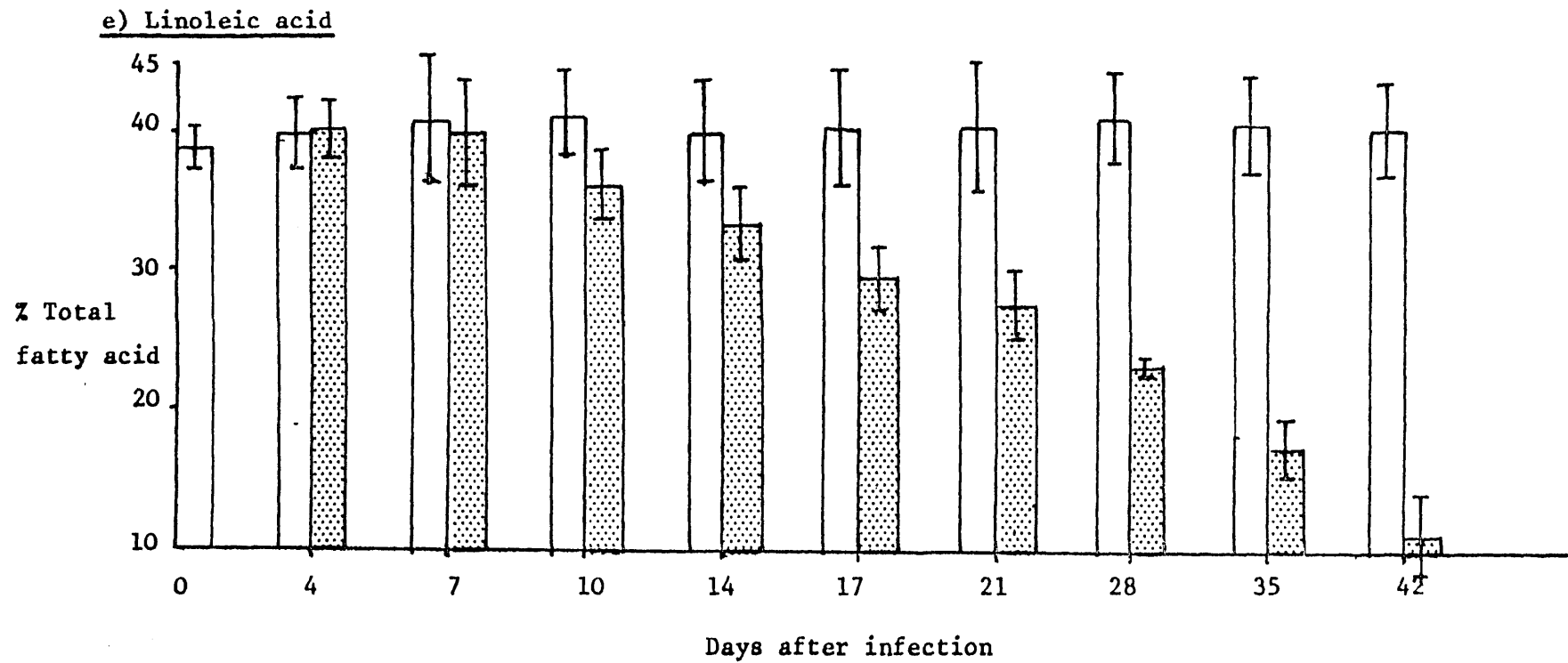
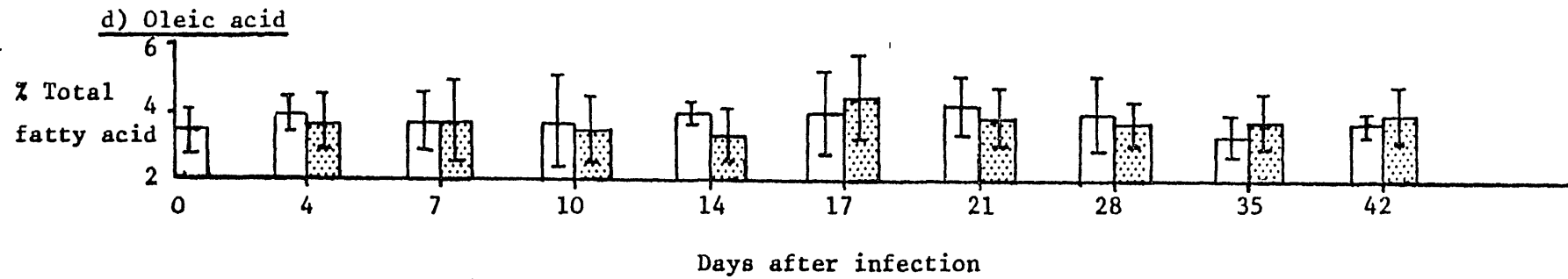
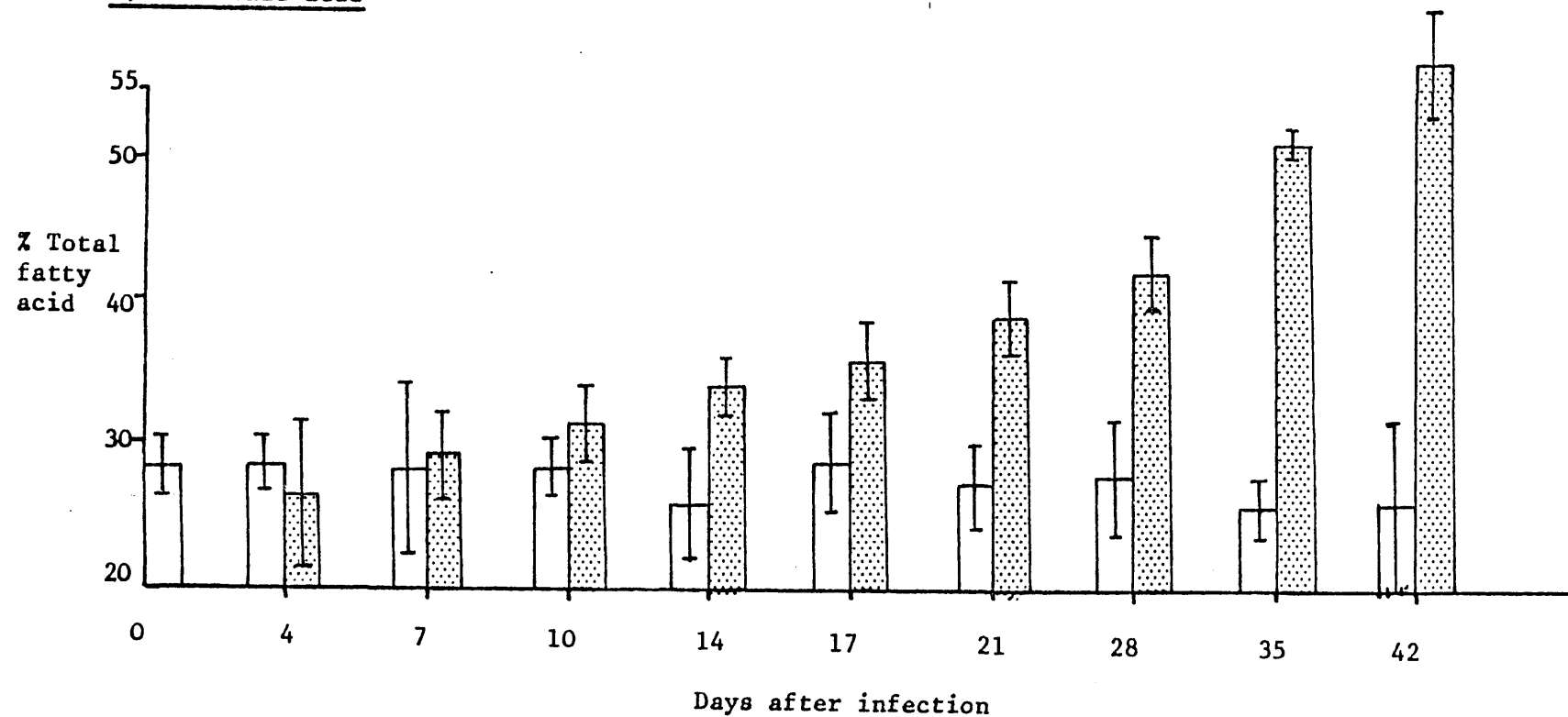


Figure 49 (cont.)

f) Linolenic acid



Unshaded Uninfected Plants

Shaded Infected Plants

I 95% Confidence Limits

Figure 50: The effect of infection by E. pisi on the fatty acid composition of PG in epidermal strips from the cultivar Onward

Figure 50

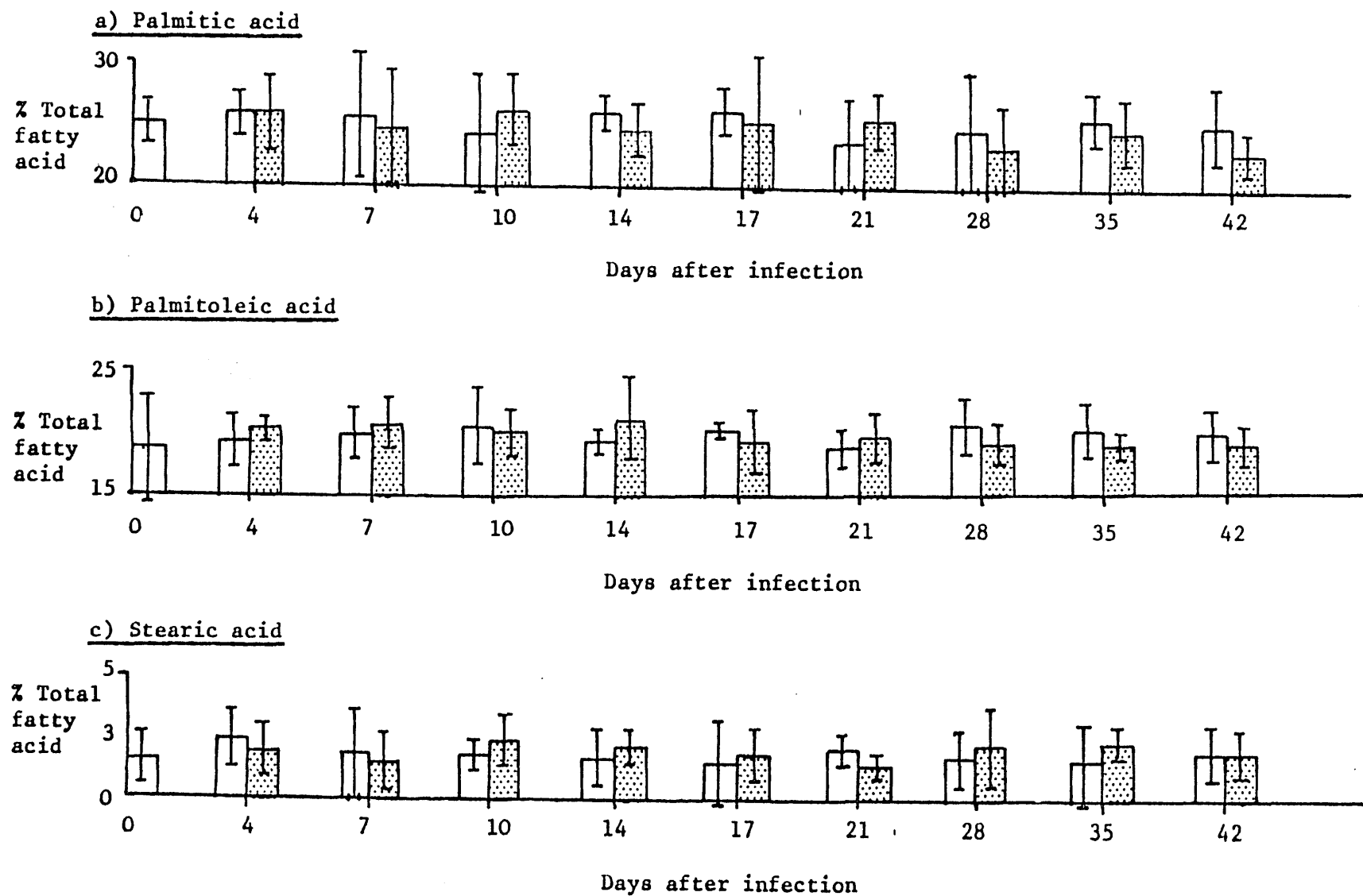
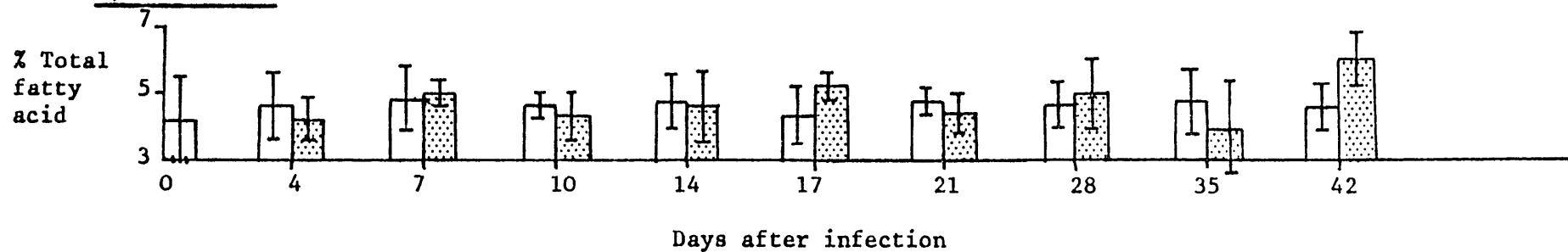
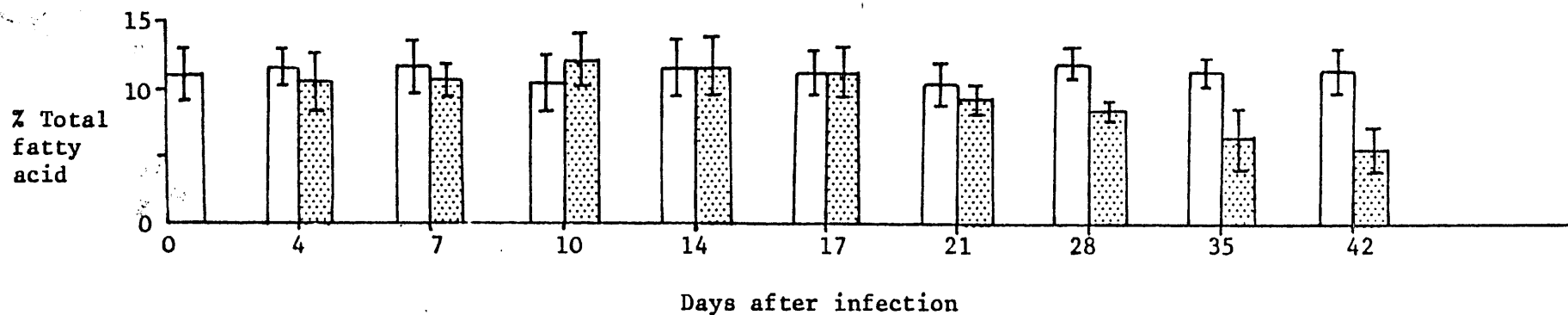


Figure 50 (cont.)

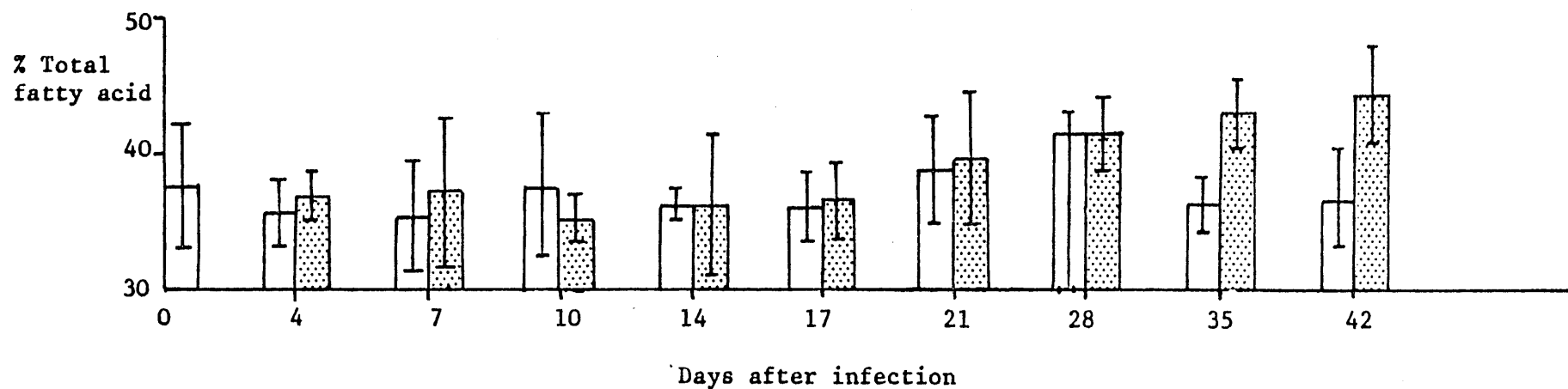
d) Oleic acid



e) Linoleic acid



f) Linolenic acid

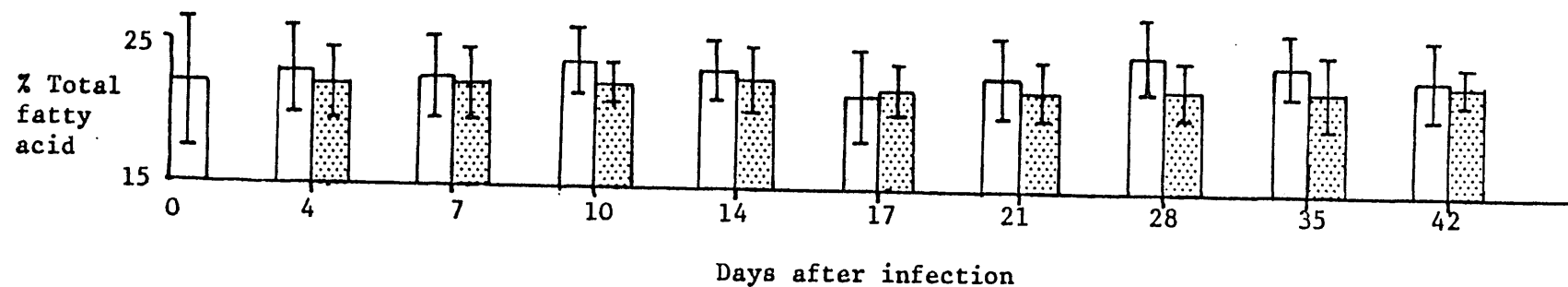


Unshaded Uninfected Plants
Shaded Infected Plants
I 95% Confidence Limits

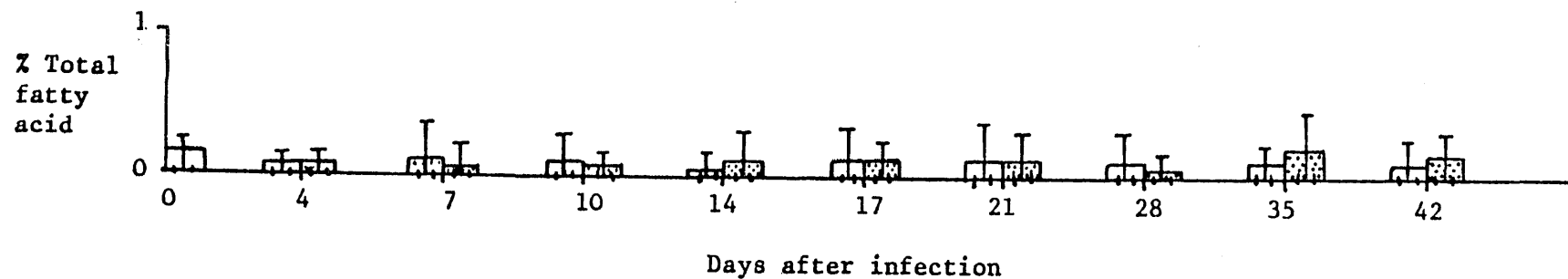
Figure 51: The effect of infection by E. pisi on the fatty acid composition of PI in epidermal strips from the cultivar Onward

Figure 51

a) Palmitic acid



b) Palmitoleic acid



c) Stearic acid

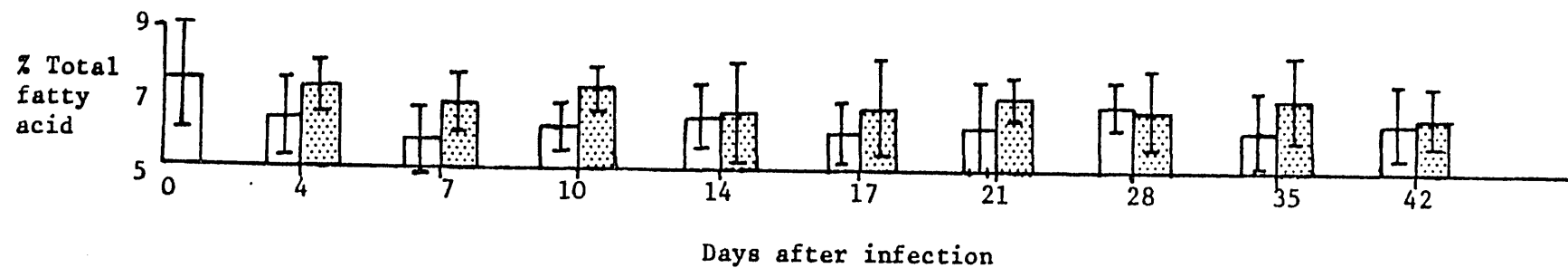


Figure 51 (cont.)

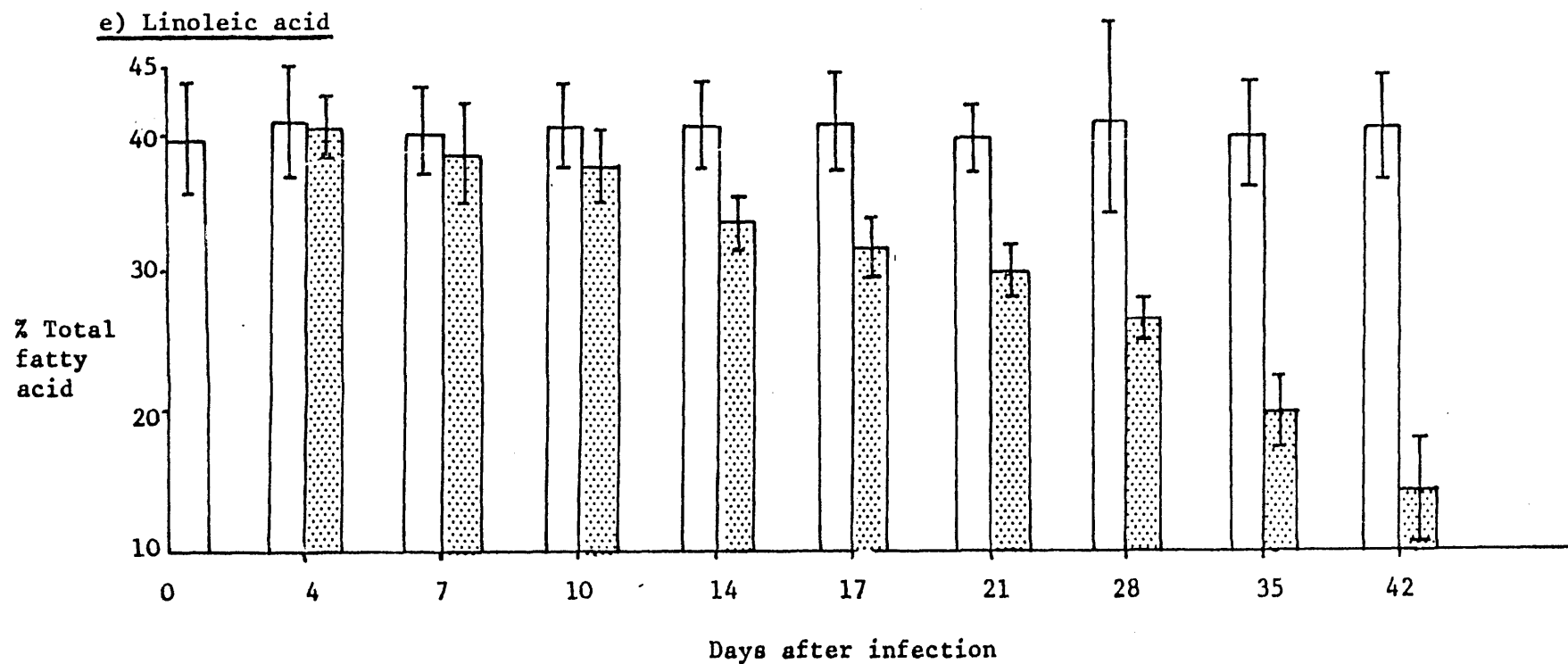
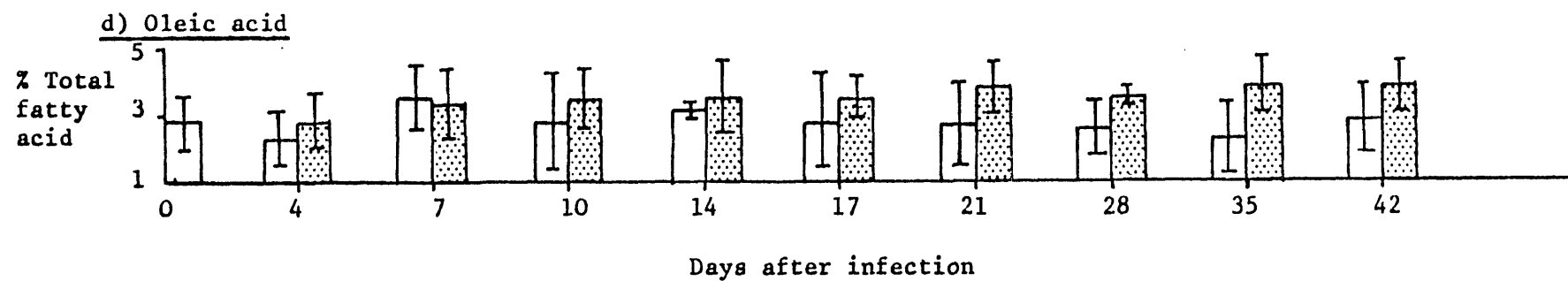
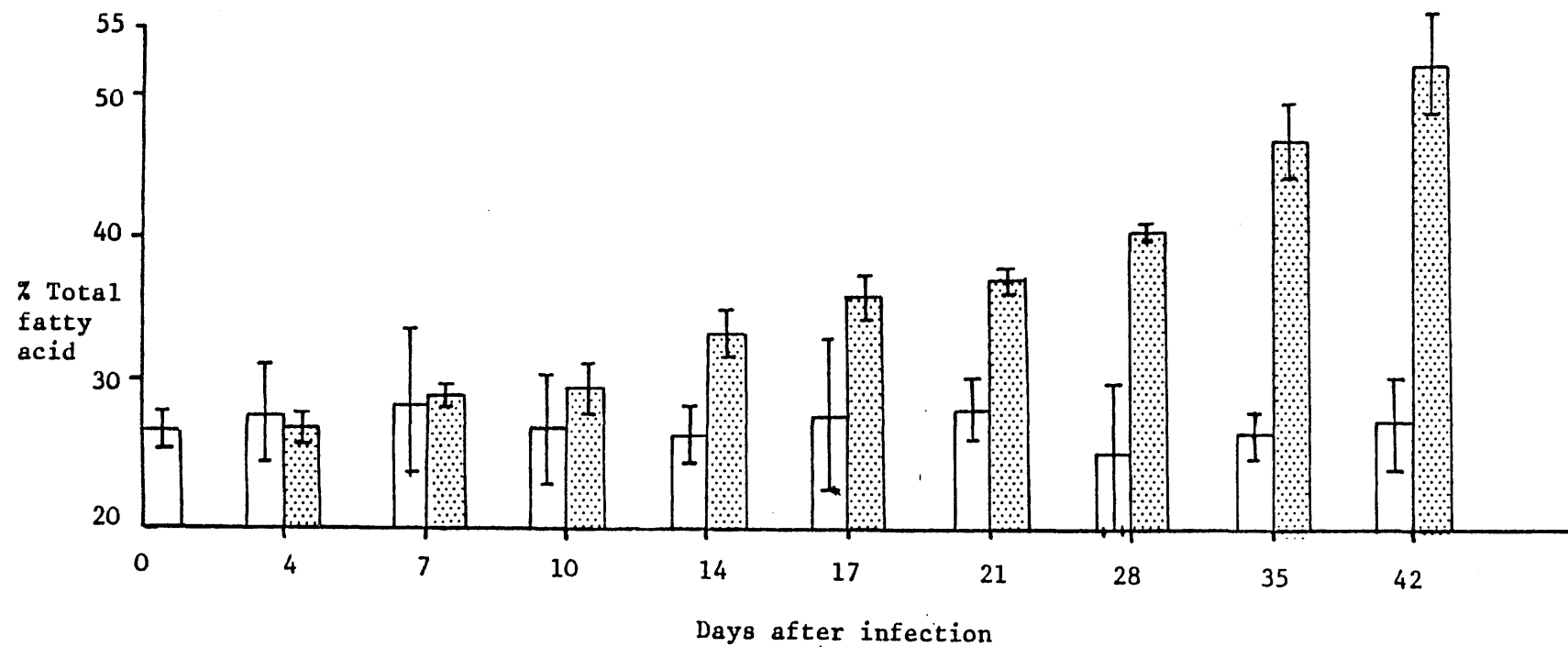


Figure 51 (cont.)

f) Linolenic acid



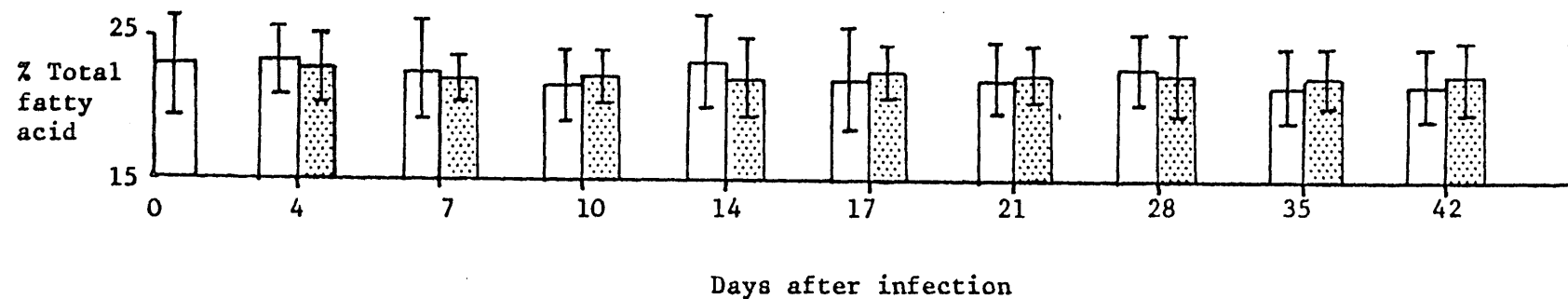
Unshaded Uninfected Plants
Shaded Infected Plants

I 95% Confidence Limits

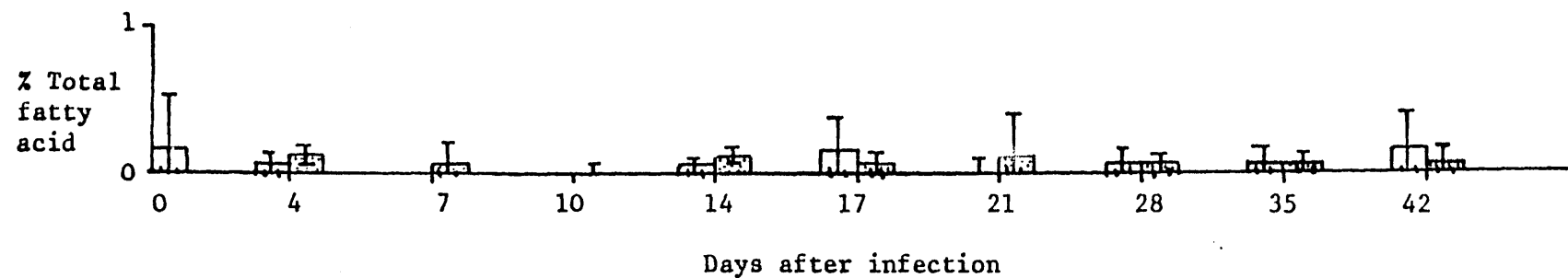
Figure 52: The effect of infection by E. pisi on the fatty acid composition of PS in epidermal strips from the cultivar Onward

Figure 52

a) Palmitic acid



b) Palmitoleic acid



c) Stearic acid

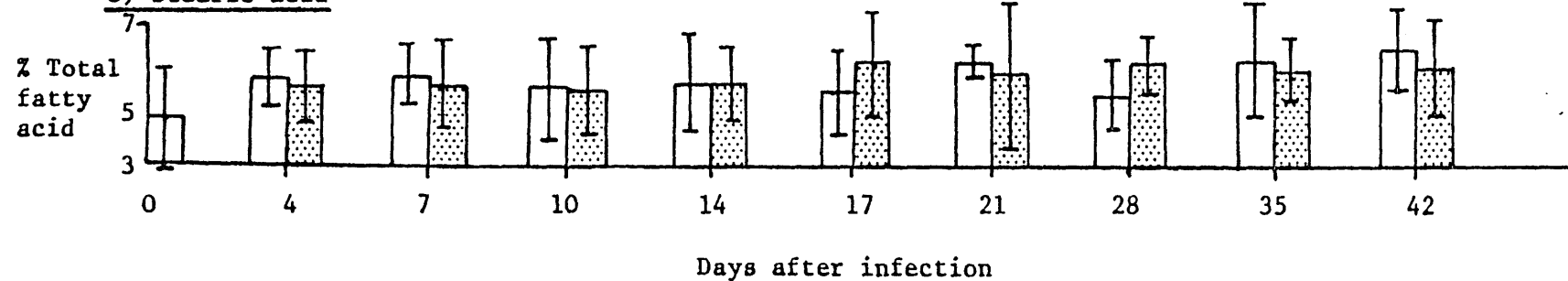


Figure 52 (cont.)

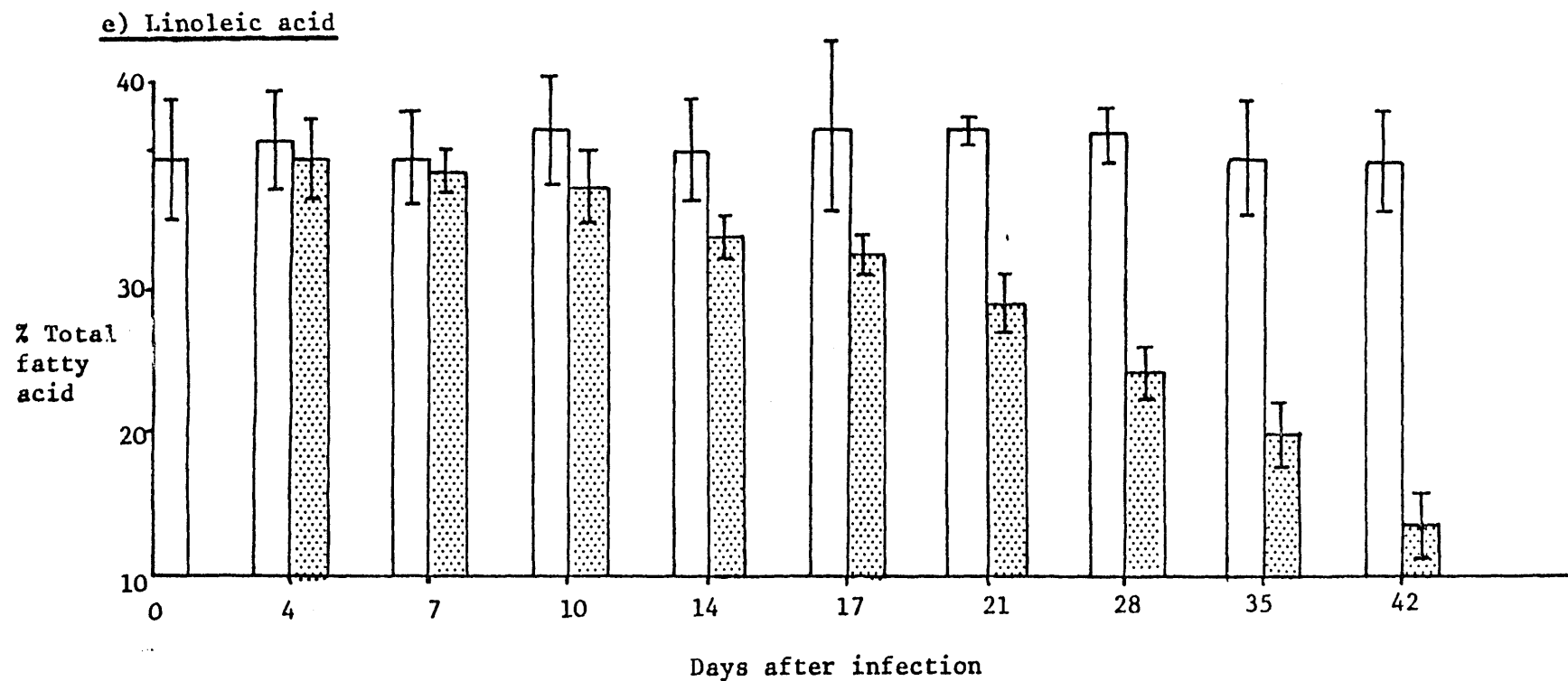
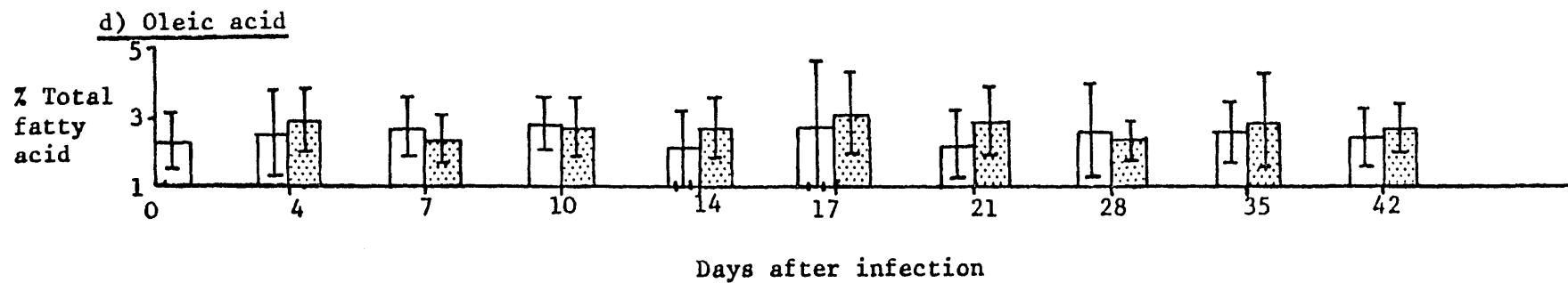
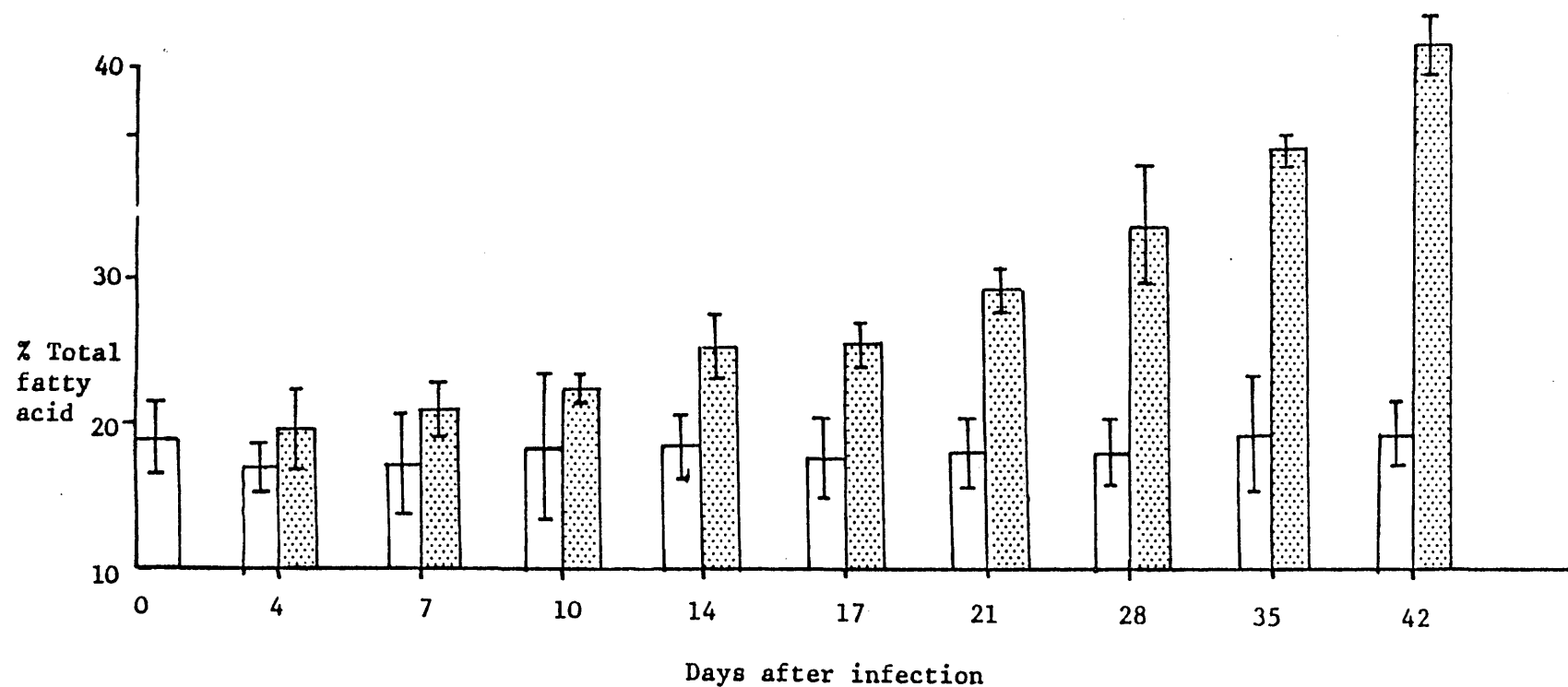


Figure 52 (cont.)

f) Linolenic acid



Unshaded Uninfected Plants

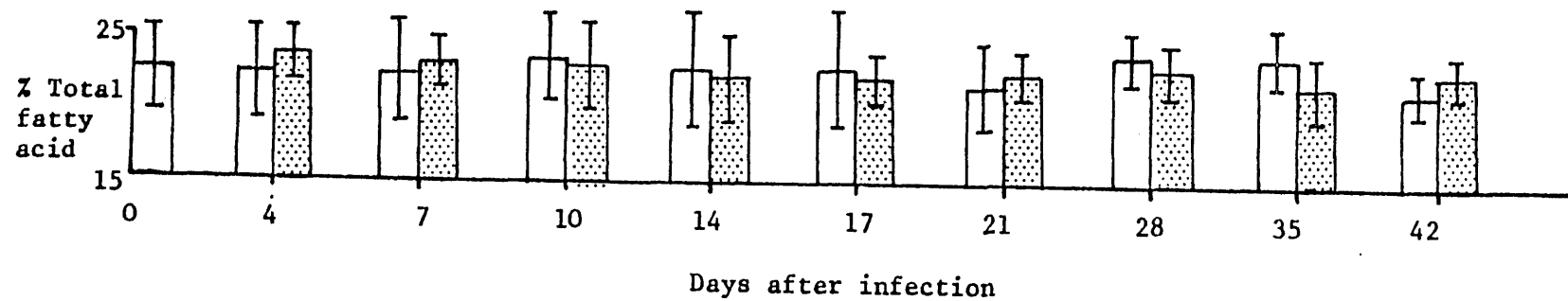
Shaded Infected Plants

I 95% Confidence Limits

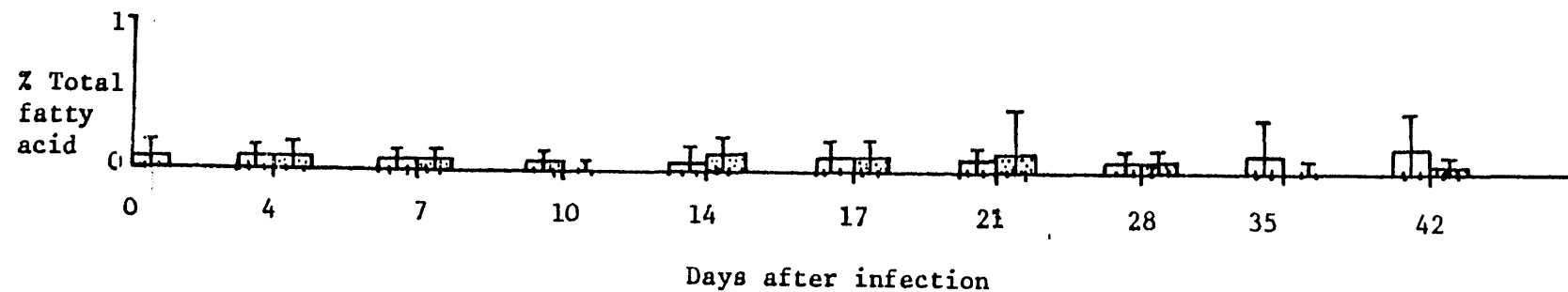
Figure 53: The effect of infection by E. pisi on the fatty acid composition of PA in epidermal strips from the cultivar Onward

Figure 53

a) Palmitic acid



b) Palmitoleic acid



c) Stearic acid

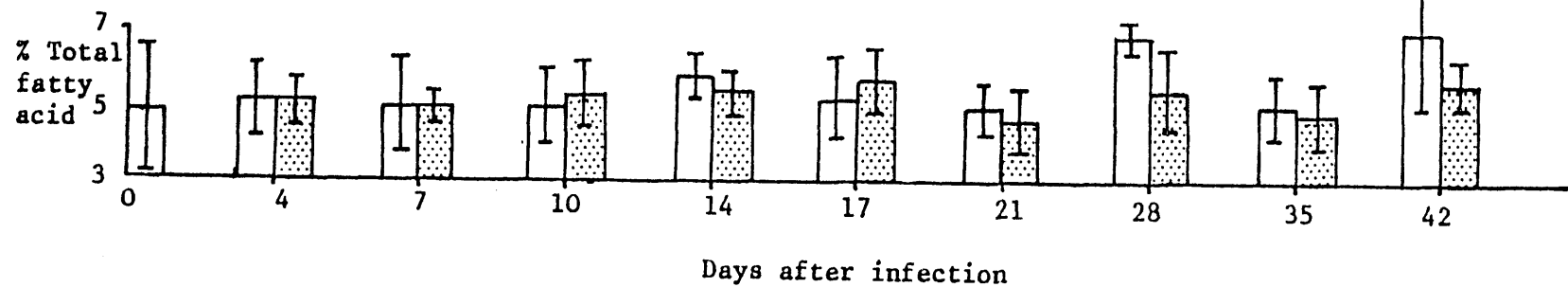
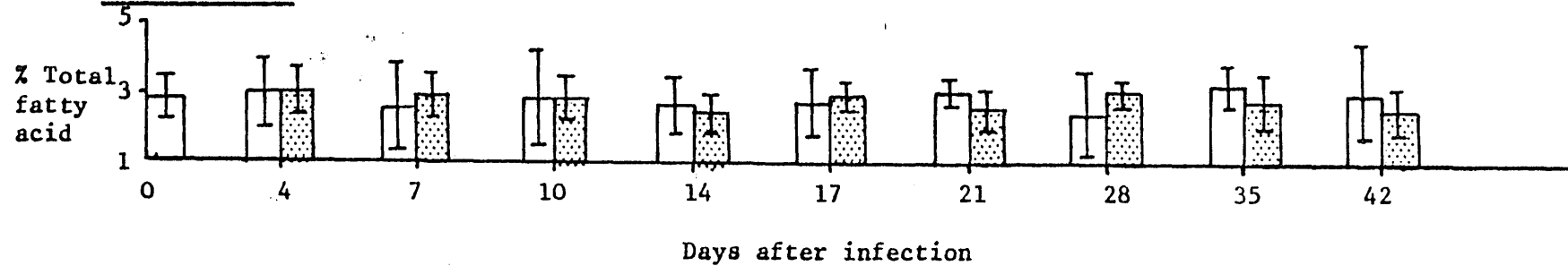


Figure 53 (cont.)

d) Oleic acid



e) Linoleic acid

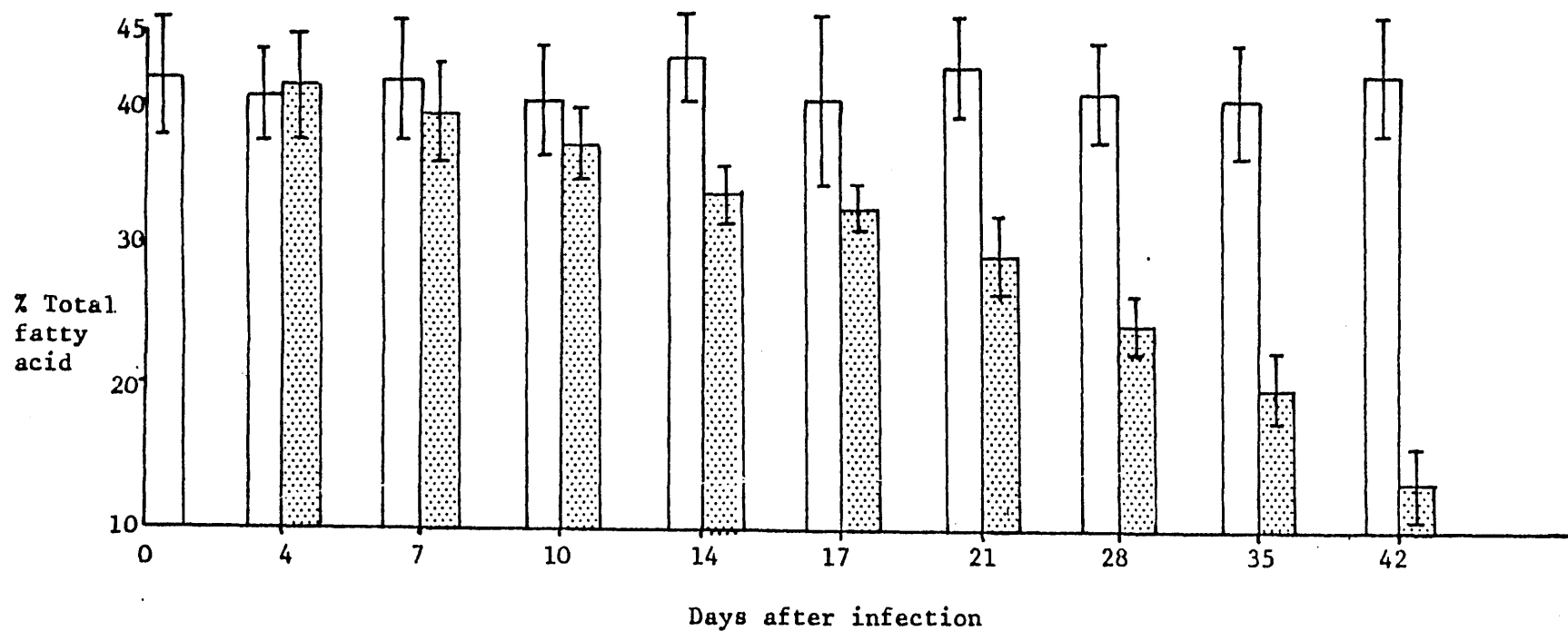
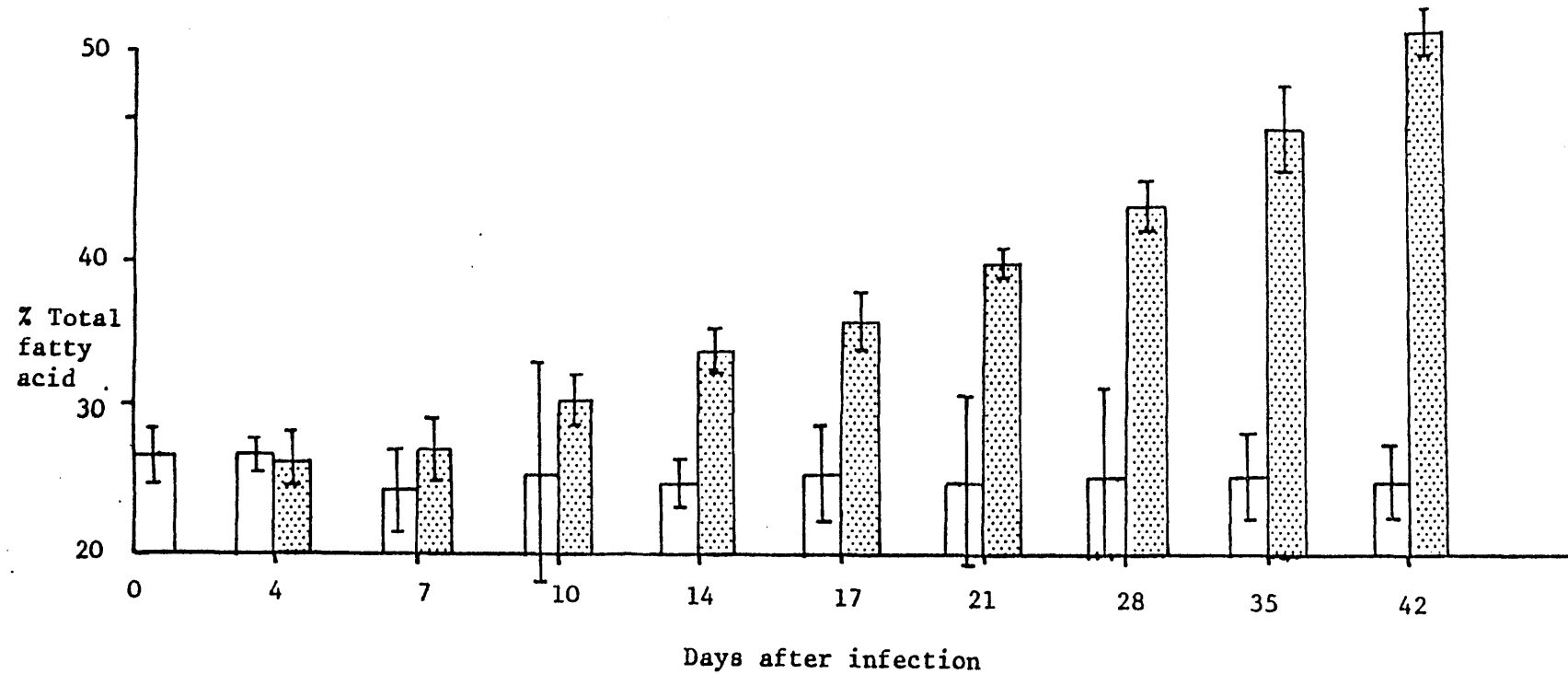


Figure 53 (cont.)

f) Linolenic acid



Unshaded Uninfected Plants

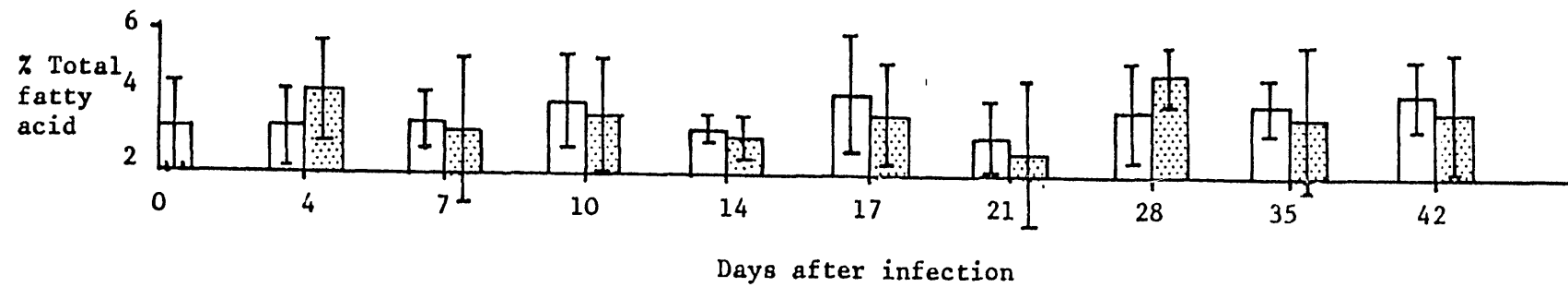
Shaded Infected Plants

I 95% Confidence Limits

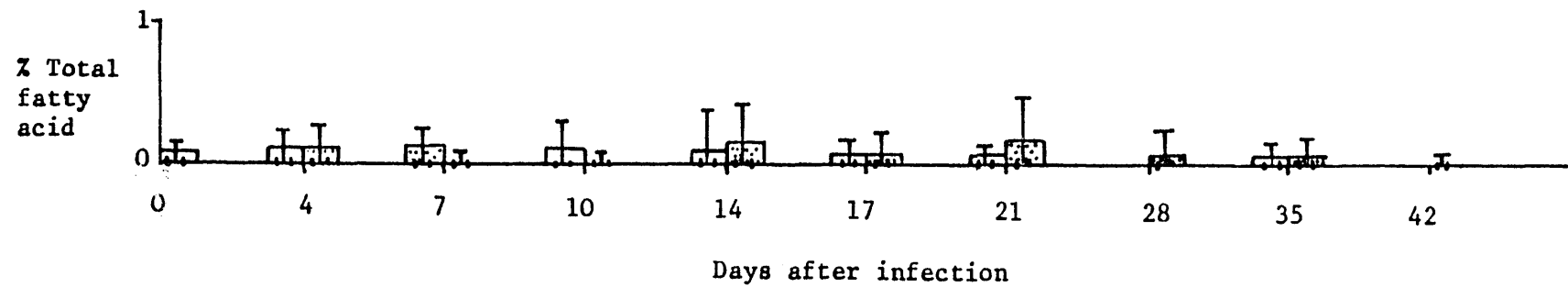
Figure 54: The effect of infection by E. pisi on the fatty acid composition of MGDG in epidermal strips from the cultivar Onward

Figure 54

a) Palmitic acid



b) Palmitoleic acid



c) Stearic acid

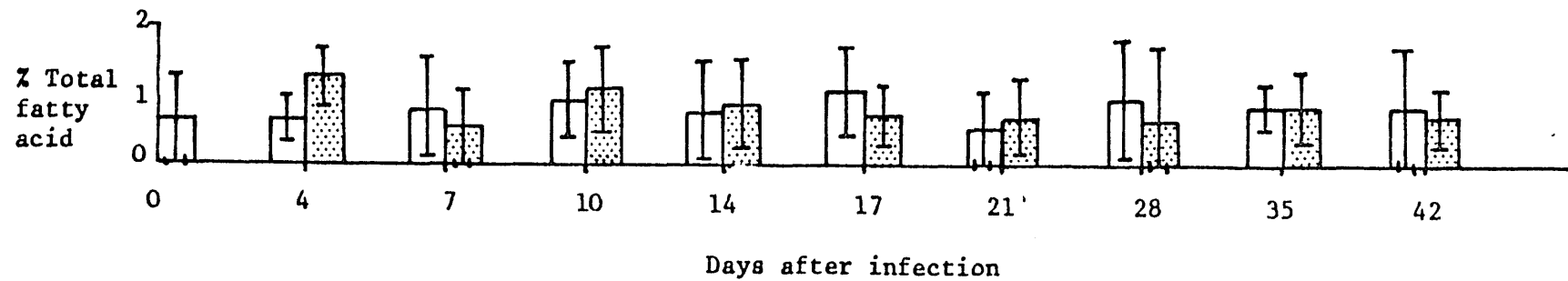
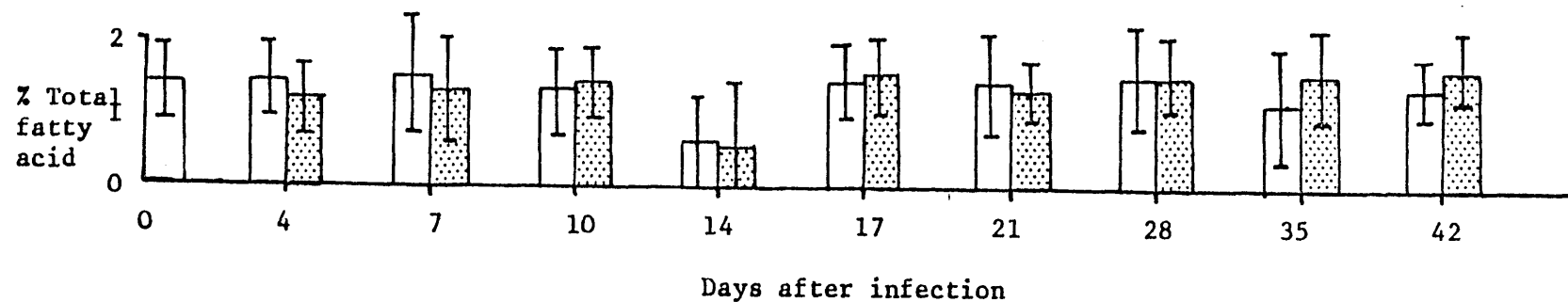
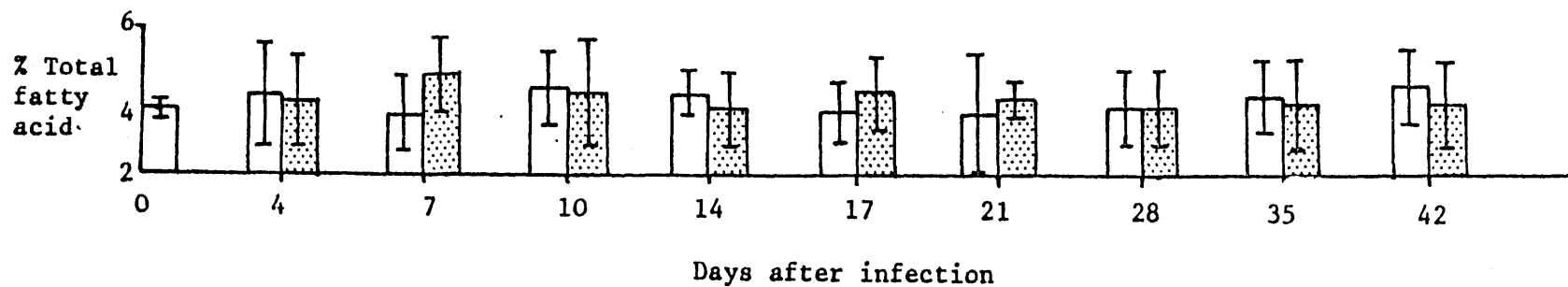


Figure 54 (cont.)

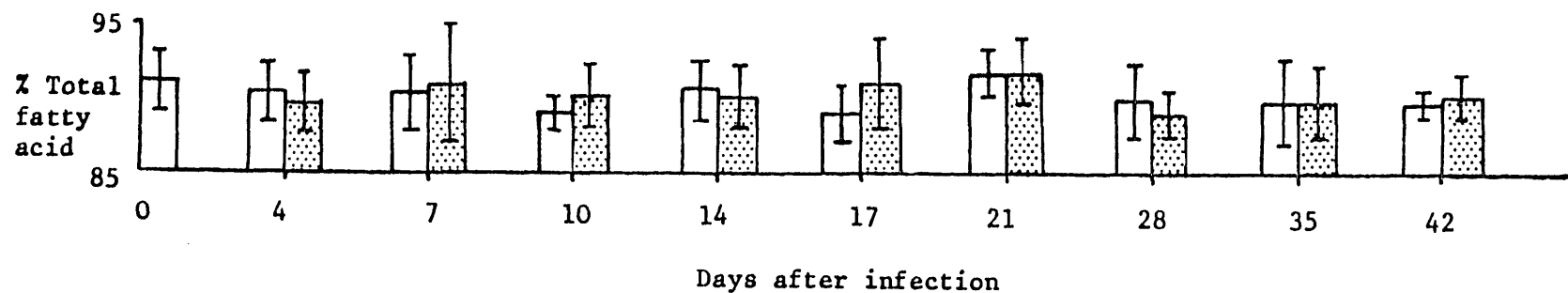
d) Oleic acid



e) Linoleic acid



f) Linolenic acid



Unshaded Uninfected Plants

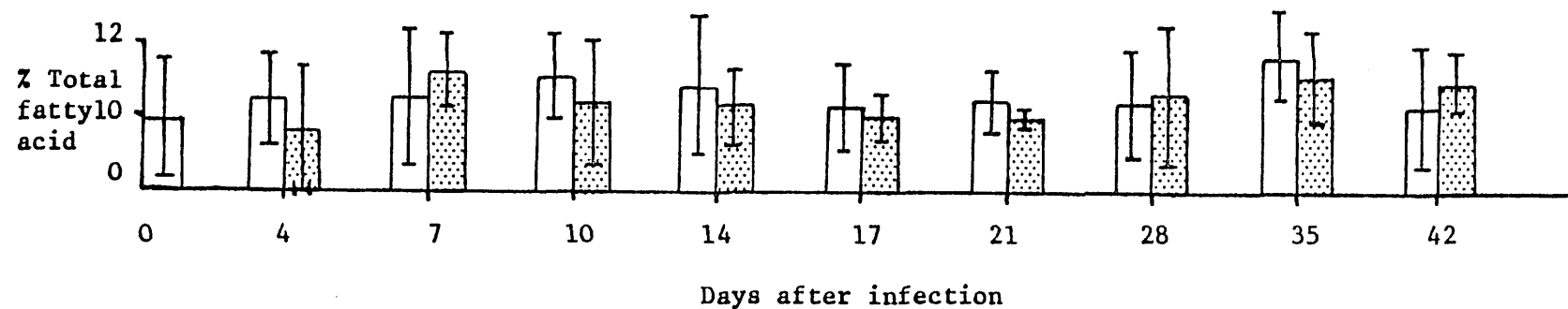
Shaded Infected Plants

I 95% Confidence Limits

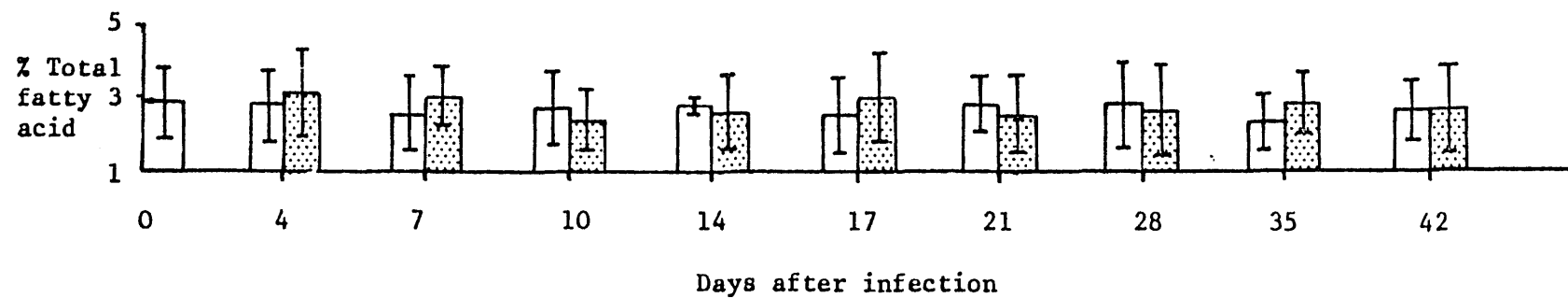
Figure 55: The effect of infection by E. pisi on the fatty acid composition of DGDG in epidermal strips from the cultivar Onward

Figure 55

a) Palmitic acid



b) Stearic acid



c) Oleic acid

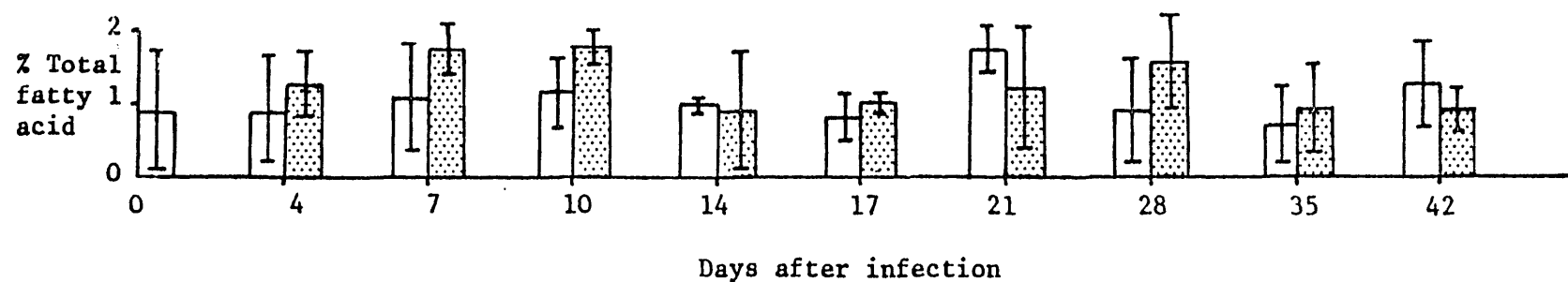
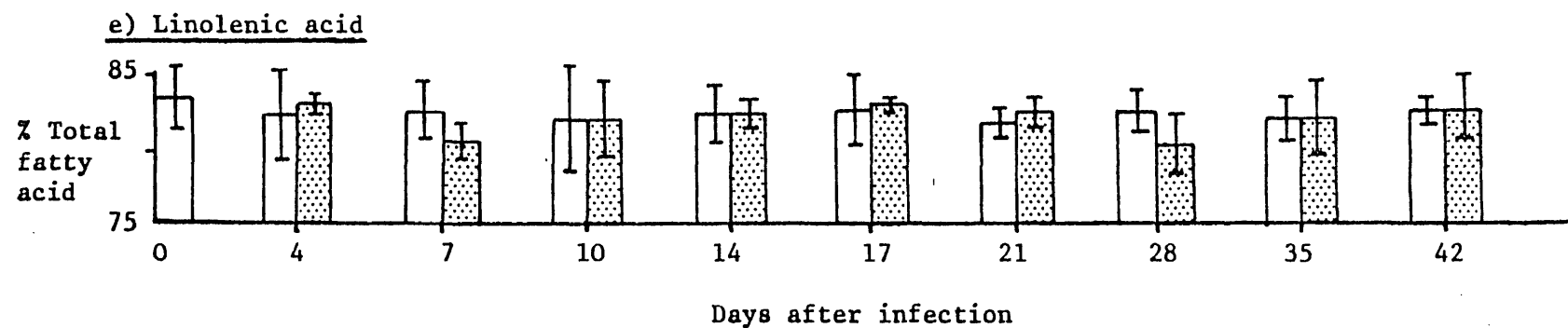
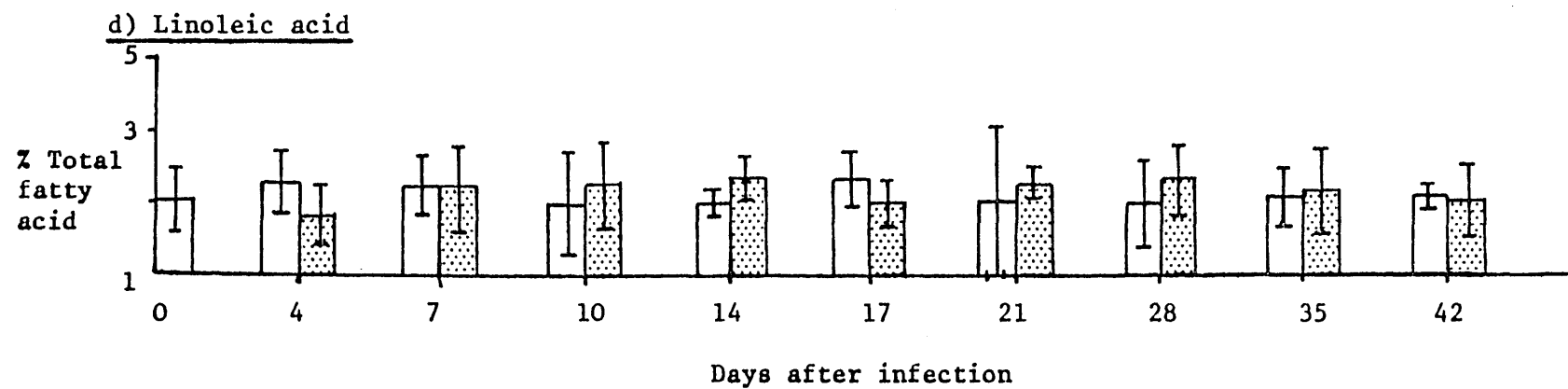


Figure 55 (cont.)



Unshaded Uninfected Plants

Shaded Infected Plants

I 95% Confidence Limits

Figure 56: The effect of infection by E. pisi on the fatty acid index from PC-bound fatty acids in epidermal strips from stems of the cultivar Onward

Figure 56

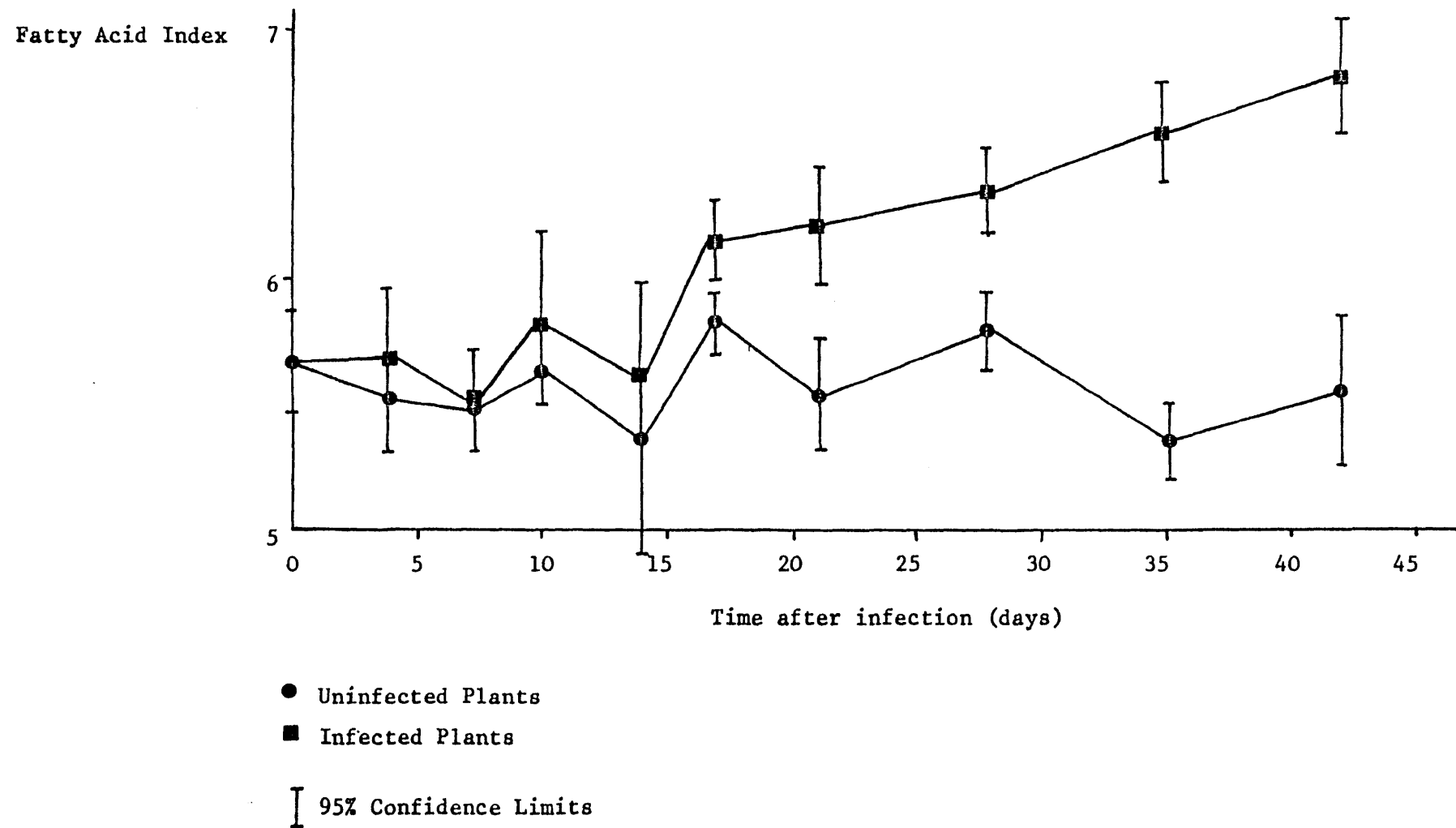


Figure 57: The effect of infection by E. pisi on the fatty acid index from PE-bound fatty acids in epidermal strips from the stems of the cultivar Onward

Figure 57

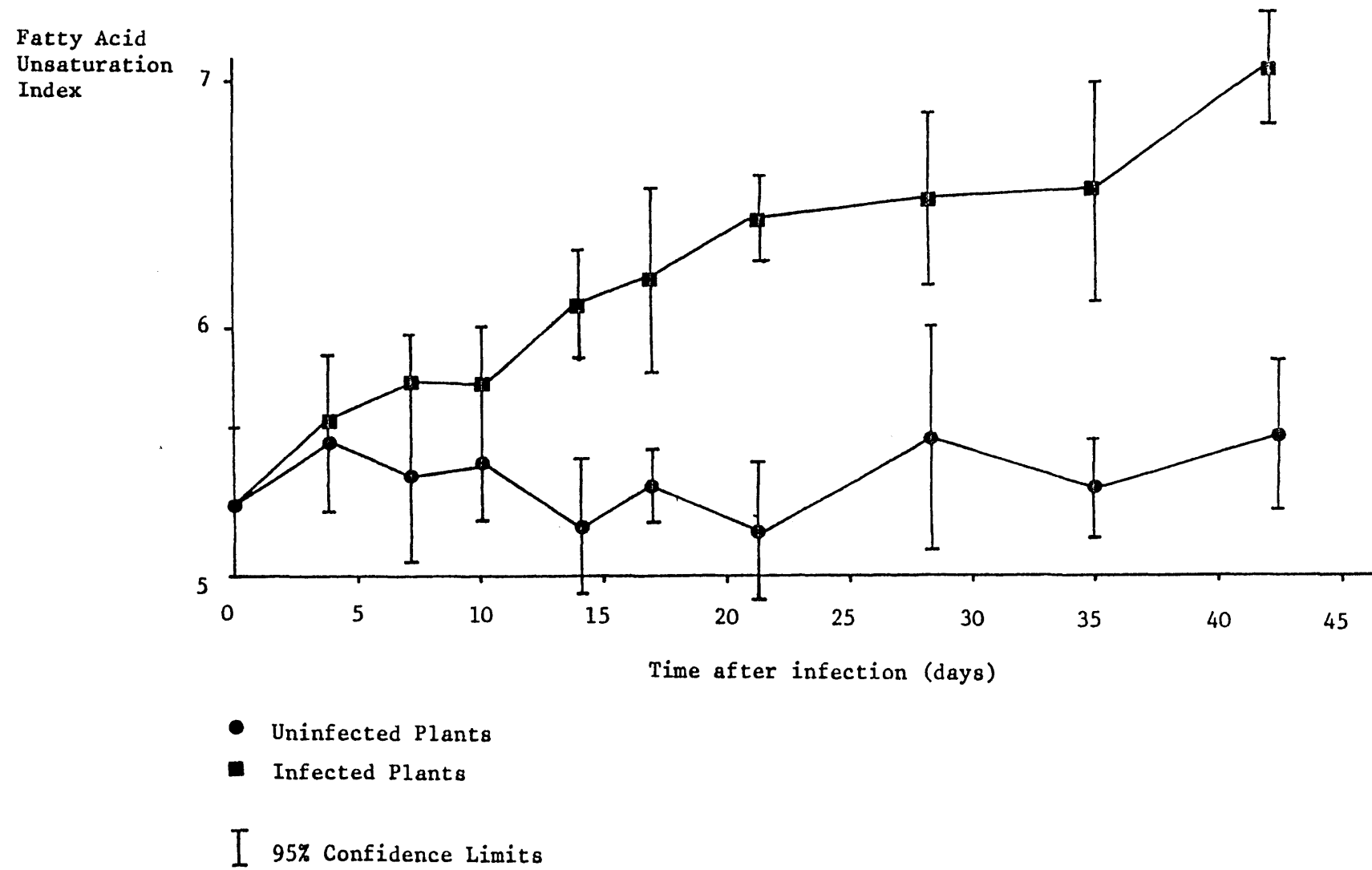


Figure 58: The effect of infection by E. pisi on the fatty acid index from PG-bound fatty acids in epidermal strips from stems of the cultivar Onward

Figure 58

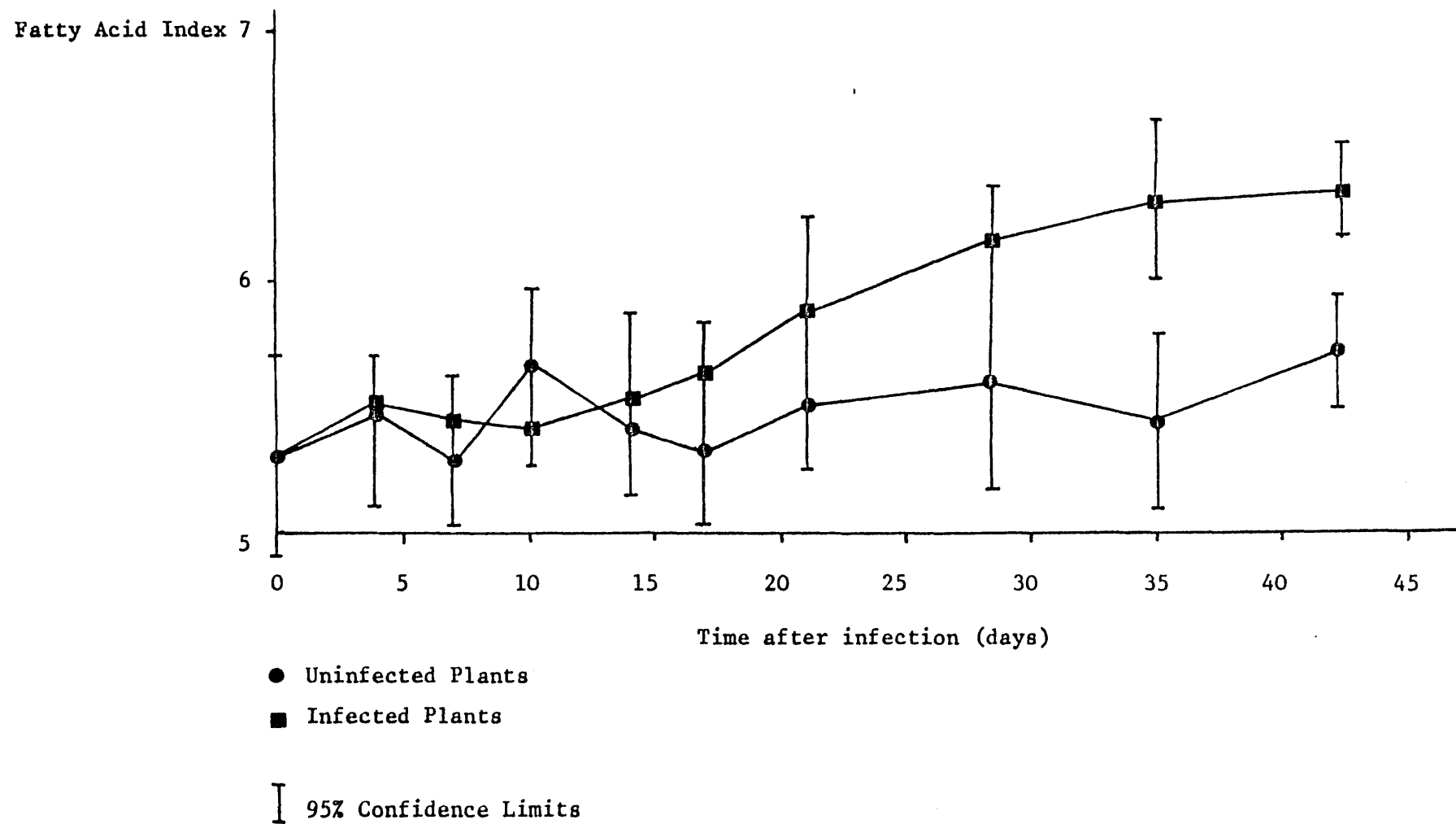


Figure 59: The effect of infection by E. pisi on the fatty acid Index from PI-bound fatty acids in epidermal strips from stems of the cultivar Onward

Figure 59

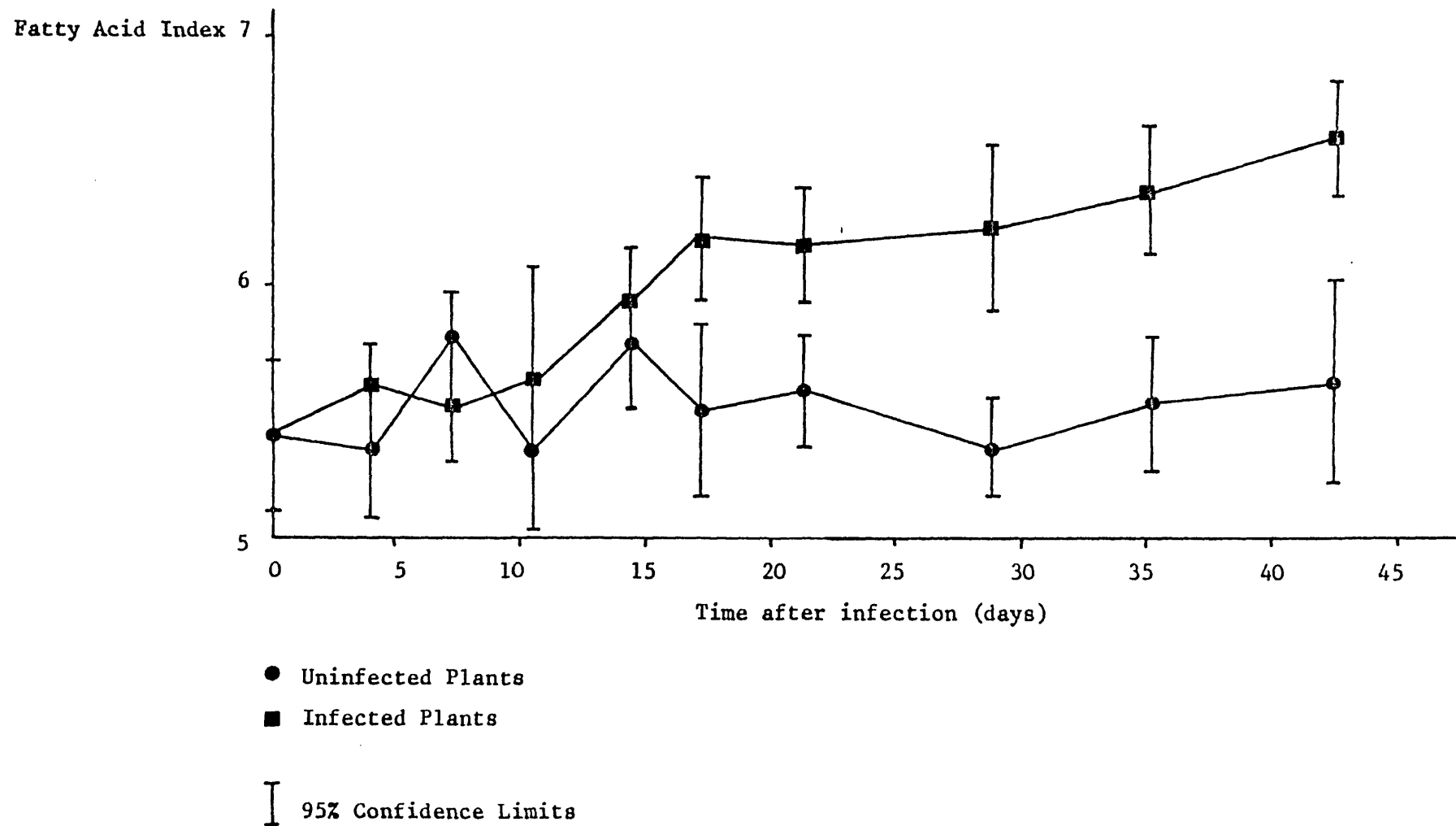


Figure 60: The effect of infection by E. pisi on the fatty acid index from PS-bound fatty acids in epidermal strips from stems of the cultivar Onward

Figure 60

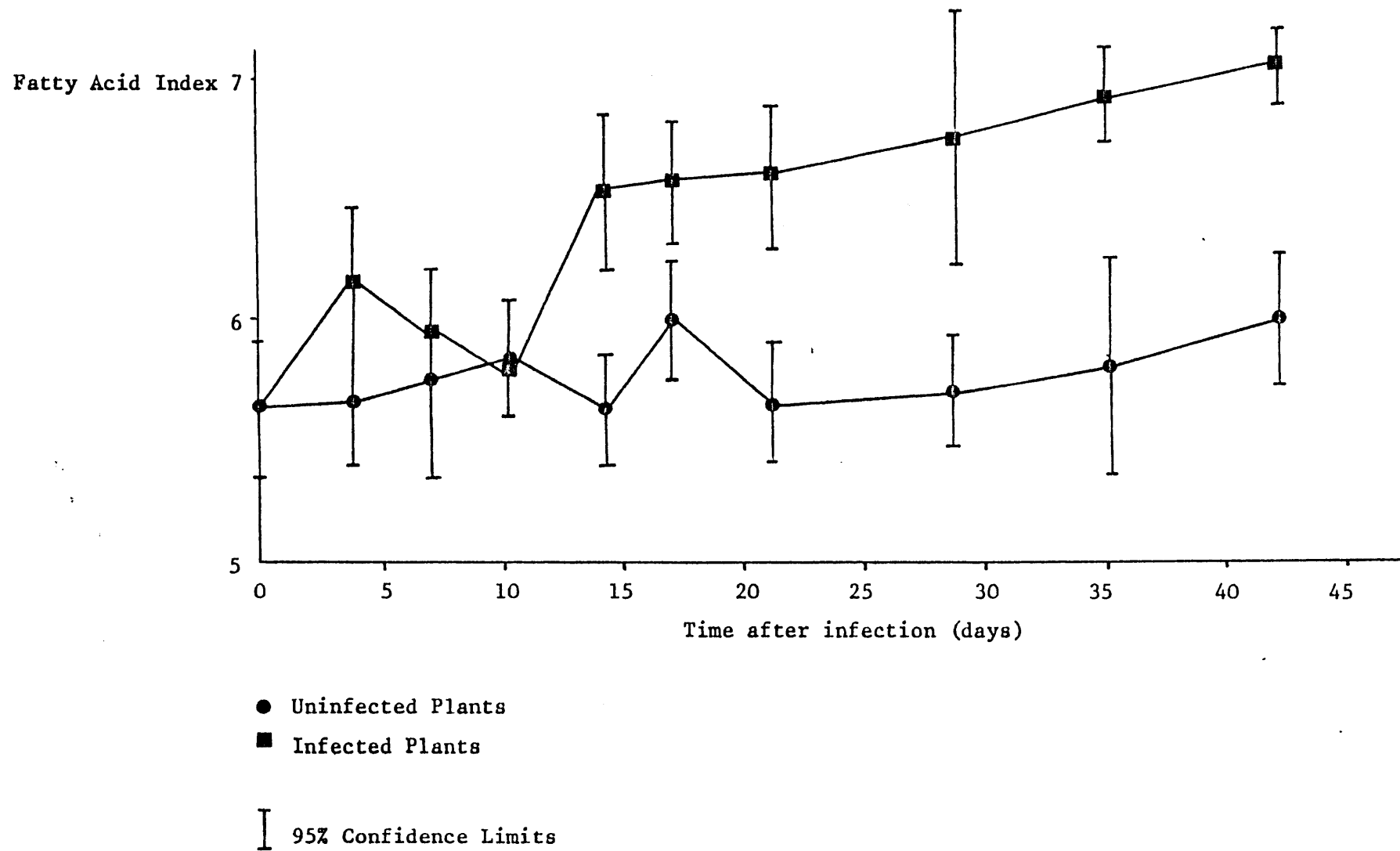


Figure 61: The effect of infection by E. pisi on the fatty acid index from PA-bound fatty acids in epidermal strips from the stems of the cultivar Onward

Figure 61

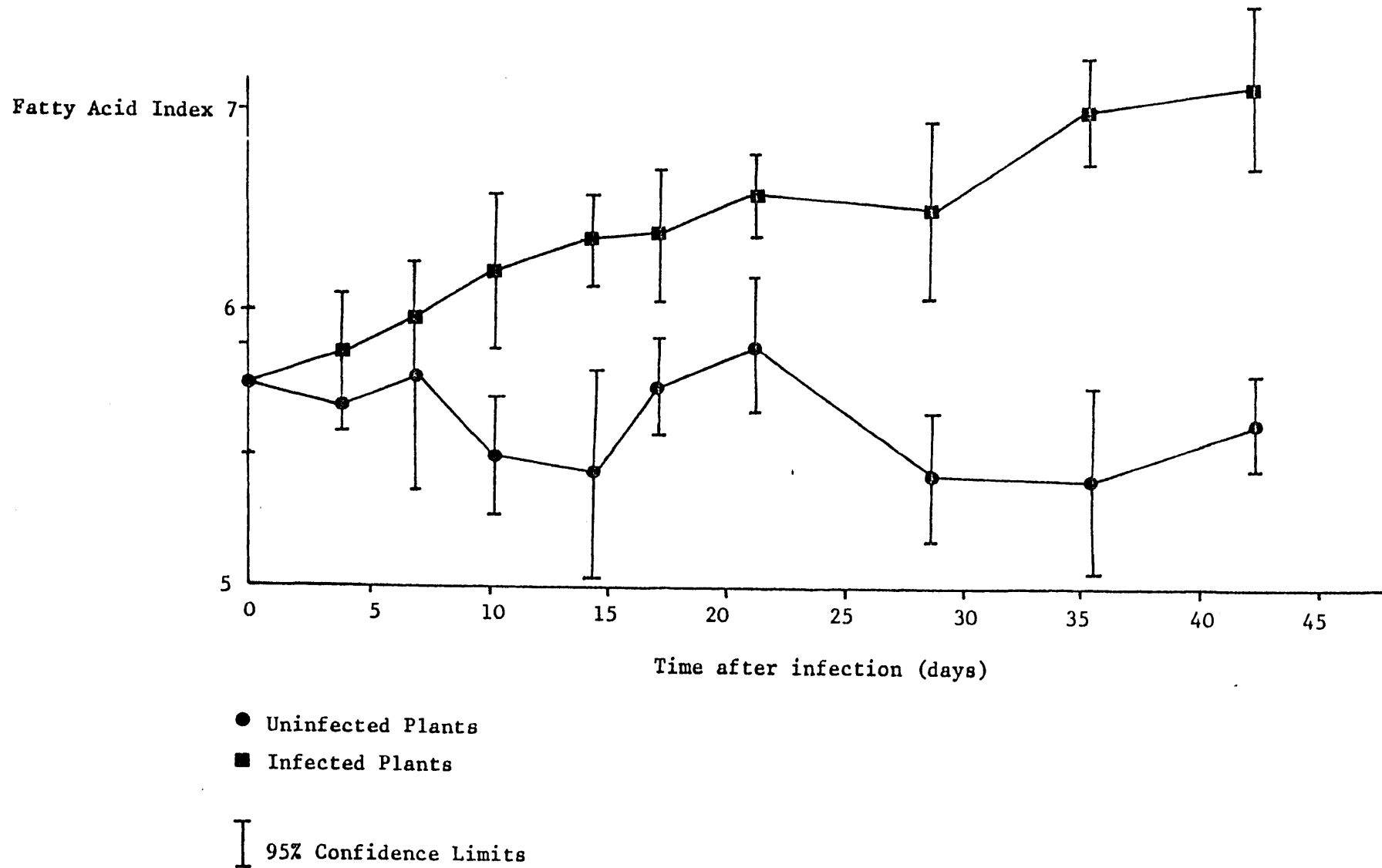


Figure 62: The effect of infection by E. pisi on the fatty acid index from MGDG-bound fatty acids in epidermal strips from the stems of the cultivar Onward

Figure 62

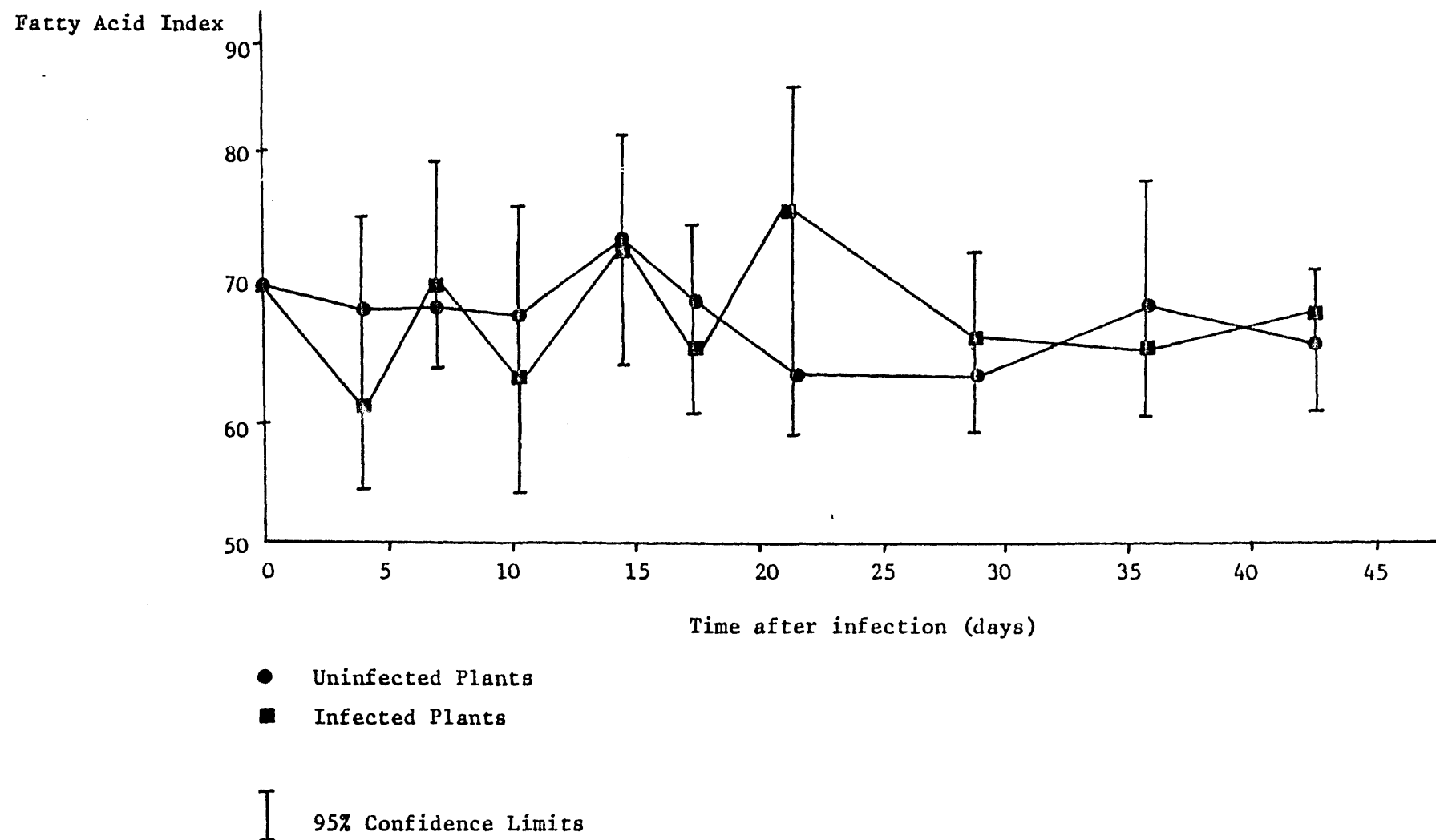
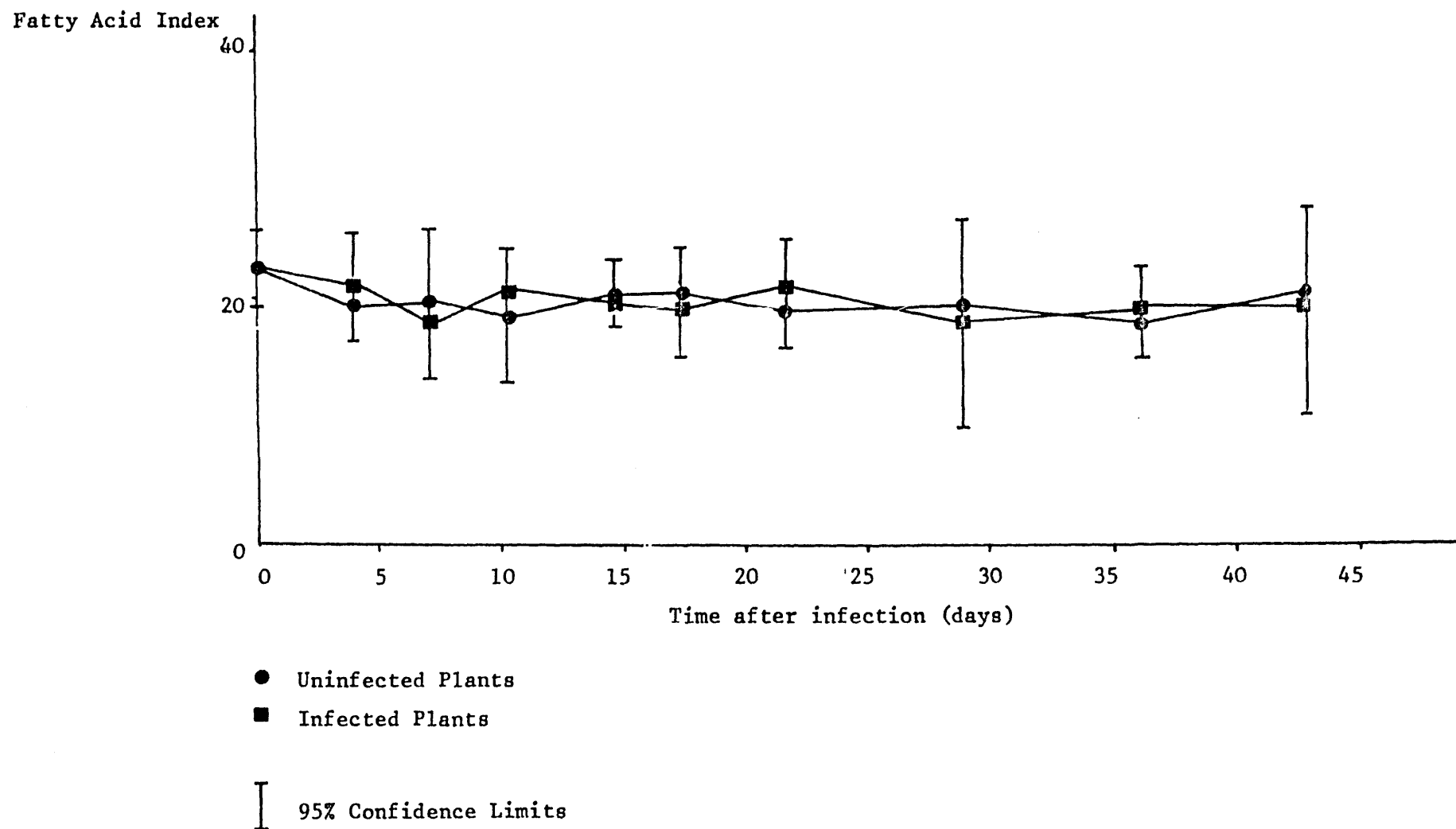


Figure 63: The effect of infection by E. pisi on the fatty acid index from DGDG-bound fatty acids in epidermal strips from the stems of the cultivar Onward

Figure 63



e) Individual Neutral Lipids

The individual neutral lipids were also assessed during the same 42 day time period. The percentage composition of the neutral lipids during this are shown in Table 21 and Figure 64 using the cultivar Onward as an example.

The percentage composition of the neutral lipids from healthy plants shows two slight changes after 42 days diglycerides which is matched by an increase in the triglycerides and free fatty acids. In infected plants there were a number of differences. There was a significant ($p = 0.05$) decrease in the amount of sterol esters, monoglycerides and diglycerides and an increase in the amount of triglycerides and free fatty acids. The decrease became statistically significant ($p = 0.05$) on the 28th day (sterol esters, diglycerides and triglycerides), on the 35th day (free fatty acids) and on the 42nd day (monoglycerides).

f) Neutral Lipid Fatty Acids

When the neutral lipid fatty acids were assessed over the 42 day infection period in epidermal cells from stems of uninfected plants the percentage composition of each fatty acid at the beginning of the experiment was as follows; lauric acid $4.0 \pm 0.6\%$, myristic acid $5.7 \pm 1.2\%$, palmitic acid $24.3 \pm 2.8\%$, palmitoleic acid $0.2 \pm 0.2\%$, stearic acid $4.6 \pm 0.8\%$, oleic acid $8.4 \pm 1.6\%$, linoleic acid $27.8 \pm 2.8\%$ and linolenic acid $23.4 \pm 4.3\%$. Over the ensuing 42 days there were no significant changes in fatty acid content in epidermal cells taken from either healthy or infected plants (Figure 65). Although statistically insignificant there was a tendency for stearic and linoleic acid levels in infected plants to be greater than, and linolenic acid to be less than those in healthy plants, especially towards the end of the sampling period. Although not calculated it is clear that the fatty acid unsaturation index is actually lower in infected plants; in sharp contrast to the situation with polar lipids.

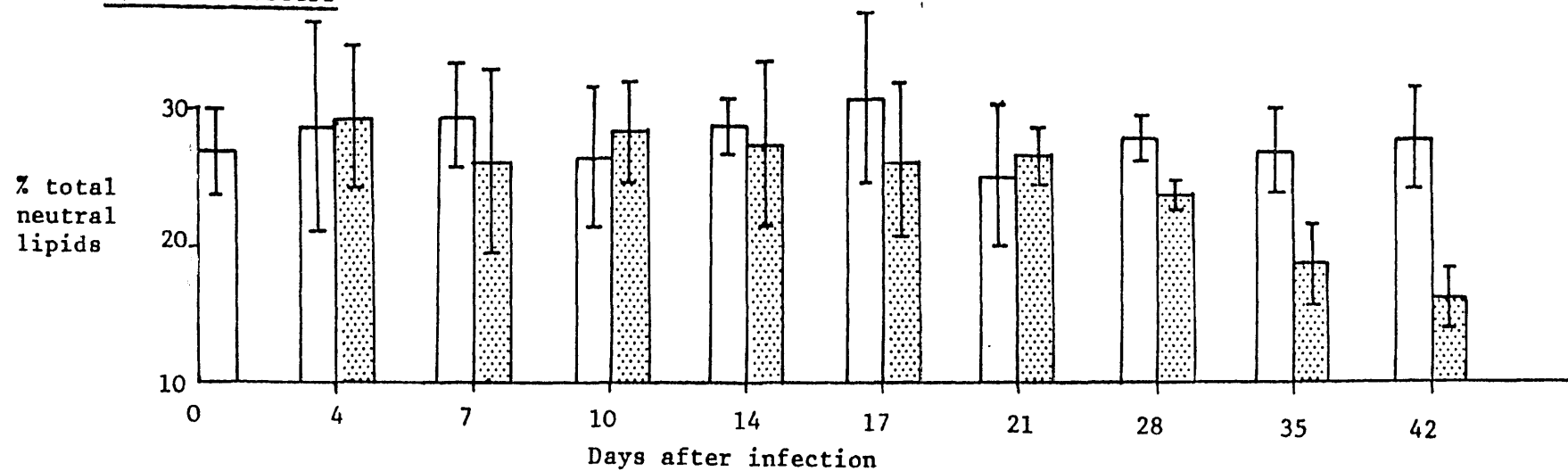
Table 21: Neutral lipids in healthy and diseased stem epidermis

Lipids	Healthy at day 0	Healthy at day 42	Infected at day 42
Sterol esters	26.7 $\pm$ 2.6%	27.7 $\pm$ 3.3%	16.0 $\pm$ 2.0%
Sterol	18.3 $\pm$ 1.3%	15.5 $\pm$ 2.3%	15.1 $\pm$ 1.4%
Monoglycerides	4.5 $\pm$ 0.9%	2.1 $\pm$ 1.0%	1.5 $\pm$ 0.9%
Diglycerides	14.9 $\pm$ 0.9%	11.2 $\pm$ 2.5%	7.9 $\pm$ 1.8%
Triglycerides	28.5 $\pm$ 1.5%	33.5 $\pm$ 4.6%	43.4 $\pm$ 1.3%
Free Fatty Acids	7.1 $\pm$ 1.2%	10.0 $\pm$ 1.4%	16.1 $\pm$ 1.3%

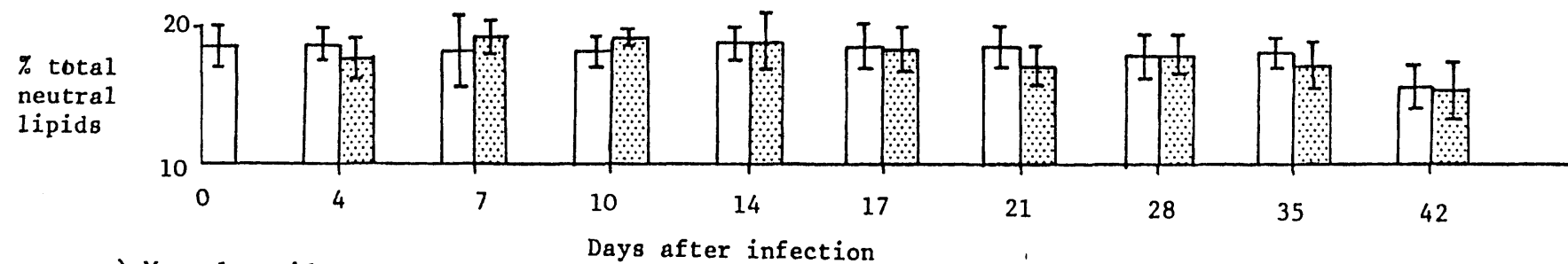
All figures are the means of four replicates quoted with 95%
confidence limits

Figure 64: The effect of infection of E. pisi on the neutral lipid composition of epidermal strips from the cultivar Onward

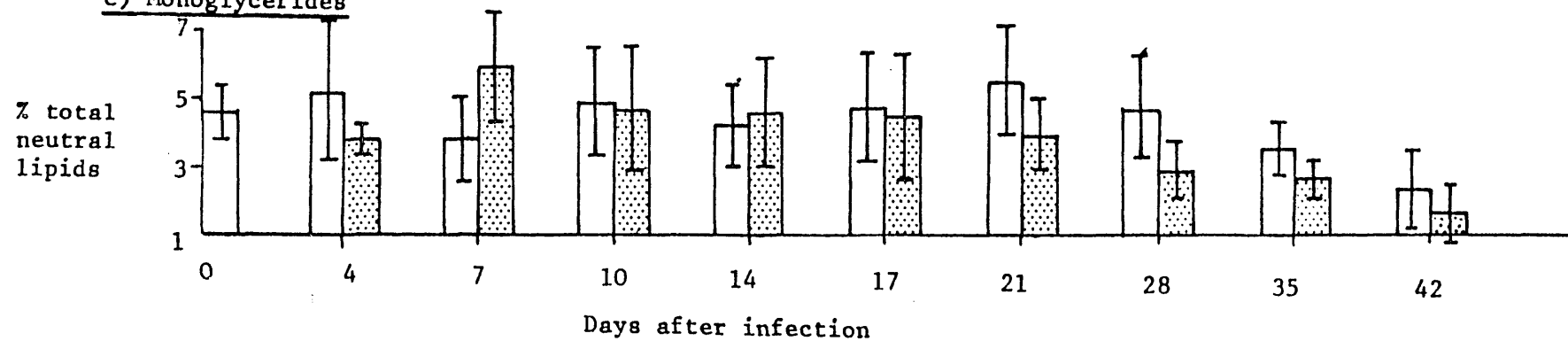
a) Sterol esters



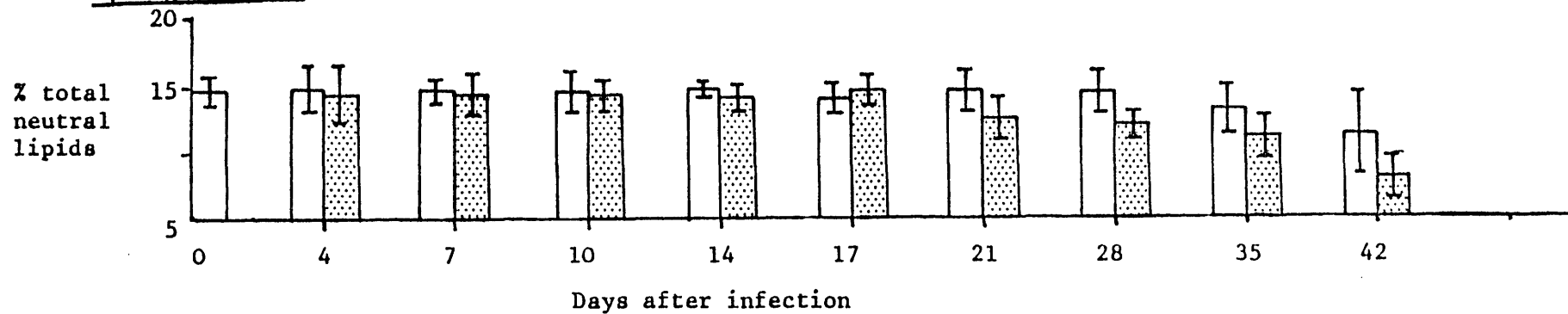
b) Sterols



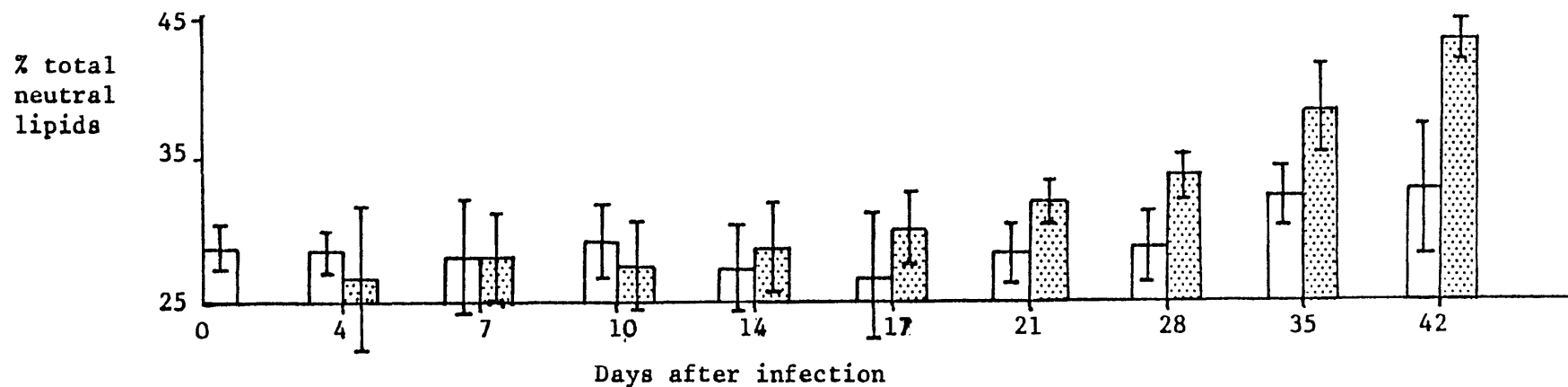
c) Monoglycerides



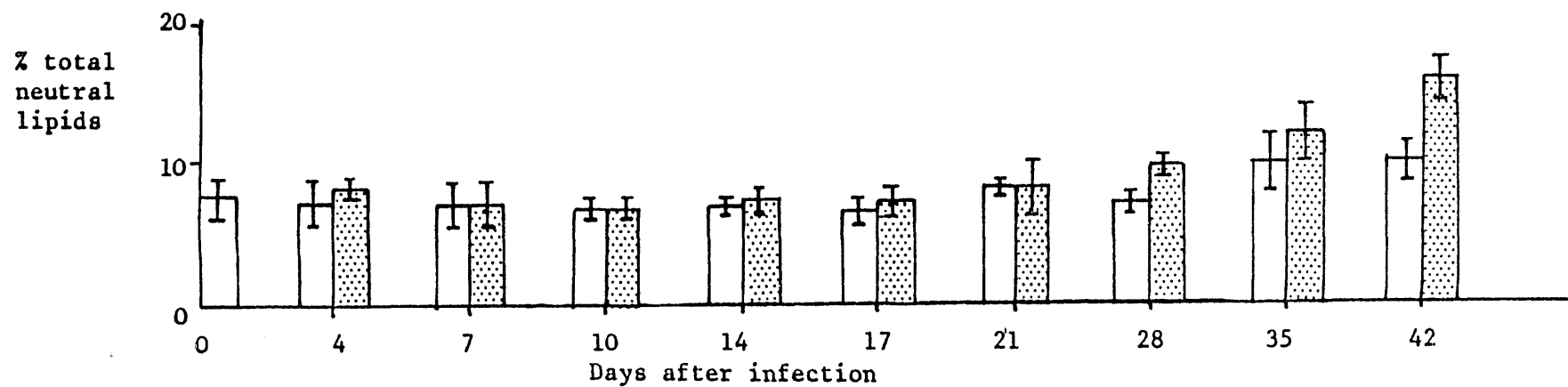
d) Diglycerides



e) Triglycerides



f) Free Fatty Acids



Unshaded Uninfected Plants

Shaded Infected Plants

I 95% Confidence Lim

Figure 65: The effect of infection by E. pisi on the neutral lipid fatty acid composition of epidermal strips from the cultivar Onward

Figure 65

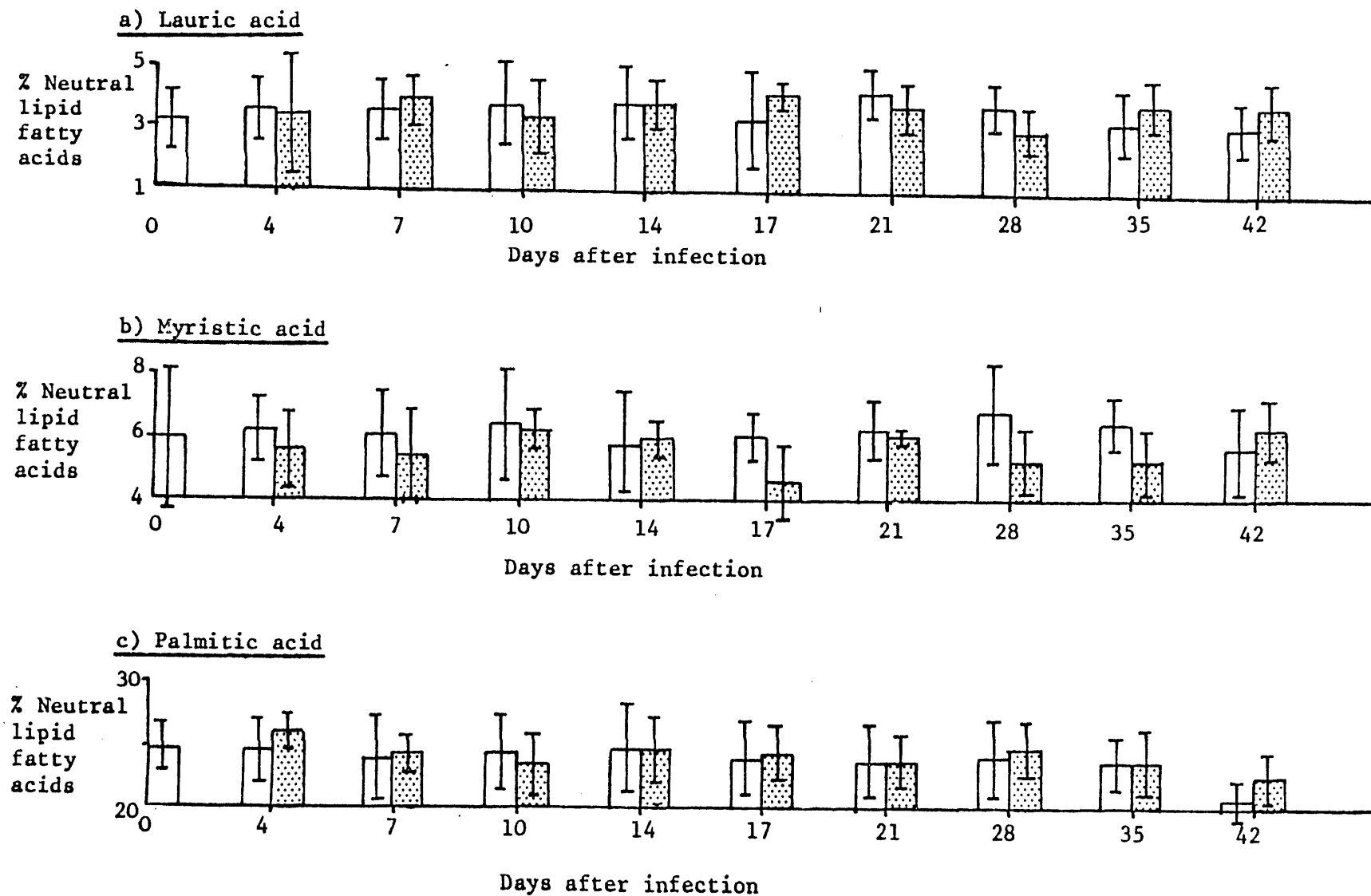
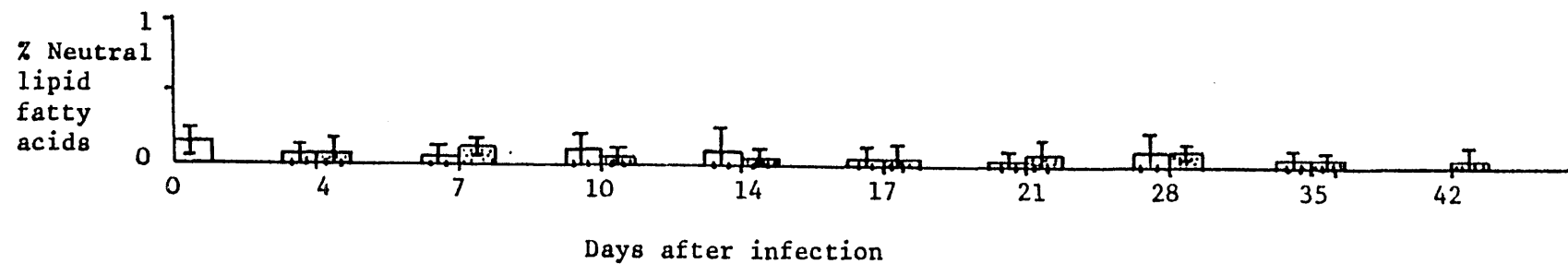
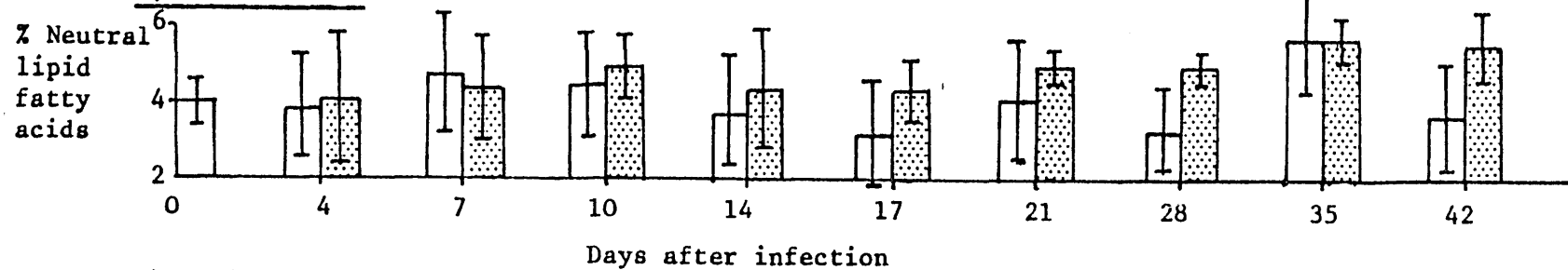


Figure 65 (cont.)

d) Palmitoleic acid



e) Stearic acid



f) Oleic acid

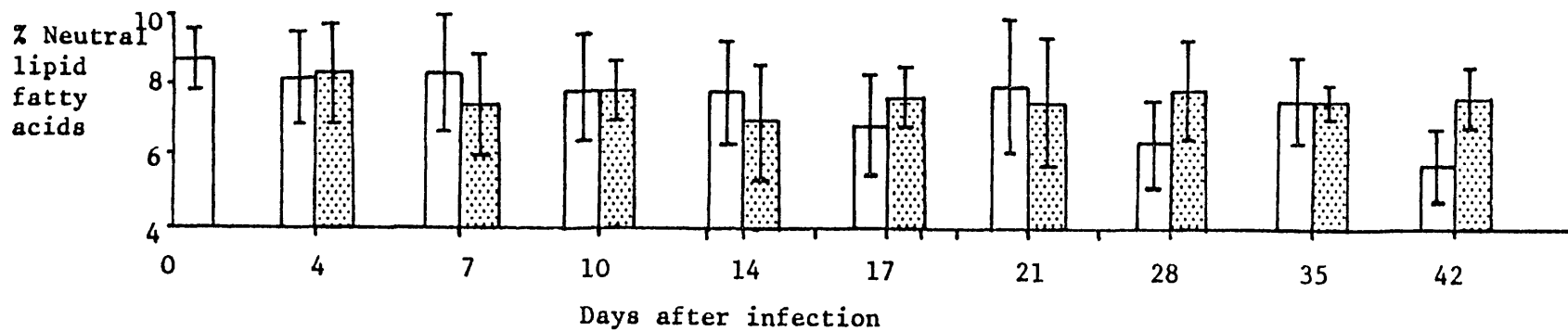
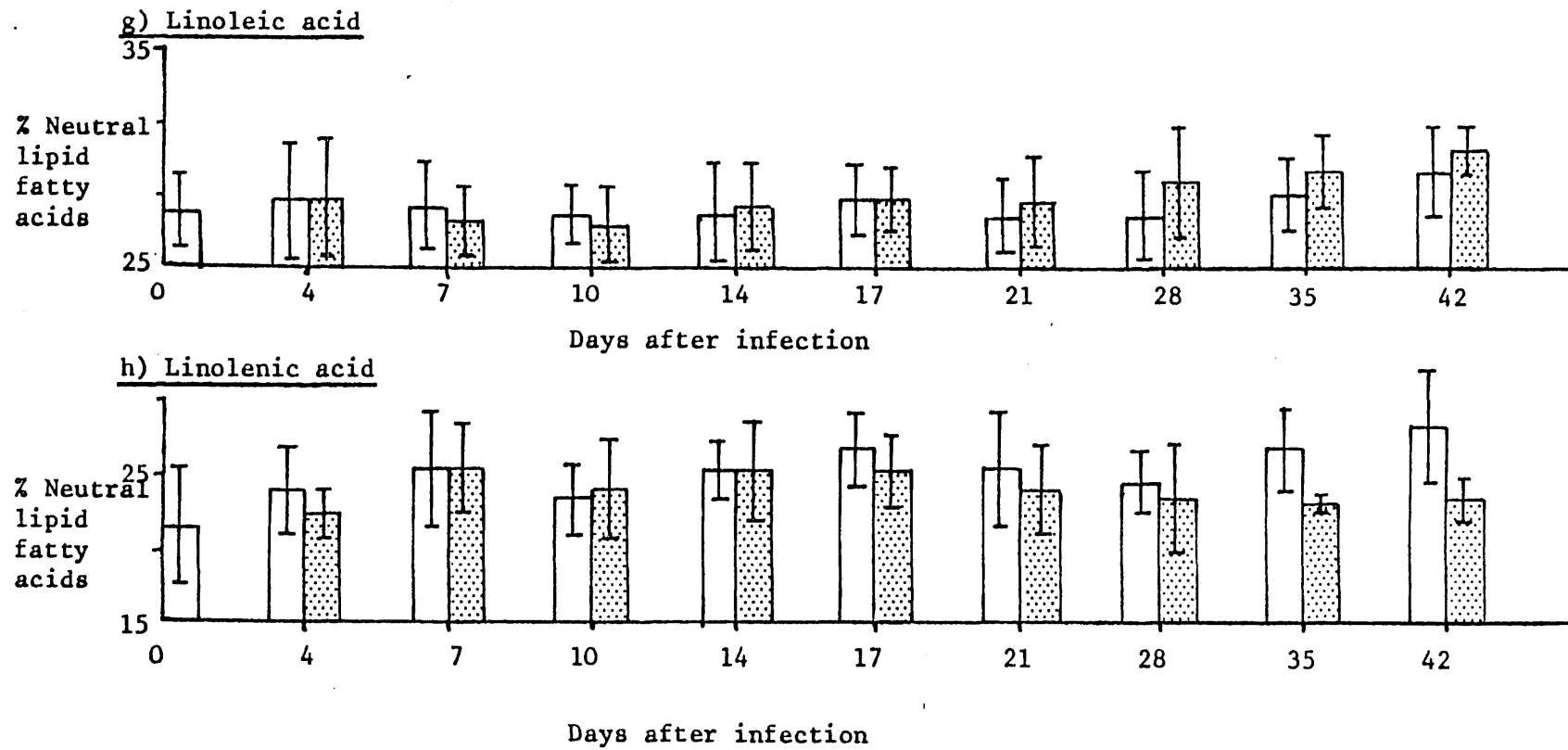


Figure 65 (cont.)



Unshaded Uninfected Plants

Shaded Infected Plants

I 95% Confidence Limits

Table 23. Percentage fatty acids of individual neutral lipids 42 days after infection

	Lipid Classes									
	Sterol esters		Monoglycerides		Diglycerides		Triglycerides		Free Fatty Acids	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
Lauric acid	3.6 ± 1.3	3.3 ± 1.4	3.2 ± 0.7	3.9 ± 1.2	3.6 ± 1.2	3.0 ± 1.5	4.7 ± 1.3	4.8 ± 1.3	3.9 ± 0.4	4.4 ± 0.7
Myristic acid	7.7 ± 0.8	7.6 ± 1.2	6.6 ± 1.4	4.7 ± 0.8	5.5 ± 0.6	6.3 ± 1.6	5.7 ± 0.6	5.9 ± 0.3	8.1 ± 1.2	7.4 ± 0.7
Palmitic acid	24.4 ± 1.6	24.0 ± 2.0	23.9 ± 2.5	24.8 ± 2.7	21.8 ± 2.6	24.6 ± 2.6	24.3 ± 2.2	22.3 ± 0.8	21.4 ± 4.3	22.6 ± 2.6
Palmitoleic acid	-	-	0.2 ± 0.3	0.2 ± 0.3	0.0 ± 0.1	0.1 ± 0.1	0.2 ± 0.5	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.2
Stearic acid	4.4 ± 0.7	5.0 ± 0.9	3.6 ± 1.0	3.3 ± 0.3	3.6 ± 1.9	4.1 ± 1.1	3.3 ± 0.5	4.7 ± 0.6	3.5 ± 2.6	4.5 ± 0.7
Oleic acid	8.3 ± 1.3	8.4 ± 0.9	8.8 ± 1.3	8.3 ± 1.2	6.7 ± 2.0	7.8 ± 1.7	8.8 ± 1.5	8.5 ± 0.9	8.9 ± 0.8	8.8 ± 0.6
Linoleic acid	27.9 ± 1.7	28.9 ± 2.9	29.7 ± 3.6	29.3 ± 4.3	30.0 ± 3.0	7.8 ± 1.7	8.8 ± 1.5	8.5 ± 0.4	8.9 ± 0.8	8.8 ± 0.6
Linolenic acid	23.5 ± 3.9	22.8 ± 2.7	25.1 ± 4.8	25.4 ± 1.4	28.4 ± 3.4	24.4 ± 2.9	23.3 ± 2.4	20.1 ± 1.8	23.2 ± 4.7	19.3 ± 2.2

All figures represent the means of 4 replicates quoted with 95% confidence limits

4.4. Lipid composition of *E. pisi* mycelium

Mycelial lipid composition was assessed at various intervals throughout a period of 42 days from initial inoculation. The reason for analysing these lipids was to test whether any changes in host epidermal lipids could be attributed to the presense of fungal lipids within the cells. While it is conceded that the fungal lipids within the epidermal cells may differ from mycelial lipids, the difficulty of obtaining a pure sample of haustorial complexes (Section 4.6) left the epiphytic vegetative hyphae as the most convenient alternative source of fungal tissue.

4.4.1. Total Lipids

Total lipids in fungal mycelium were assessed by relating the weight of the lipids to the dry weight of mycelium (Figure 66). The total amount of lipid in the mycelium did not change significantly ($P \approx 0.05\%$) during the 42 day infection period. After 4 days total lipids constituted $276 \pm 84 \mu\text{g g}^{-1}$ dry weight, polar lipids were $147.6 \pm 4 \mu\text{g g}^{-1}$ dry weight and neutral lipids constituted the difference viz $128.8 \pm 18.6 \mu\text{g g}^{-1}$ dry weight. None of the lipid classes changed significantly throughout the 42 day period (Figure 66).

4.4.2. Polar Lipids

The composition of the mycelial polar lipid 4 days after inoculation was as follows:- PC $22.2 \pm 1.6\%$, PE $29.4 \pm 1.9\%$, PG $11.3 \pm 0.6\%$, PI $5.8 \pm 0.8\%$, PS $4.9 \pm 0.8\%$, PA $3.4 \pm 0.8\%$, MGDG $11.5 \pm 1.3\%$ and DGDG $11.4 \pm 0.8\%$. These values did not change significantly ($p = 0.05$) throughout the 42 day period (Figure 67).

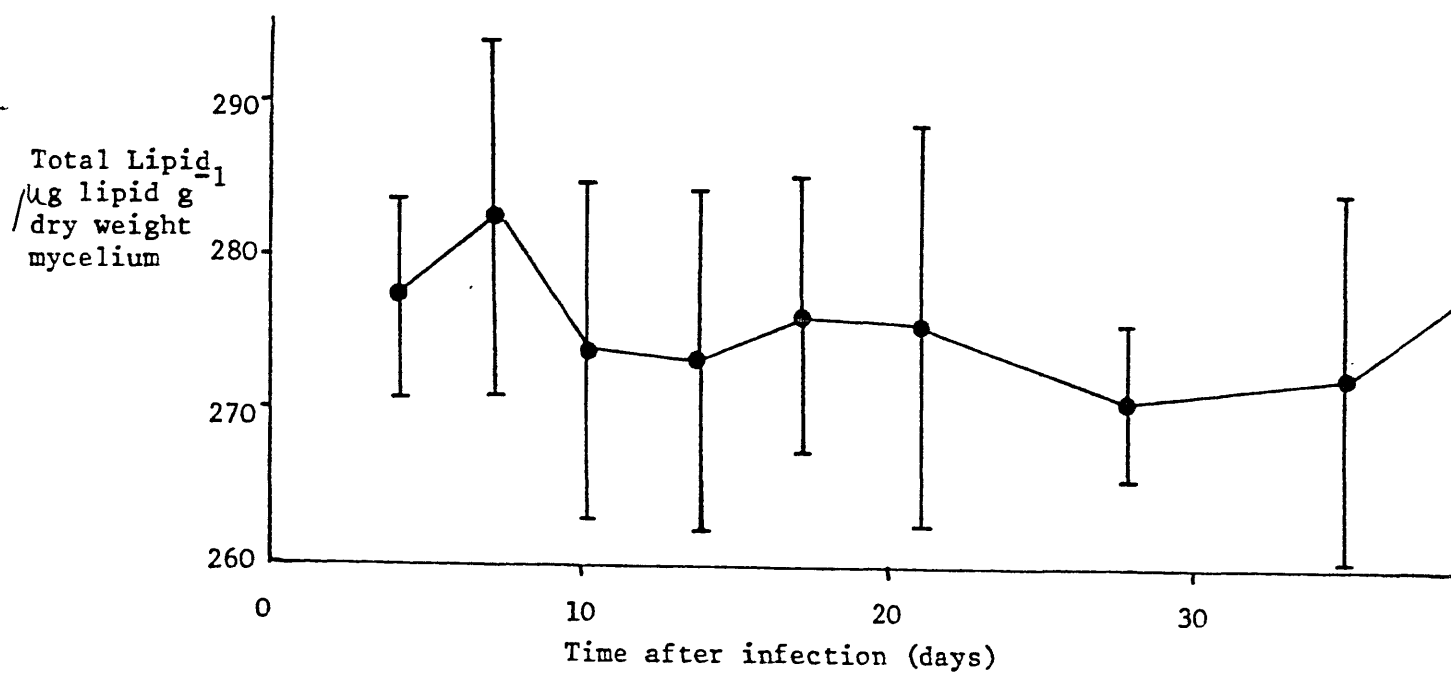
The composition of the mycelial polar lipid-bound fatty acids 4 days after inoculation was as follows; myristic acid $3.1 \pm 1.7\%$, palmitic acid $13.1 \pm 1.4\%$, palmitoleic acid $3.3 \pm 0.6\%$, stearic acid $7.1 \pm 0.8\%$, oleic acid $12.3 \pm 1.0\%$, linoleic acid $5.7 \pm 1.4\%$, linolenic acid $21.0 \pm 2.8\%$, arachidic acid $4.0 \pm 1.3\%$, arachidonic acid $8.7 \pm 0.7\%$ and lignoceric acid $1.6 \pm 0.6\%$ (Figure 68). These values did not change significantly ($p = 0.05$) throughout the 42 day infection period.

Closer analysis of each of the component polar lipids revealed that each of the phospholipids had a similar composition in terms of fatty acids at the start of the 42 day period (Table 24). The two glycolipids differed in that the linolenic acid content was much higher, constituting about 60% of the total (Table 24). With the single exception of palmitic acid in DGDG each of the other component fatty acids was present in lower amounts than in the phospholipids. There were no changes observed in any of the fatty acid compositions of any polar lipid over the 42 day period of observation.

Figure 66: Total mycelial lipid content at various stages during a 42 day infection period

Figure 66

a) Total Lipid



b) Total Polar Lipids

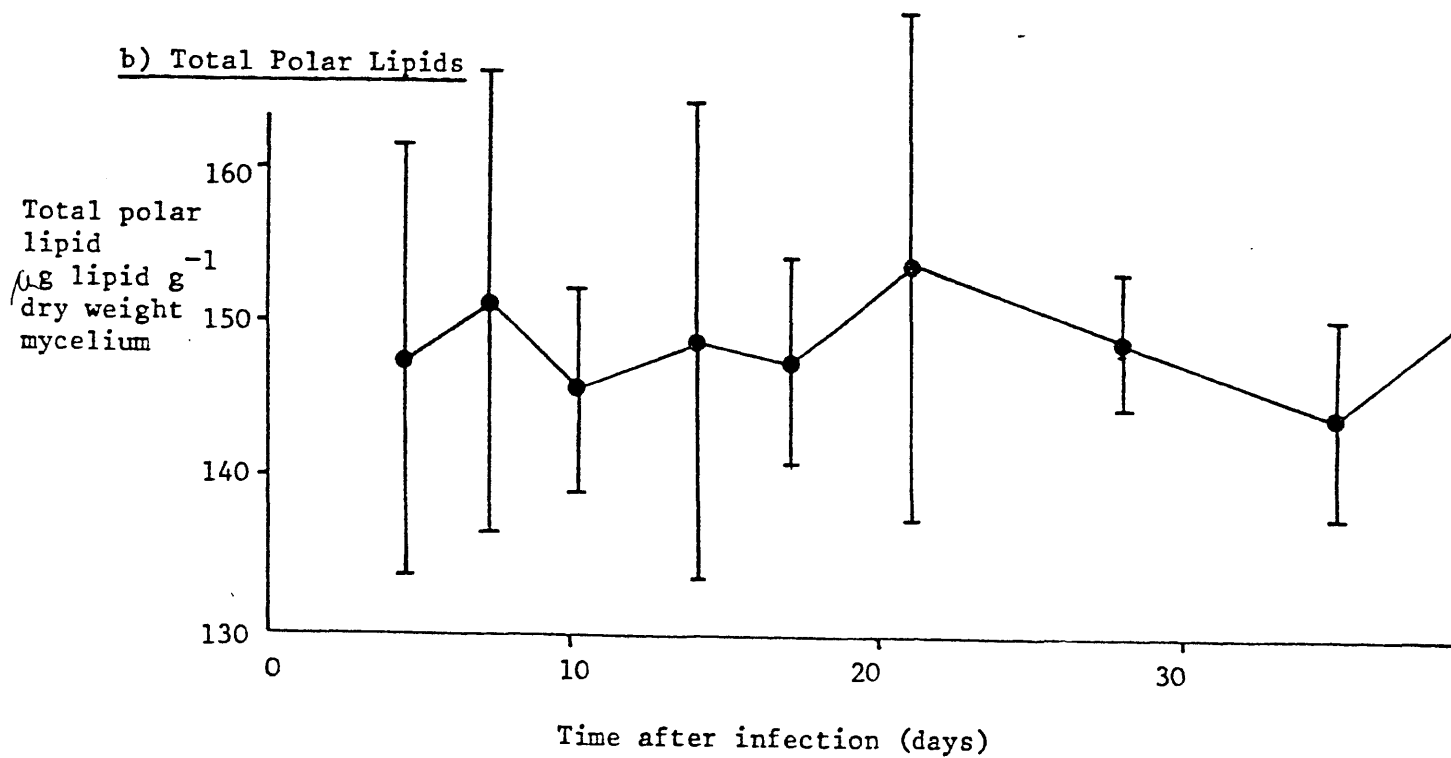


Figure 66 (cont)

c) Total Neutral Lipid



I 95% Confidence Limits

Figure 67: Polar lipid composition of fungal hypha for 42 days after infection

Figure 67

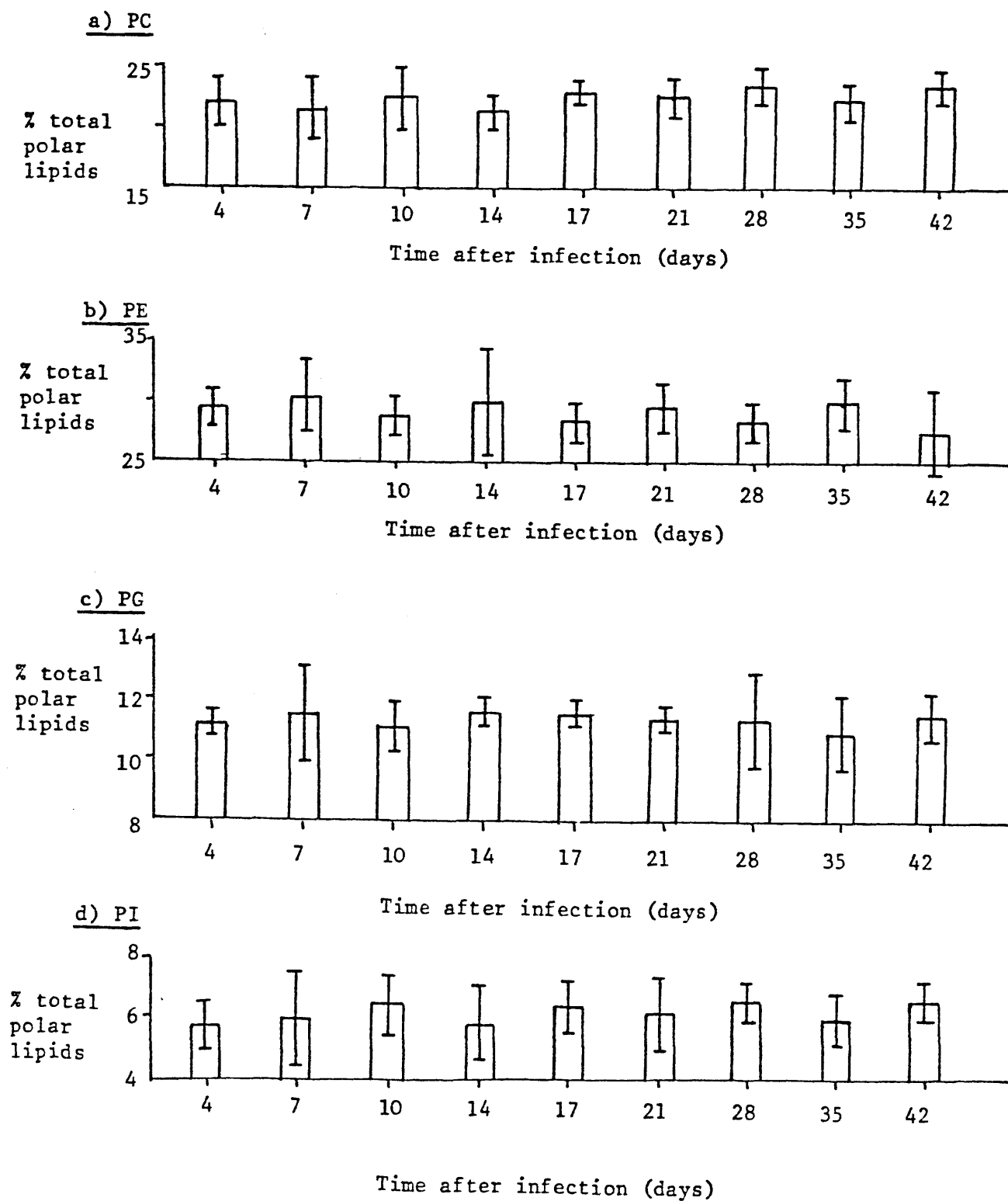
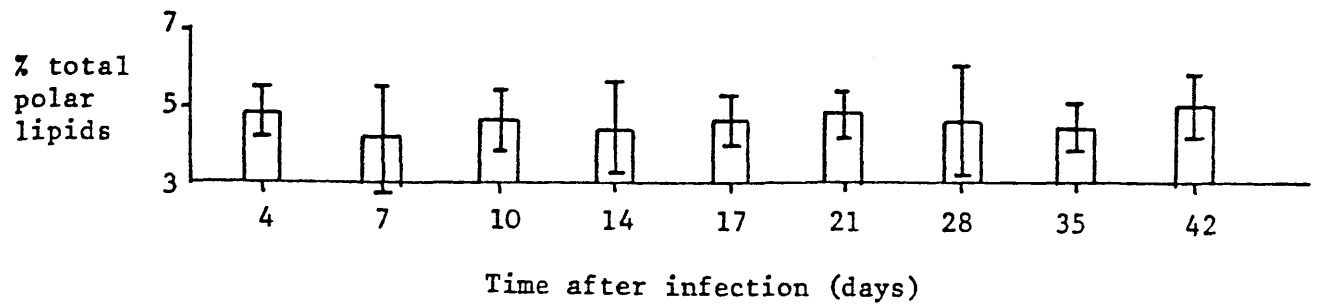
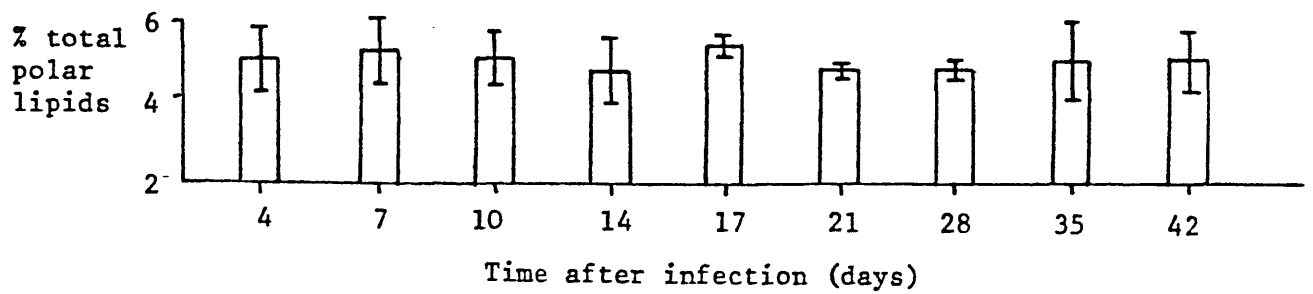


Figure 67 (cont.)

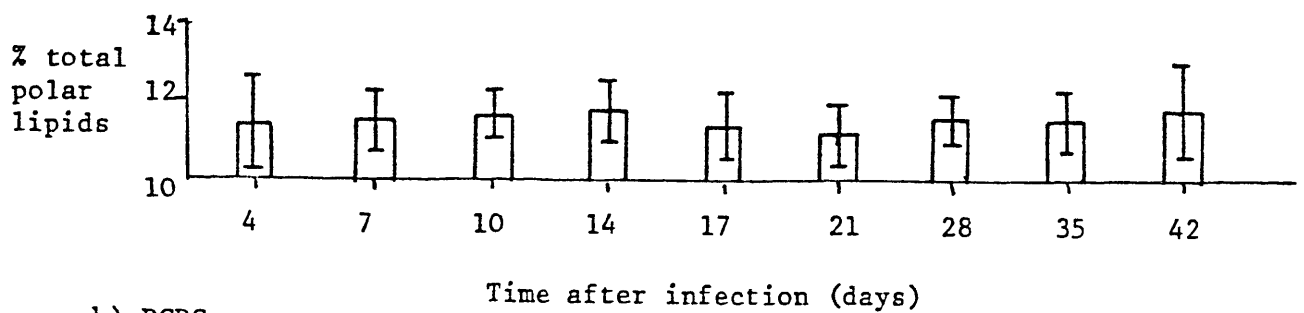
e) PS



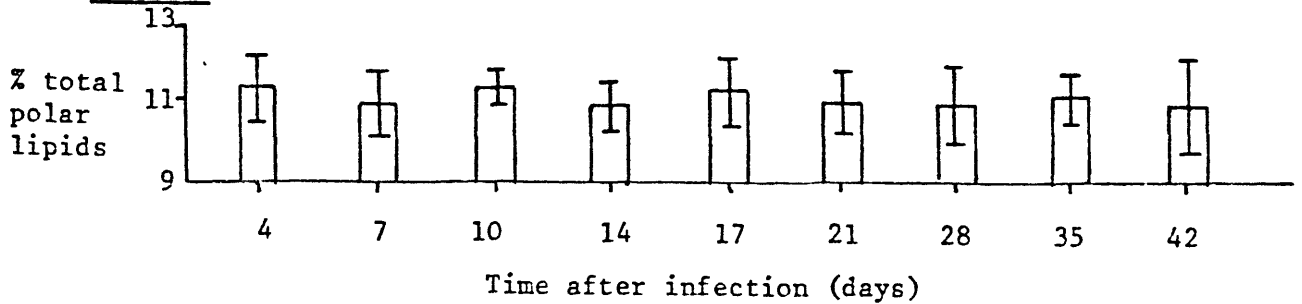
f) PA



g) MGDG



h) DGDG



I 95% Confidence Limits

4.4.3. Neutral Lipids

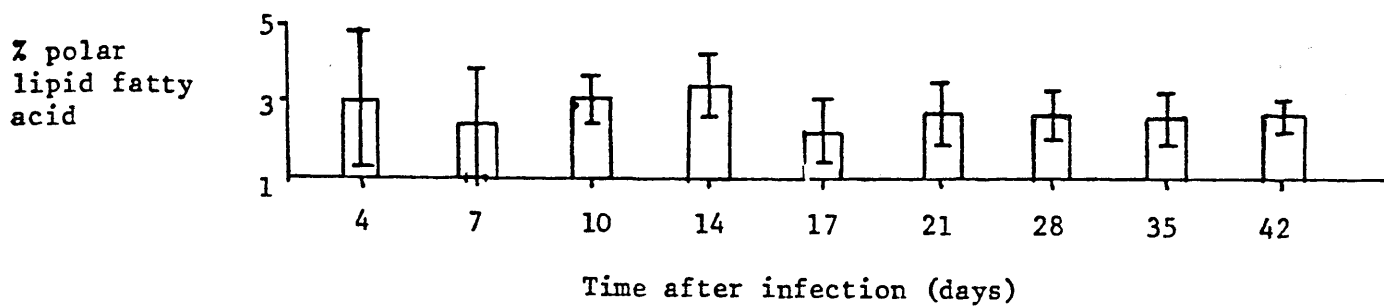
The composition of the mycelial neutral lipid 4 days after inoculation was as follows:- sterol esters $14.6 \pm 1.6\%$, sterols $28.0 \pm 2.7\%$, monoglycerides $3.0 \pm 1.1\%$, diglycerides $5.4 \pm 1.7\%$, triglycerides $40.5 \pm 4.1\%$, and free fatty acids $8.3 \pm 1.2\%$ (Figure 69). These values did not change significantly ($p = 0.05$) throughout the 42 day infection period.

The composition of the total mycelial neutral lipid bound fatty acids 4 days after infection was as follows; myristic acid $3.4 \pm 1.7\%$, palmitic acid $13.1 \pm 1.8\%$, palmitoleic acid $0.4 \pm 0.7\%$, stearic acid $4.0 \pm 0.5\%$, oleic acid $3.0 \pm 1.6\%$, linoleic acid $22.5 \pm 2.5\%$, linolenic acid $38.5 \pm 2.2\%$, arachidic acid $3.2 \pm 1.2\%$, arachidonic acid $9.5 \pm 1.2\%$ and lignoceric acid $2.1 \pm 0.8\%$. These values did not change significantly ($p = 0.05$) throughout the 42 day infection period (Figure 70). Compared with the fatty acids of the polar lipid fraction (Figure 69) the content of palmitoleic, stearic, oleic and linoleic acids was lower and that of linolenic acid was appreciably higher.

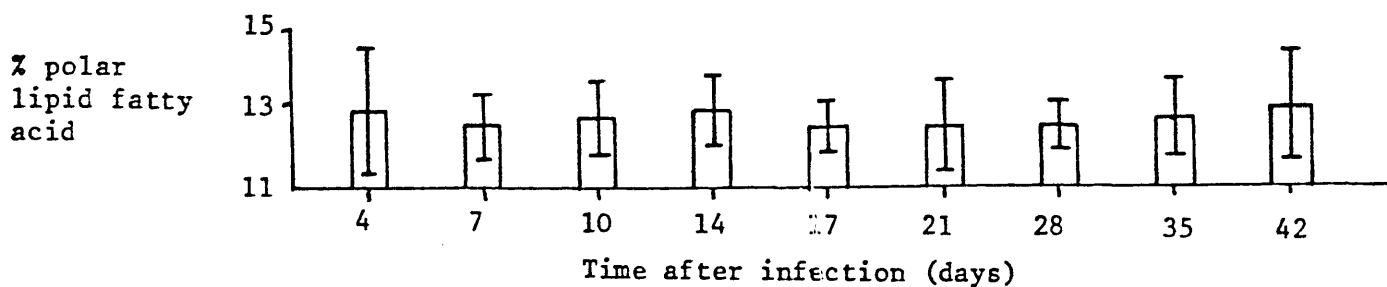
Figure 68: Fatty acid composition of the polar lipid fraction at various stages during a 42 day infection period

Figure 68

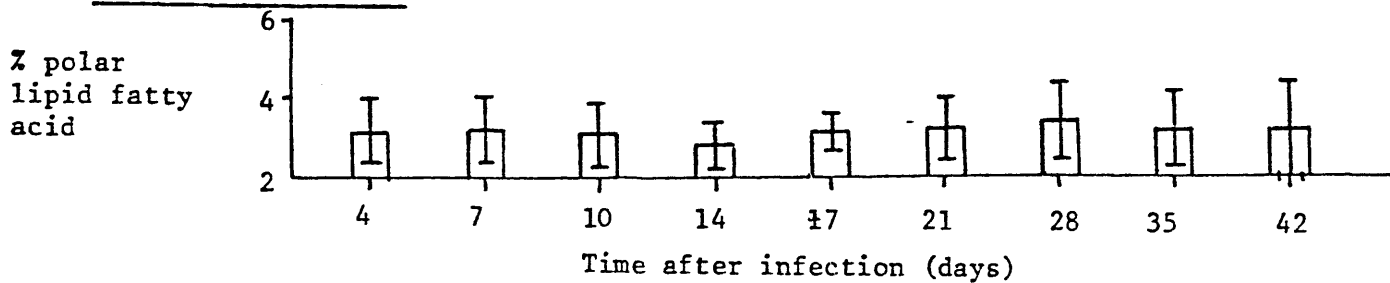
a) Myristic acid



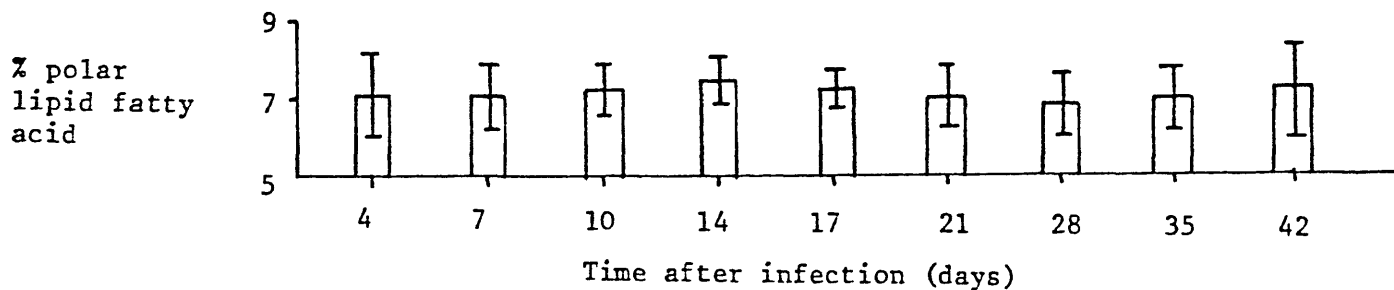
b) Palmitic acid



c) Palmitoleic acid



d) Stearic acid



e) Oleic acid

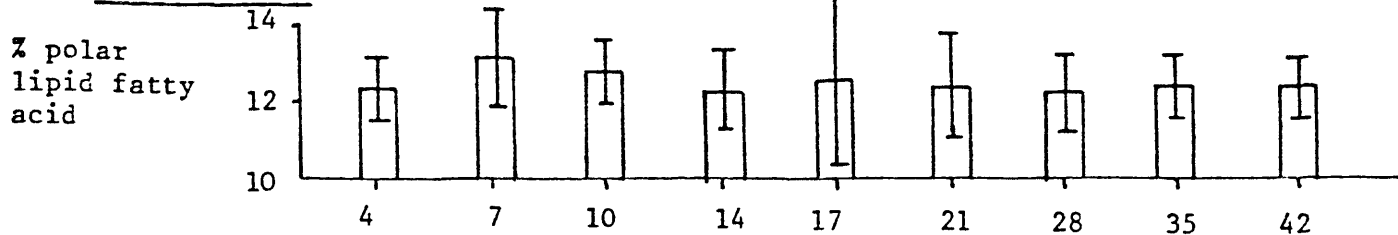
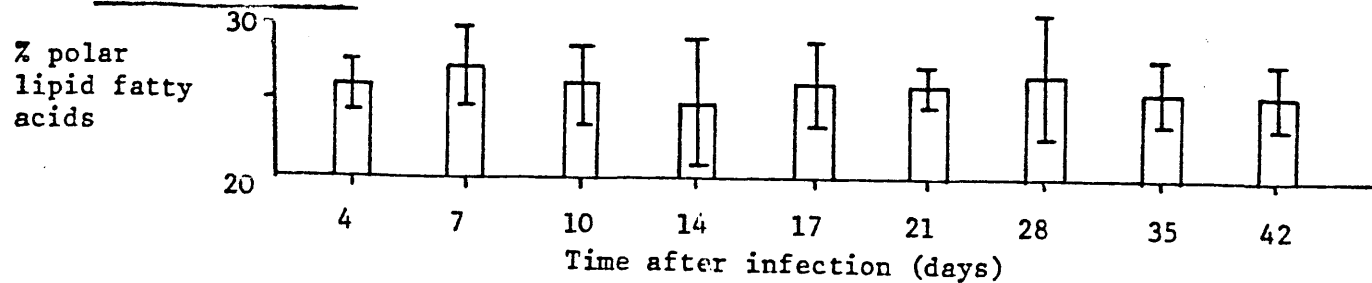
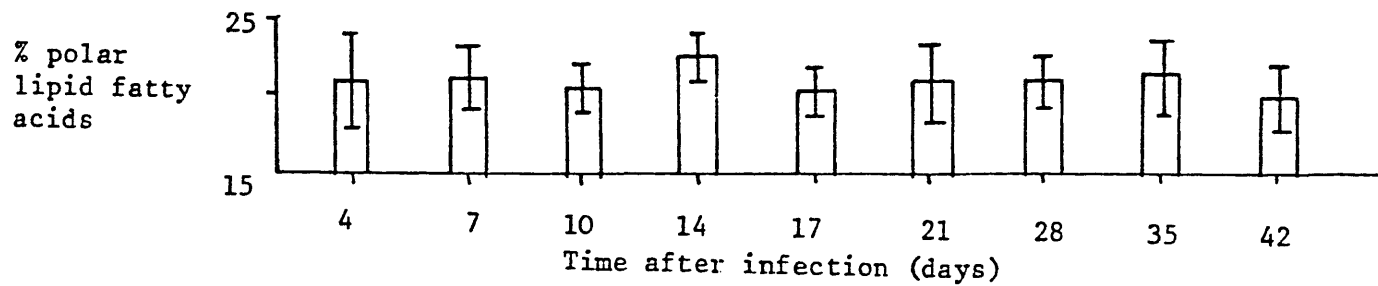


Figure 68 (cont.)

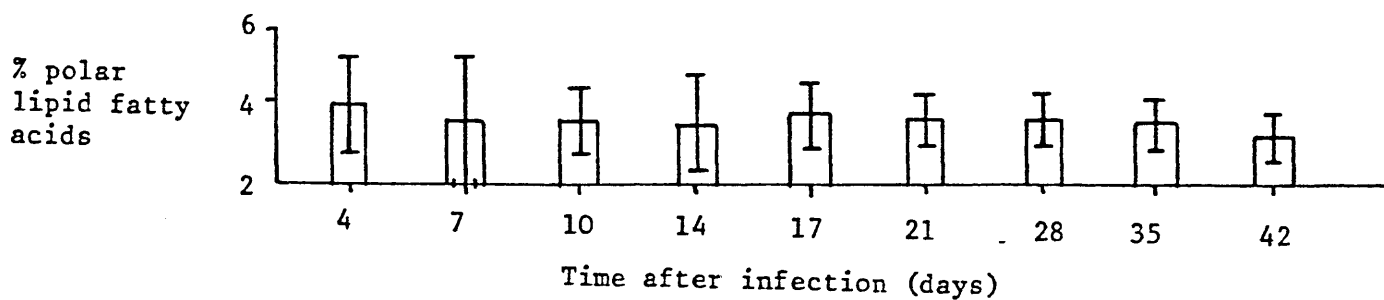
f) Linoleic acid



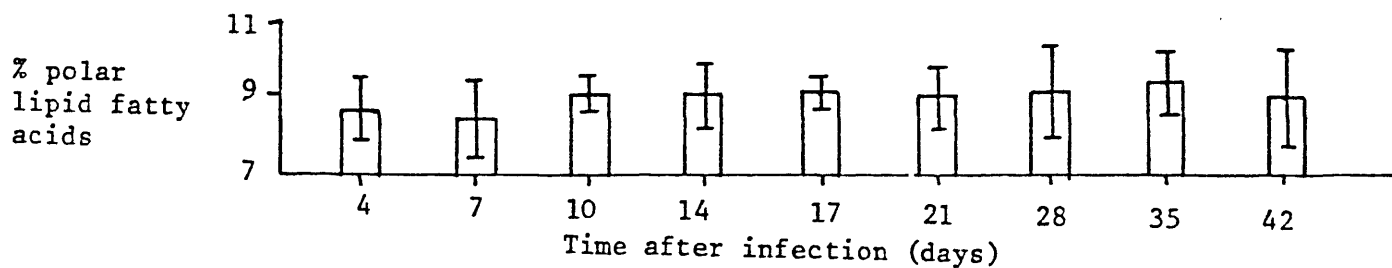
g) Linolenic acid



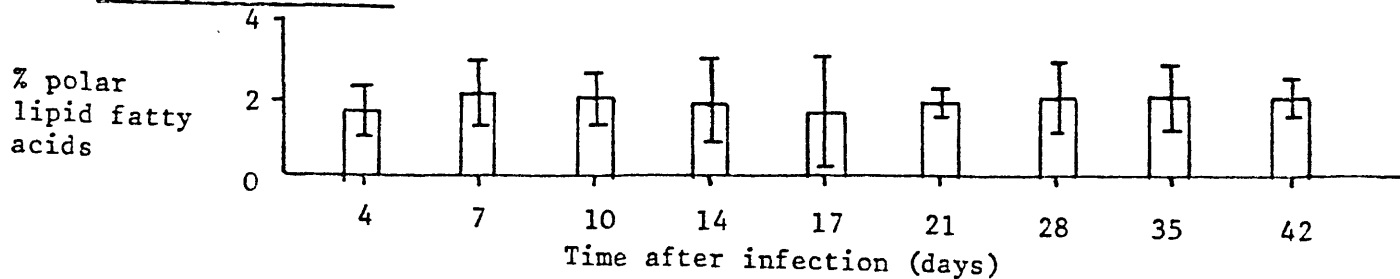
h) Arachidic acid



i) Arachidonic acid



j) Lignoceric acid

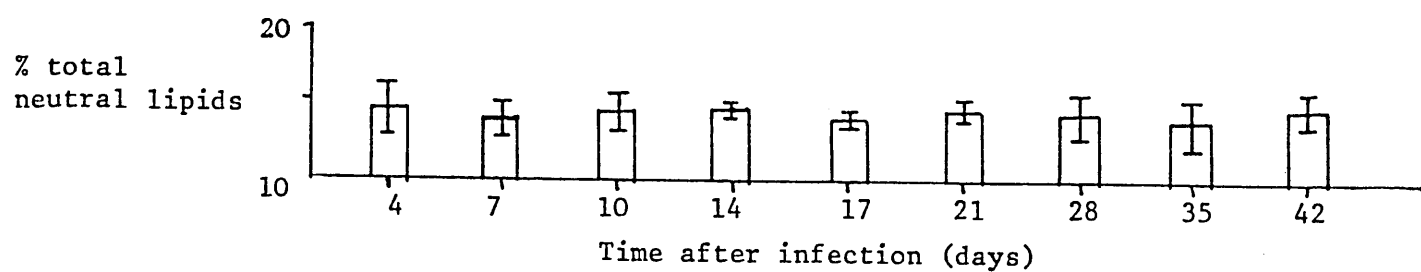


I 95% Confidence Limits

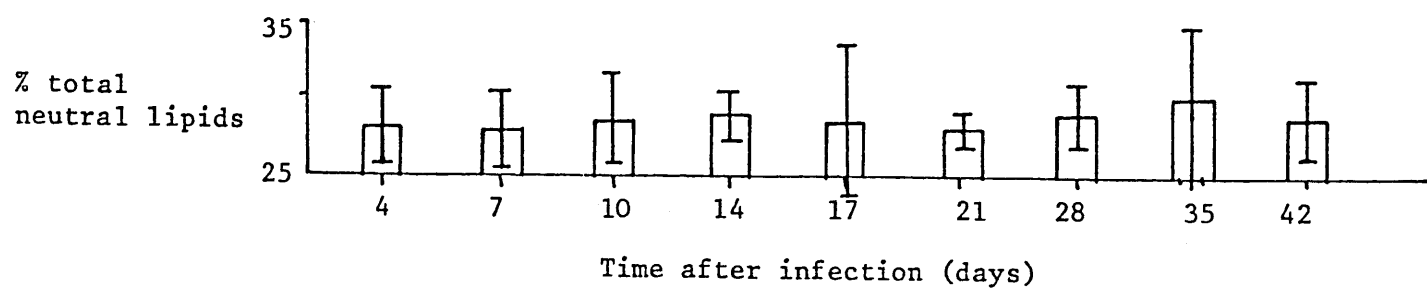
Figure 69: Neutral Lipid composition of fungal hypha for 42 days after infection

Figure 69

a) Sterol esters



b) Sterols



c) Monoglycerides

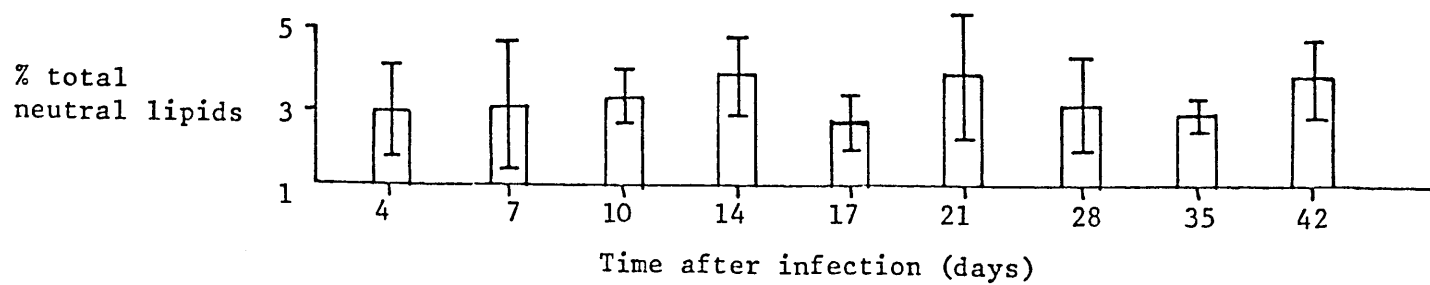
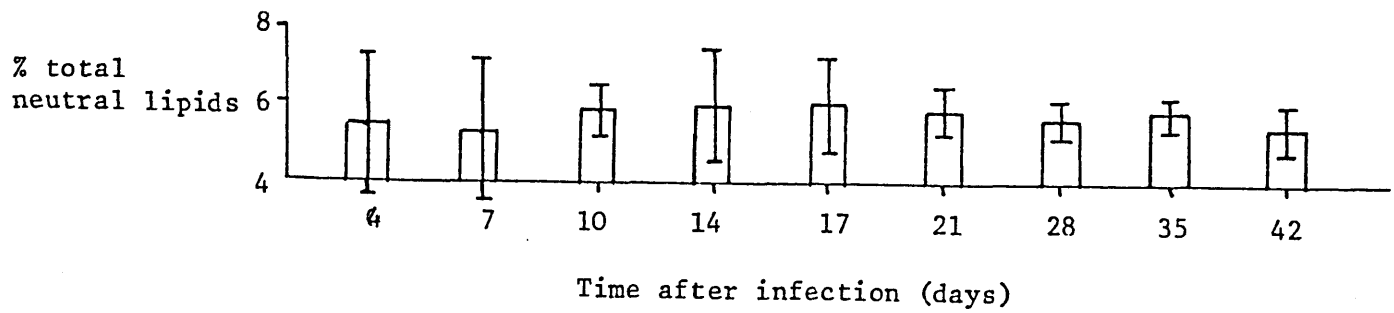
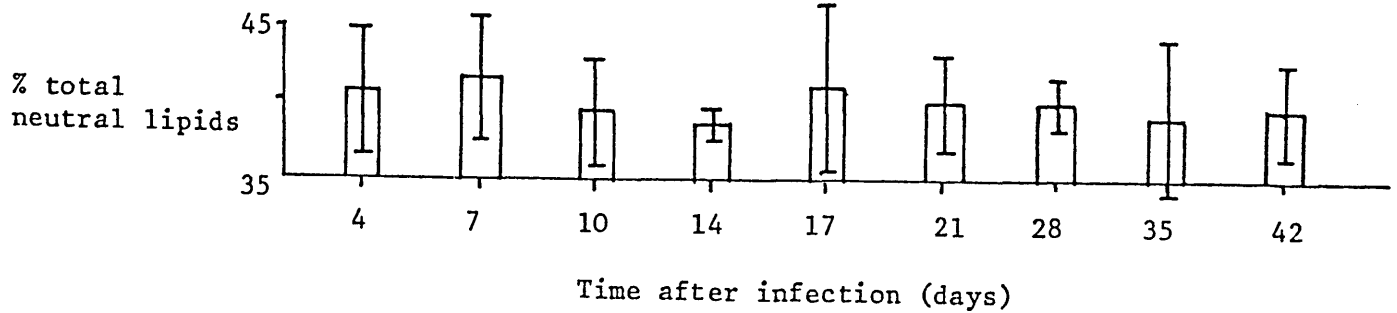


Figure 69 (cont.)

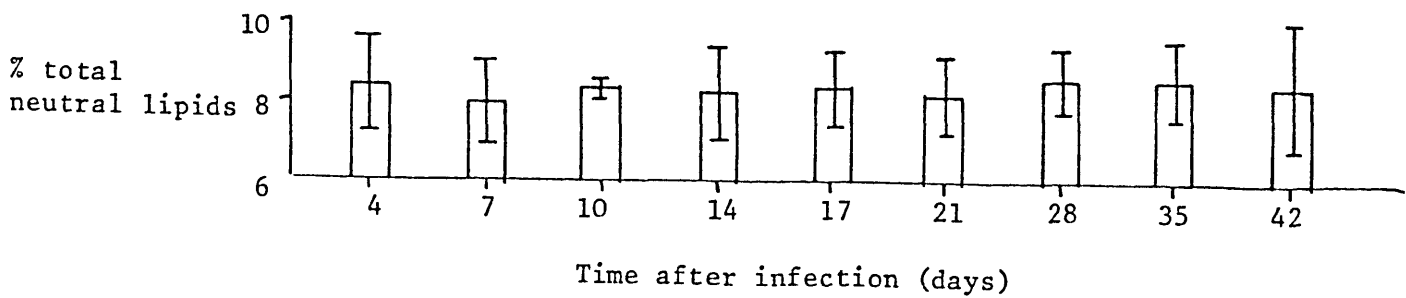
d) Diglycerides



e) Triglycerides



f) Free Fatty Acids

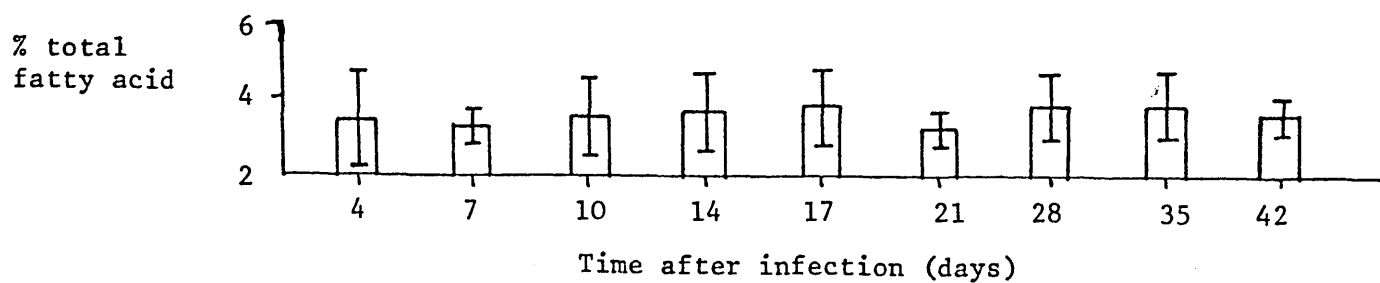


┌ 95% Confidence Limits

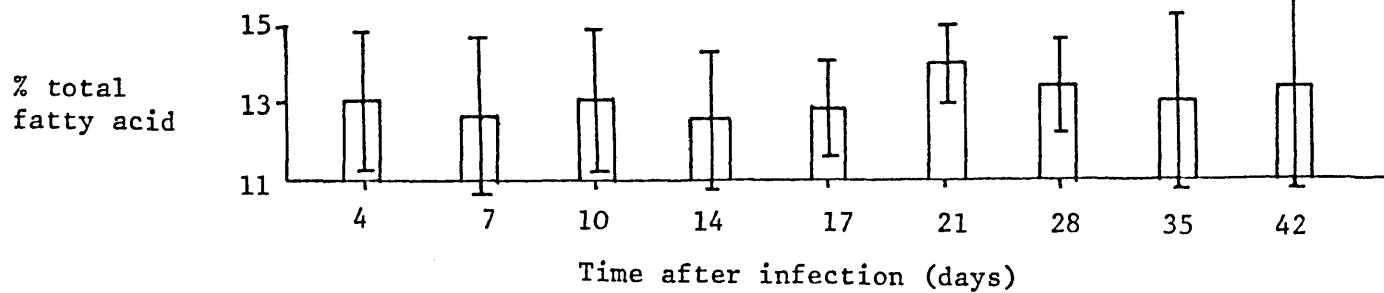
Figure 70: Fatty acid composition of the mycelial neutral lipid fraction at various stages during a 42 day infection period

Figure 70

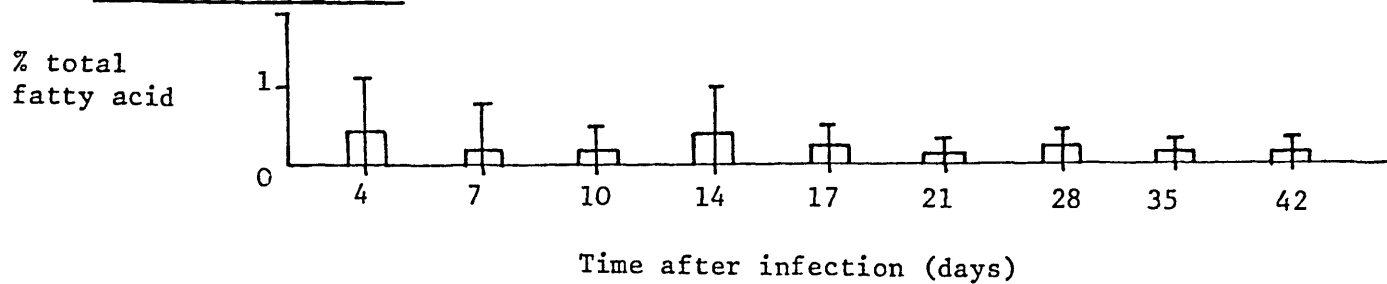
a) Myristic acid



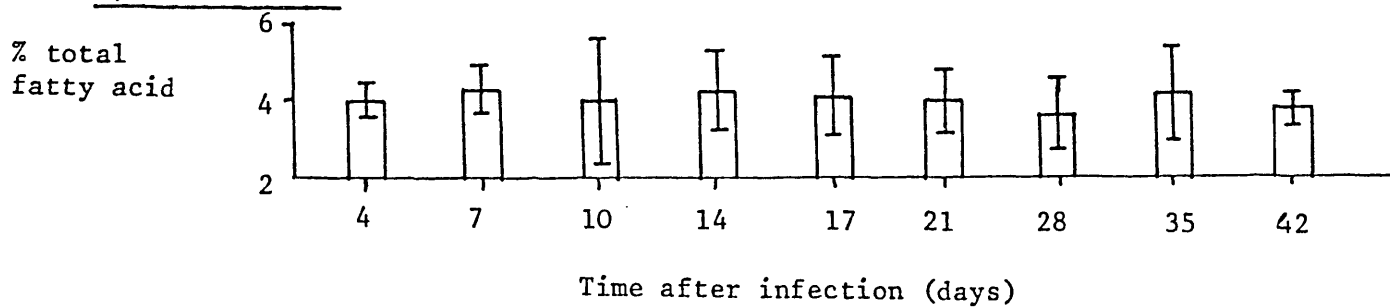
b) Palmitic acid



c) Palmitoleic acid



d) Stearic acid



e) Oleic acid

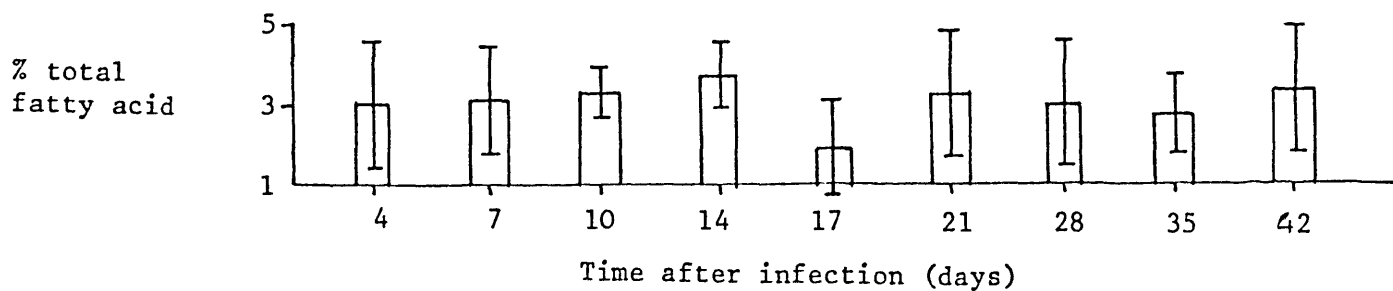
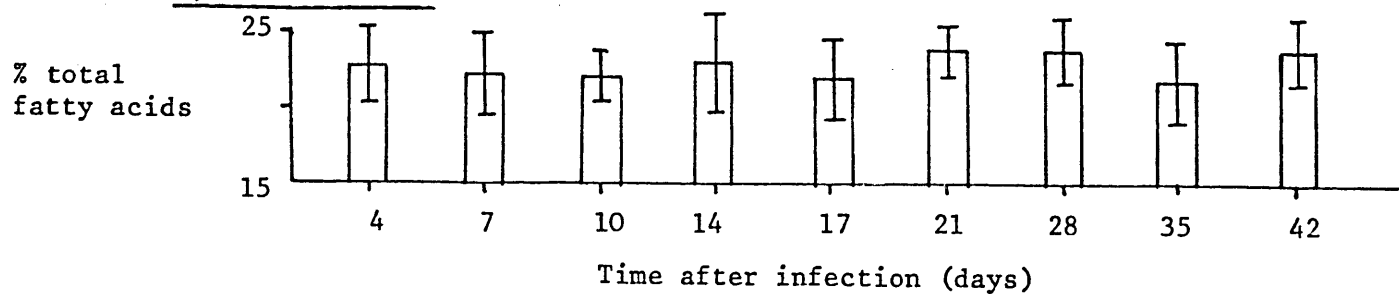
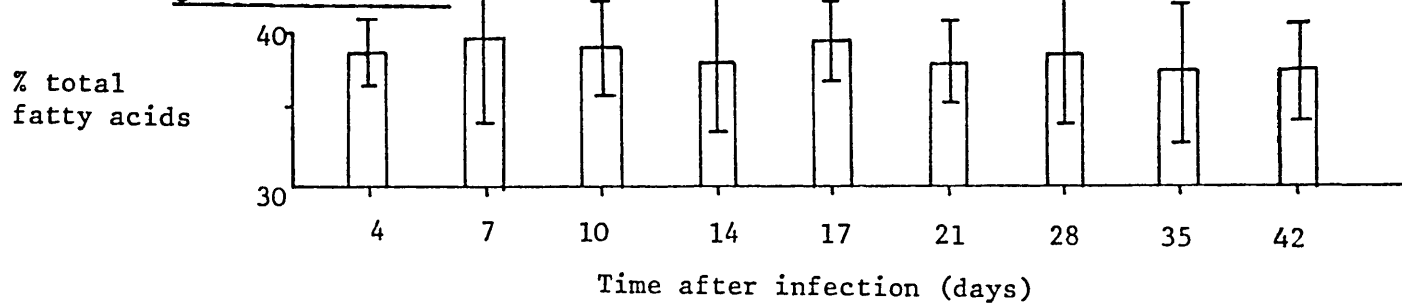


Figure 70 (cont.)

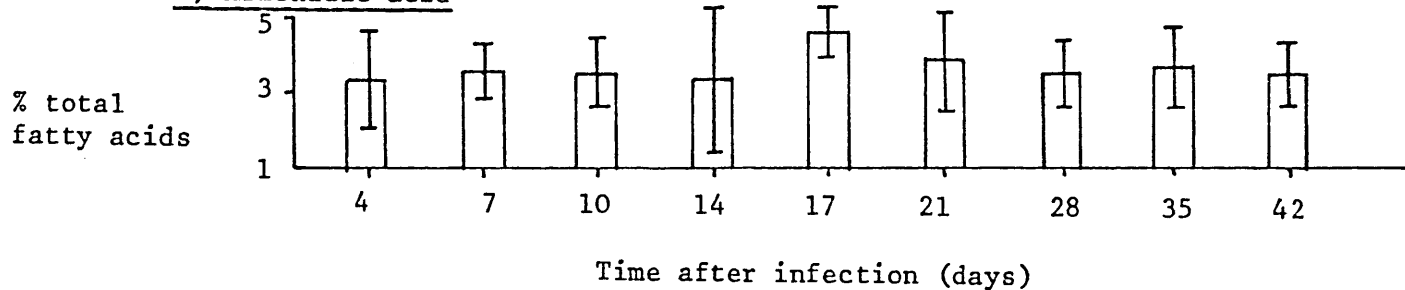
f) Linoleic acid



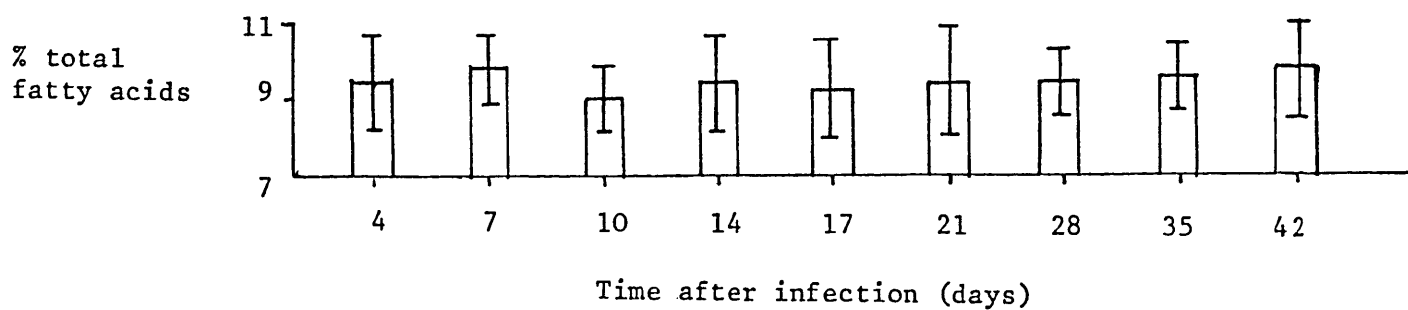
g) Linolenic acid



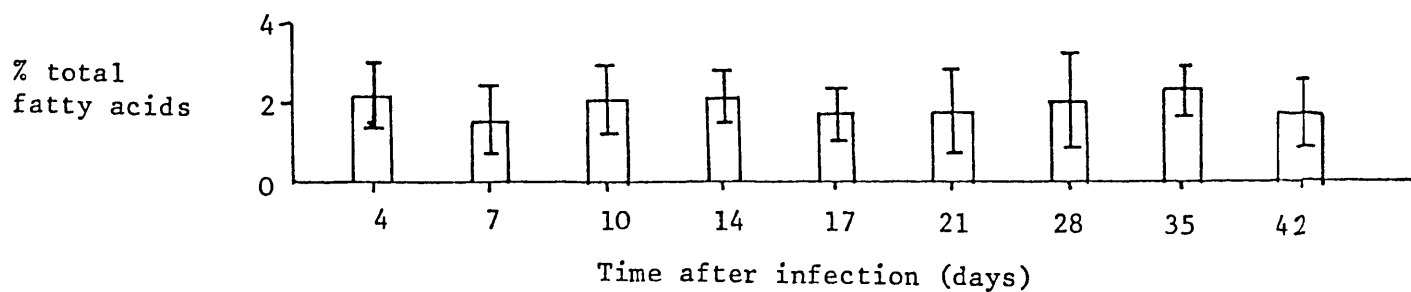
h) Arachidic acid



i) Arachidonic acid



j) Lignoceric acid



I 95% Confidence Limits

Table 24: Percentage fatty acid composition of individual polar lipid classes of fungal mycelium

Fatty Acid	PC	PE	PG	Lipid Class PI	PS	PA	MGDG	DGDG
Myristic acid	2.7 ± 0.6	2.7 ± 1.7	2.8 ± 1.4	3.0 ± 1.2	3.4 ± 0.8	3.1 ± 1.2	2.0 ± 0.4	1.4 ± 0.8
Palmitic acid	12.6 ± 0.4	12.9 ± 1.1	13.3 ± 0.9	12.7 ± 1.7	12.7 ± 1.0	13.3 ± 1.1	6.1 ± 0.9	11.9 ± 2.1
Palmitoleic acid	3.5 ± 0.5	2.5 ± 0.9	3.6 ± 1.2	3.1 ± 0.8	3.2 ± 0.9	3.3 ± 0.3	0.2 ± 0.1	0.1 ± 0.1
Stearic acid	7.1 ± 1.0	7.0 ± 0.3	6.9 ± 0.8	7.7 ± 0.7	7.0 ± 0.6	7.3 ± 0.4	3.5 ± 0.7	3.8 ± 0.7
Oleic acid	12.2 ± 1.4	11.6 ± 1.9	12.3 ± 0.9	12.7 ± 1.3	11.7 ± 0.4	12.7 ± 0.3	4.8 ± 1.0	4.7 ± 0.9
Linoleic acid	27.3 ± 1.8	27.0 ± 4.0	25.3 ± 6.4	25.0 ± 3.2	26.8 ± 3.1	26.8 ± 1.7	10.7 ± 1.1	13.5 ± 1.7
Linolenic acid	20.4 ± 0.9	20.9 ± 1.9	21.0 ± 3.3	21.2 ± 2.1	2.12 ± 0.4	19.8 ± 0.9	66.0 ± 0.7	56.7 ± 4.0
Arachidic acid	3.5 ± 1.3	3.4 ± 0.9	3.9 ± 0.6	3.4 ± 1.2	3.9 ± 0.9	3.0 ± 0.4	1.0 ± 0.5	1.8 ± 0.8
Arachidonic acid	8.8 ± 0.6	9.7 ± 0.2	8.2 ± 1.2	9.2 ± 1.0	8.4 ± 1.1	9.2 ± 1.0	5.6 ± 0.8	5.3 ± 1.3
Lignoceric acid	1.8 ± 0.8	2.3 ± 0.2	1.7 ± 1.2	1.9 ± 0.9	1.6 ± 0.6	1.5 ± 0.8	-	0.3 ± 0.7

All figures represent the means of 4 replicates quoted with 95% confidence limits

Table 25 Percentage fatty acids composition of individual neutral lipid classes of fungal mycelium

Fatty Acid	Sterol esters	Monoglycerides	Lipid Classes Diglycerides	Triglycerides	Free Fatty Acids
Myristic acid	3.2 ± 1.5	3.7 ± 0.6	3.6 ± 0.6	3.1 ± 1.0	4.1 ± 0.4
Palmitic acid	13.0 ± 1.1	12.8 ± 1.3	13.2 ± 2.1	13.2 ± 1.2	10.0 ± 1.7
Palmitoleic acid	0.2 ± 0.2	0.2 ± 0.3	0.3 ± 0.6	0.3 ± 0.6	0.9 ± 0.6
Stearic acid	4.0 ± 0.4	4.0 ± 0.2	4.0 ± 0.9	4.5 ± 0.6	7.6 ± 3.9
Oleic acid	3.6 ± 0.7	3.0 ± 1.0	3.1 ± 1.1	2.9 ± 0.9	8.9 ± 1.3
Linoleic acid	22.1 ± 2.9	22.5 ± 1.6	22.3 ± 1.2	22.1 ± 2.6	14.1 ± 2.2
Linolenic acid	39.3 ± 4.7	37.9 ± 0.5	39.7 ± 1.1	38.6 ± 4.6	31.9 ± 9.3
Arachidic acid	3.4 ± 1.0	3.7 ± 1.1	3.5 ± 0.8	3.5 ± 0.8	5.7 ± 0.4
Arachidonic acid	9.5 ± 0.9	9.8 ± 0.8	9.1 ± 1.1	10.1 ± 0.5	14.4 ± 1.5
Lignoceric acid	1.5 ± 0.5	2.4 ± 0.7	1.2 ± 0.6	1.1 ± 0.7	3.8 ± 0.8

All figures represent the means of 4 replicates quoted with 95% confidence limits

4.5. Lipid composition in infected and uninfected pea plants (cultivar Onward and plants grown in the dark)

Previous studies have shown a variety of effects of senescence on the lipid composition of plants. Galliard⁽¹⁹⁷³⁾ has shown that there is a net decrease in the amount of membrane lipid and a net increase in free fatty acids. He found that the remaining lipid bound fatty acids showed a net increase in fatty acid saturation in the early stages of senescence due to the breakdown of chloroplast lipids. El Hussein (1977) was able to show that senescence causes a significant increase in the levels of unsaturation in the fatty acids from PC, PE, PI and PS in dark grown wheat plants. These are precisely the changes found in diseased plants in these experiments and it was thought that a series of experiments looking at lipid composition in diseased plants and in prematurely senesced plants would yield some information as to the nature of the observed changes in lipid composition.

For these experiments plants were grown and infected as described in Section 3.1. The plants grown in the dark were grown under exactly the same conditions as the other plants except that from the day of infection onwards, they were kept in the dark.

The lipid composition of epidermal cells was assessed over the 42 day infection period at 7 day intervals.

4.5.1. Total Lipids

Figure 71 shows that there was no difference in the total amount of lipid found in the stem epidermal strips in uninfected plants, infected plants or in plants grown in the dark even after 42 days. There was however a significant change in the composition of the lipids in plants grown in the dark with a significant decrease in the amount of polar lipid together with a significant increase in the amount of neutral lipid. This difference began to be observed from the 28th day after the plants were put in the dark onwards (Figure 71).

4.5.2. Individual Polar Lipids

There were no changes in the level of the individual polar lipid classes during the 42 days of this experiment. The percentages were the same as were found in the previous experiment (Figure 45).

When the total polar lipid bound fatty acids were assessed the results for uninfected plants and infected plants over the 42 day period confirmed the results in the previous experiment (Figure 46). The results for plants grown in the dark mirrored the results for infected plants except that the levels of linoleic acid decreased much faster at an early stage and this was paralleled by an increase in the amount of linolenic acid (Figure 72).

This pattern was consistently found for the fatty acids bound to the individual polar lipids, this is illustrated in Figure 73 by using PC as a typical example but the pattern was similar for PE, PI, PS and PA.

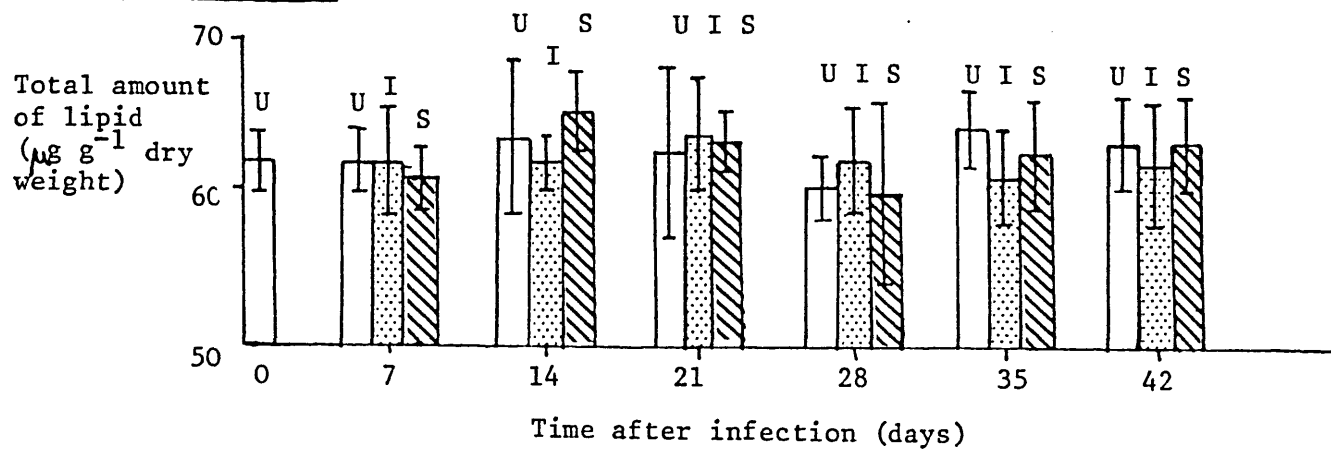
The results for MGDG- and DGDG-bound fatty acids in uninfected plants, infected plants and plants grown in the dark showed no significant change throughout the 42 day infection period. The results were the same as those illustrated in (Figure 54, 55) previous section.

The results for PG bound fatty acids are shown in Figure 74. Once again the only changes were found in linoleic and linolenic acid and the only differences between infected plants and plants grown in the dark was that the changes occurred earlier in the plants grown in the dark.

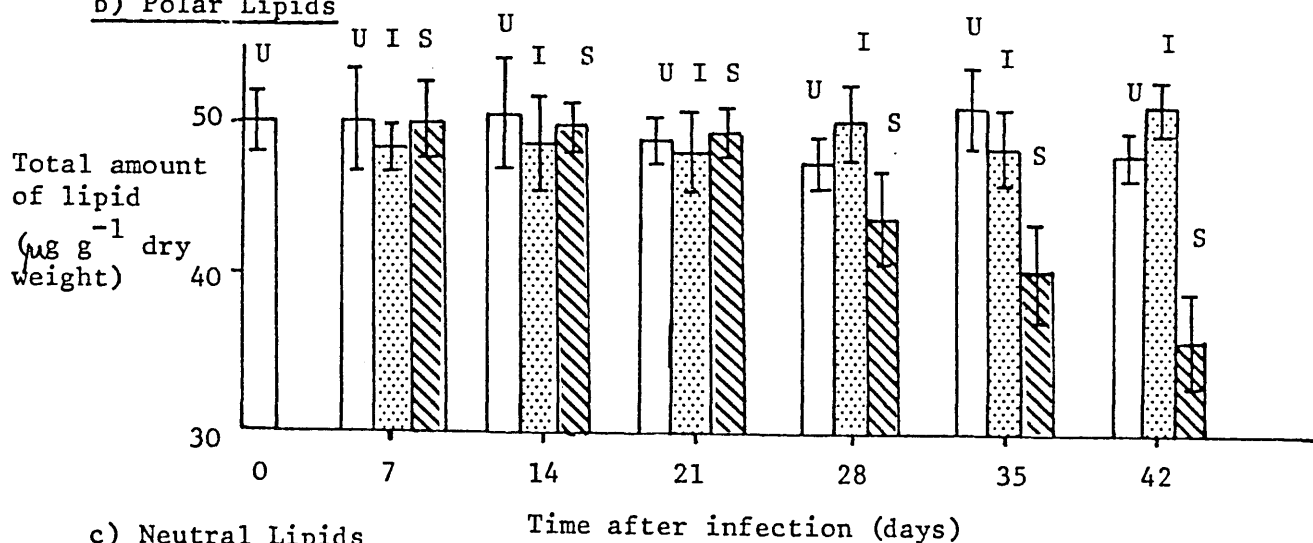
Figure 71: The effect of infection by E. pisi and growth in the dark on the lipid composition in epidermal cells from the cultivar Onward

Figure 71

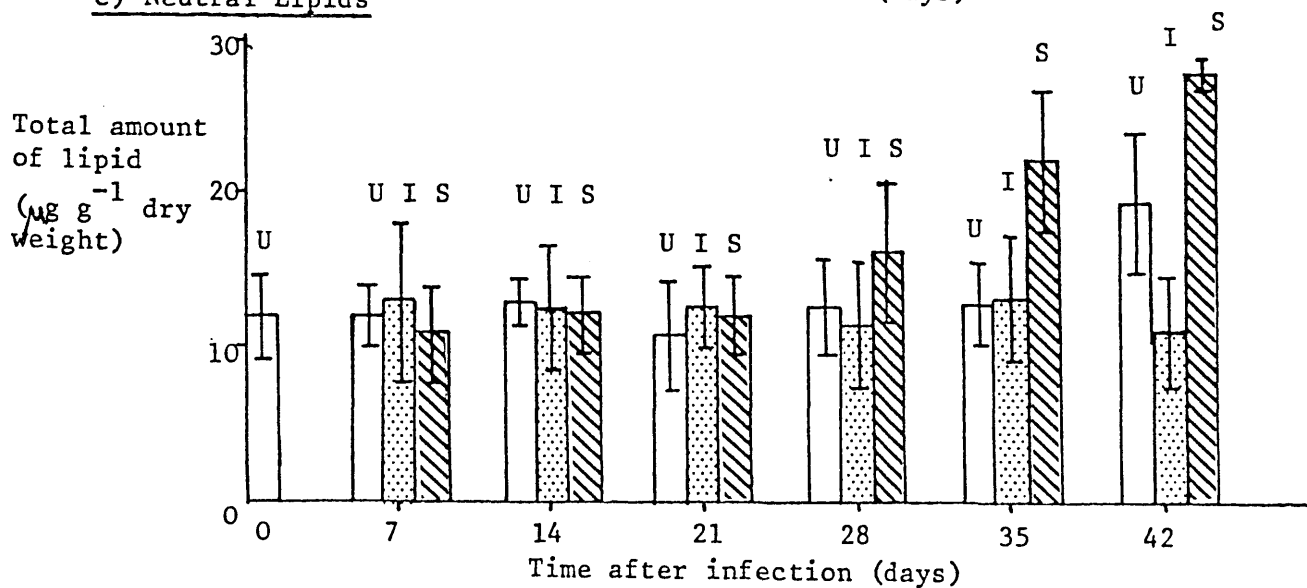
a) Total Lipids



b) Polar Lipids



c) Neutral Lipids



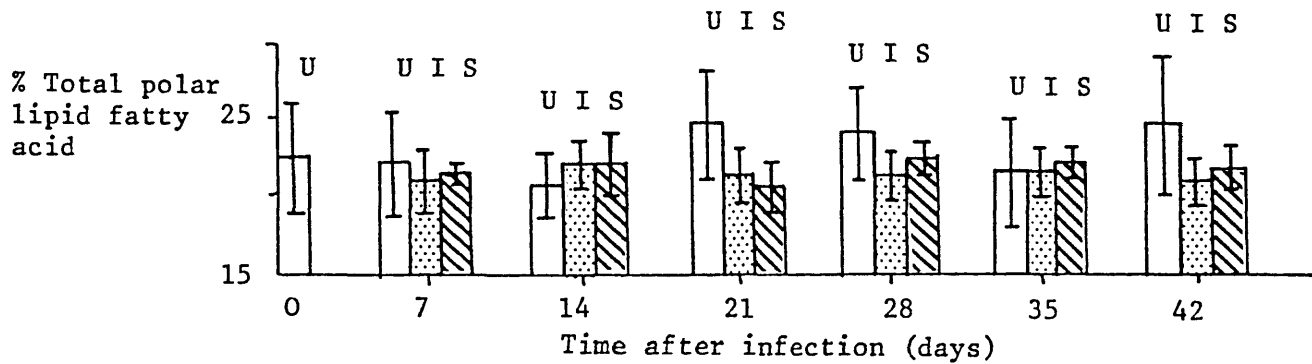
U Uninfected Plants
 I Infected Plants
 S Plants grown in the dark

95% Confidence Limits

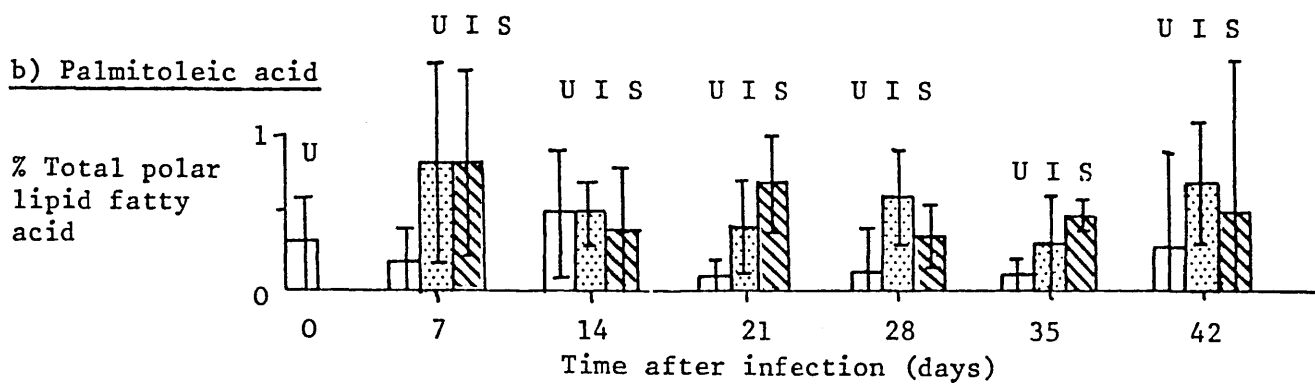
Figure 72: The effect of infection with E. pisi and growth in the dark on the polar lipid fatty acid composition in epidermal cells in the pea cultivar Onward

Figure 72

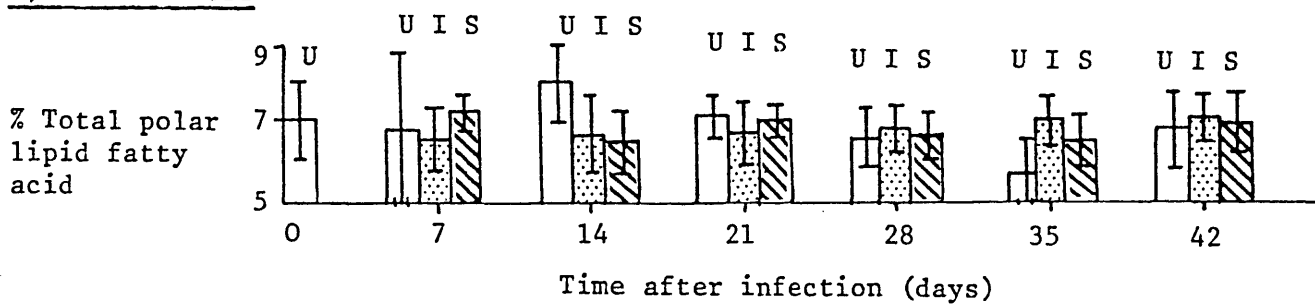
a) Palmitic acid



b) Palmitoleic acid



c) Stearic acid



d) Oleic acid

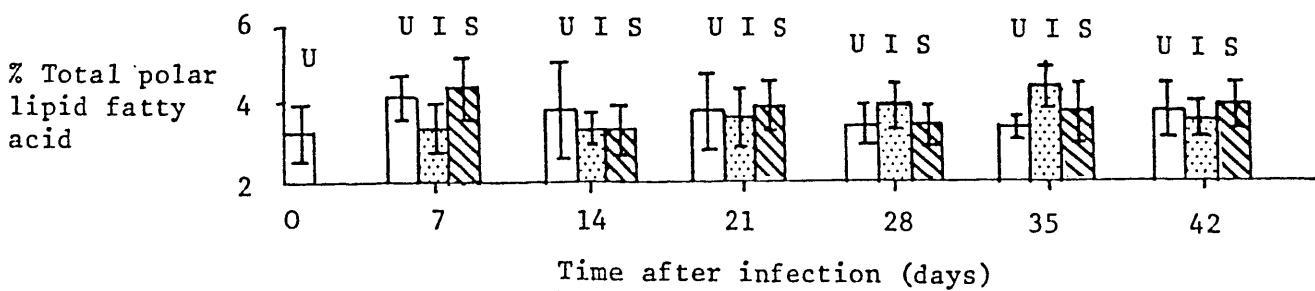
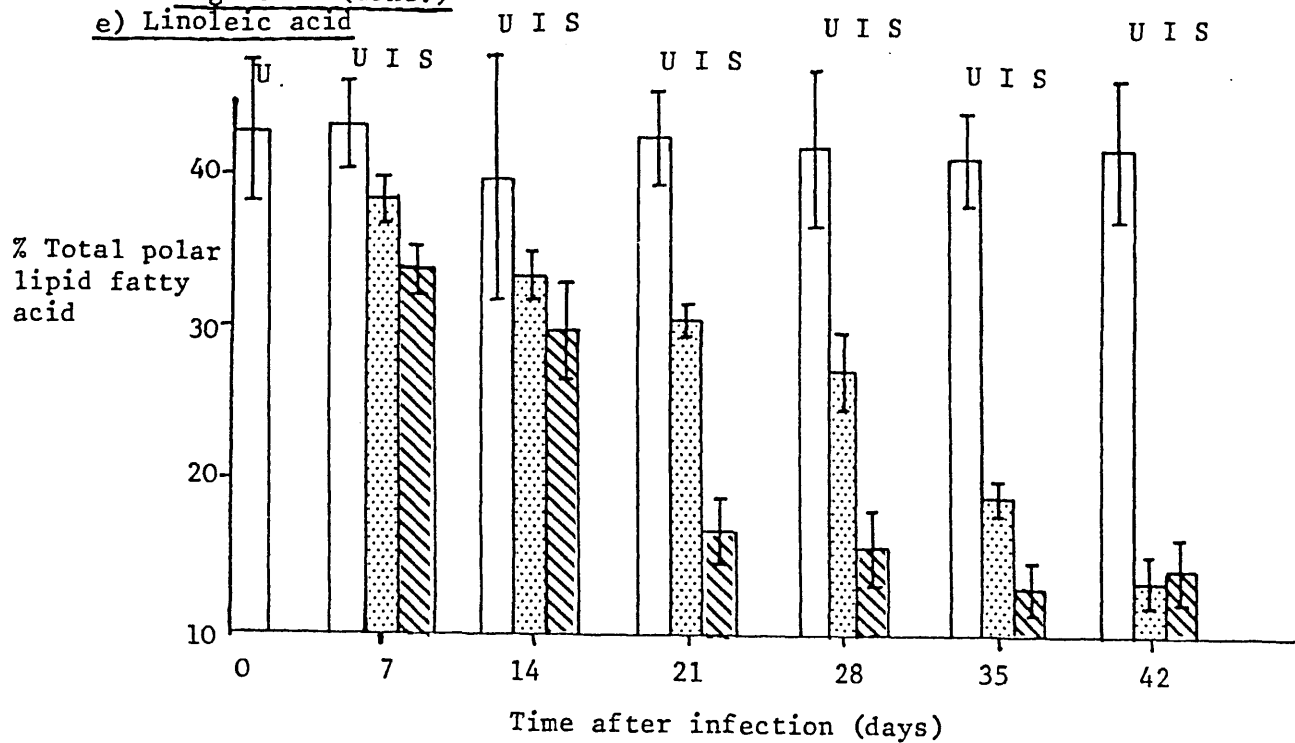
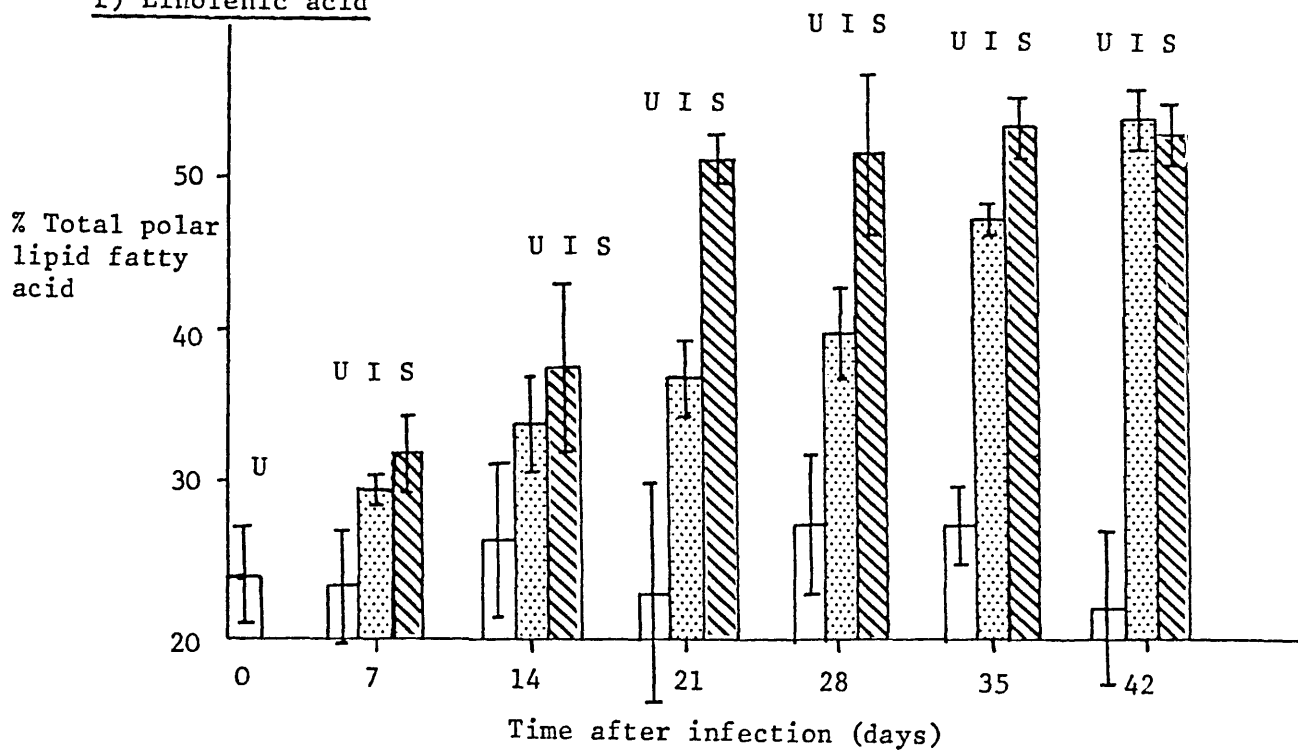


Figure 72 (cont.)
e) Linoleic acid



f) Linolenic acid



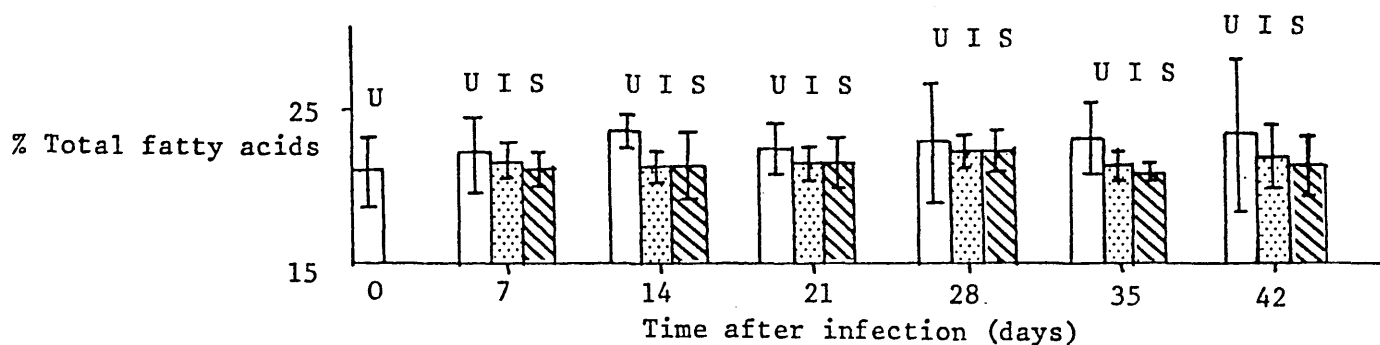
U Uninfected Plants
I Infected Plants
S Plants grown in the dark

┌ 95% Confidence Limits

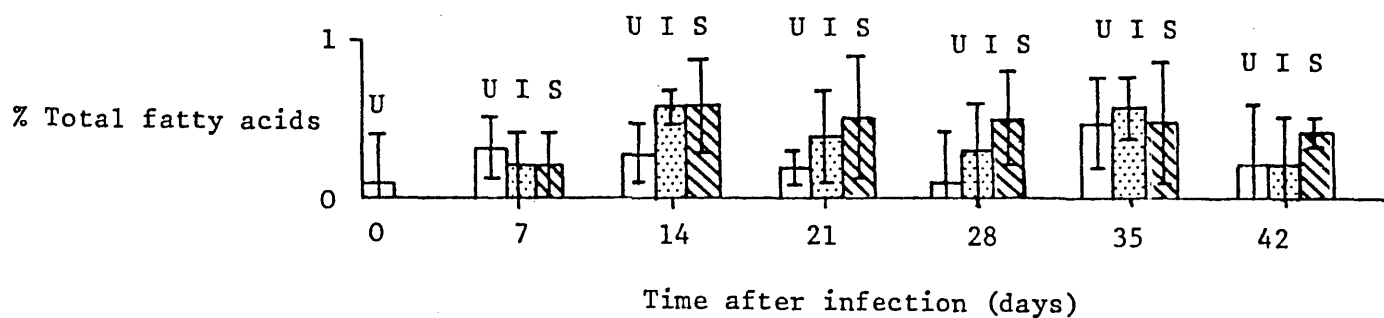
Figure 73: The effect of infection with E. pisi and growth in the dark on the fatty acid composition in PC from epidermal cells in the pea cultivar Onward

Figure 73

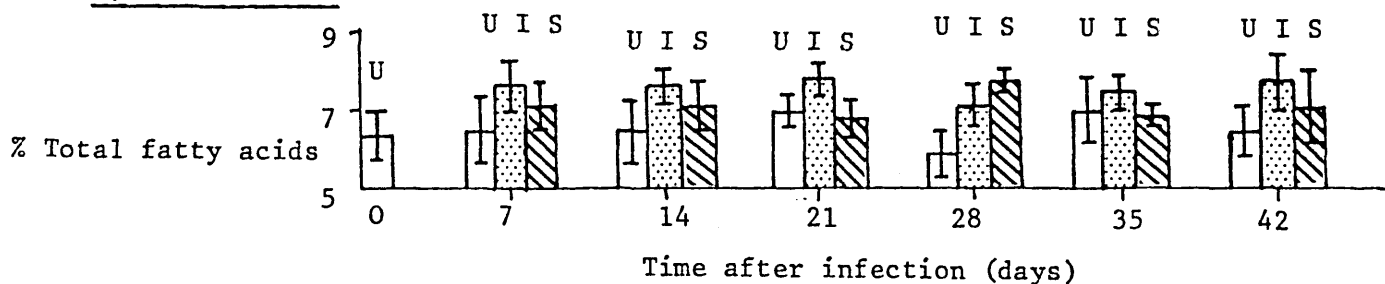
a) Palmitic acid



b) Palmitoleic acid



c) Stearic acid



d) Oleic acid

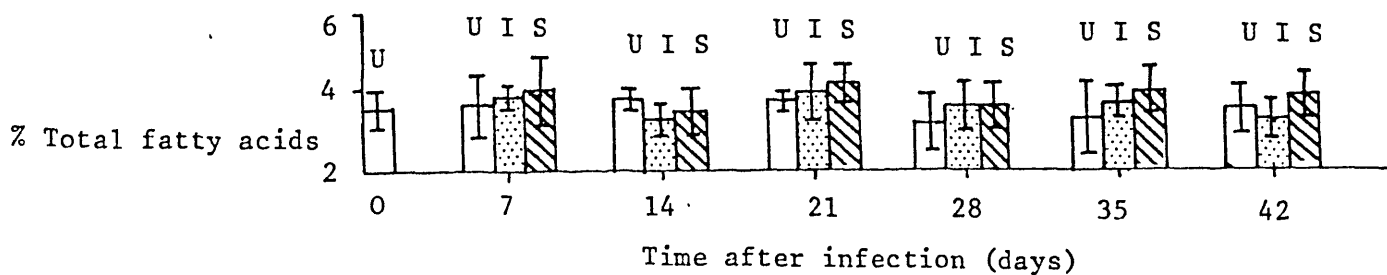
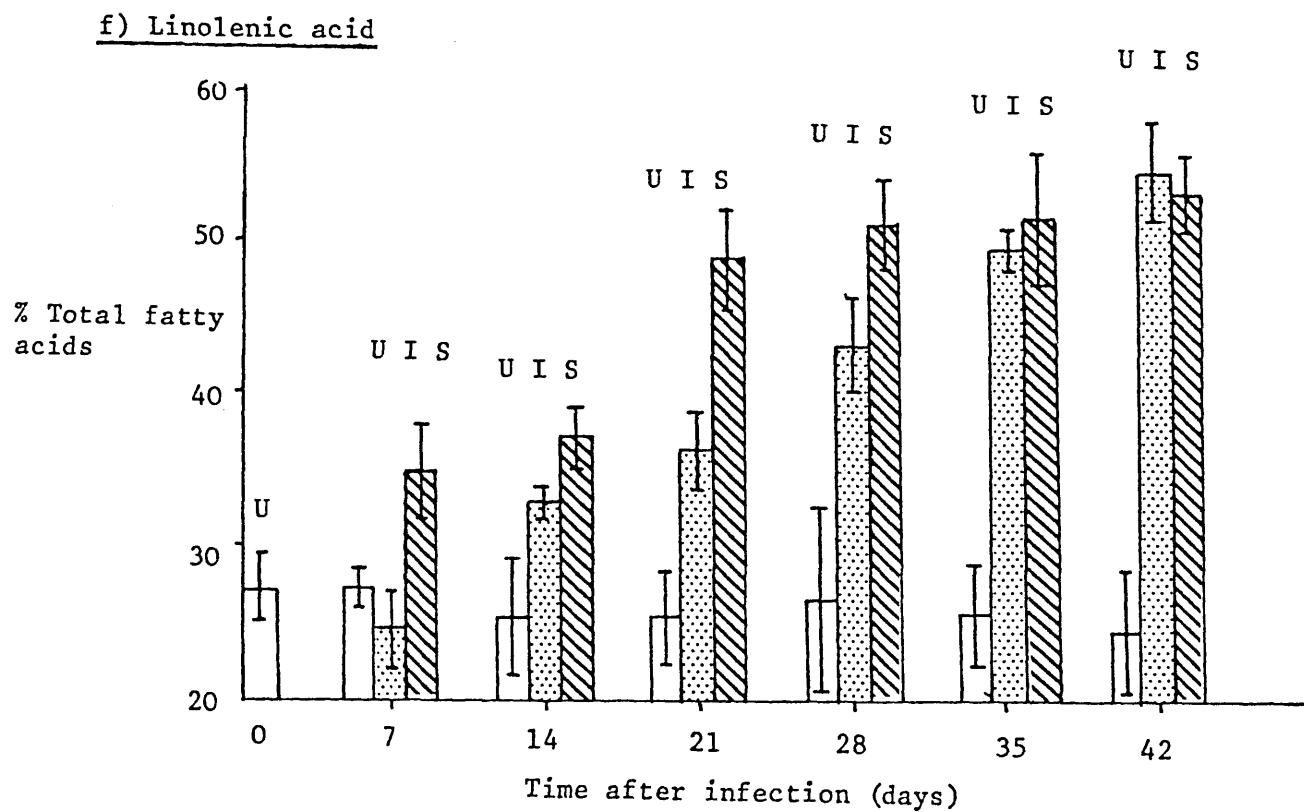
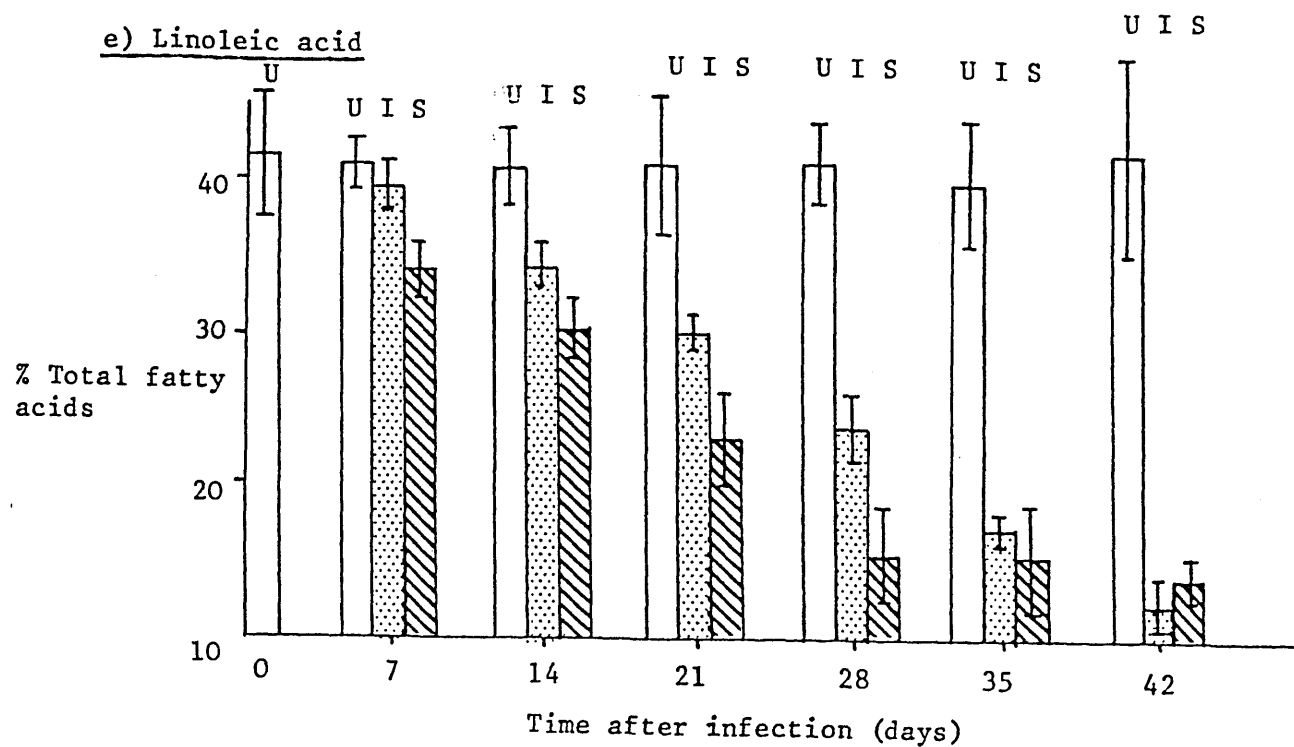


Figure 73 (cont.)



U Uninfected Plants
 I Infected Plants
 S Plants grown in the dark

I 95% Confidence Limits

Figure 74: The effect of infection with E. pisi and growth in the dark on the fatty acid composition in PG from epidermal cells in the pea cultivar Onward

Figure 74

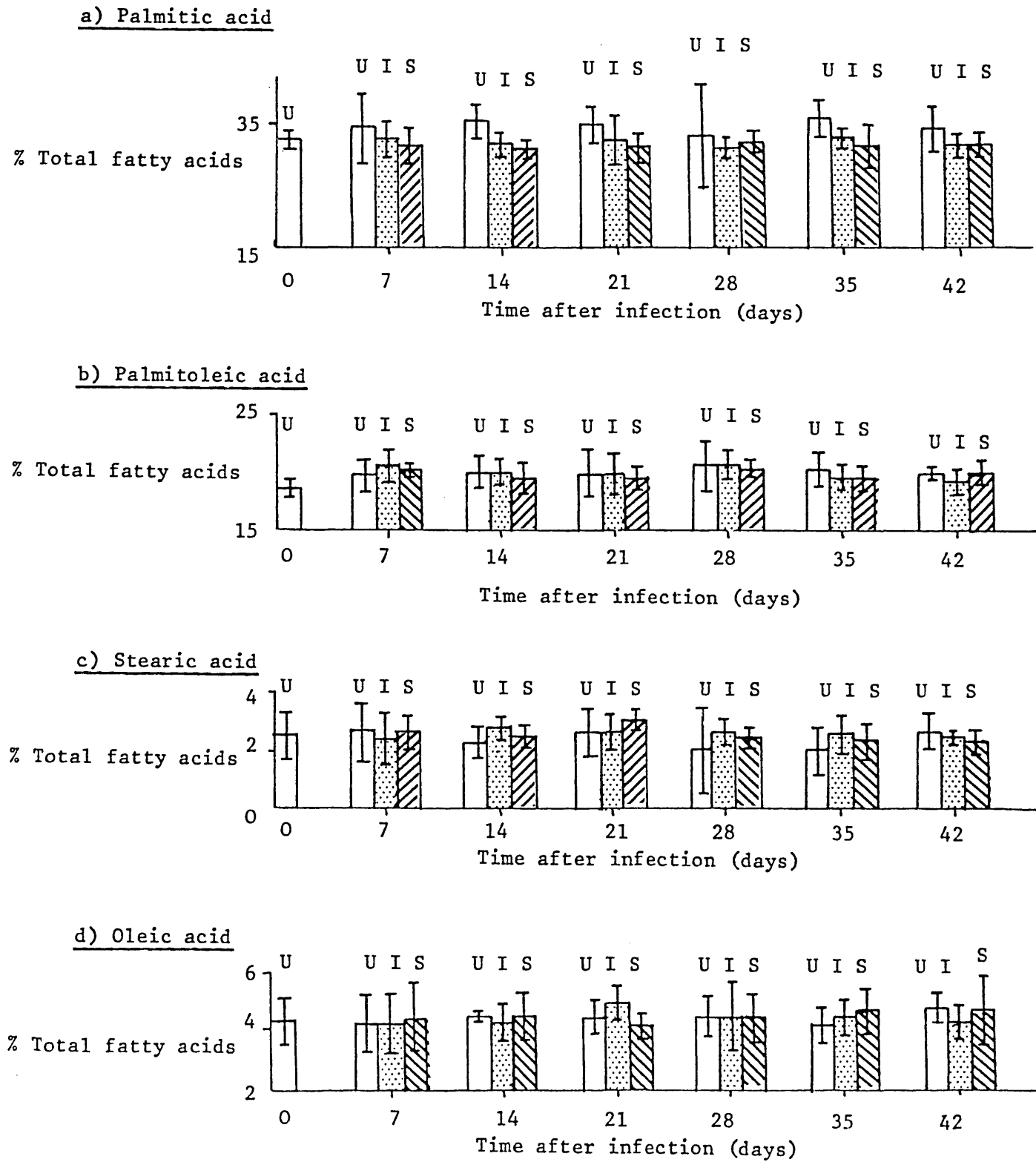
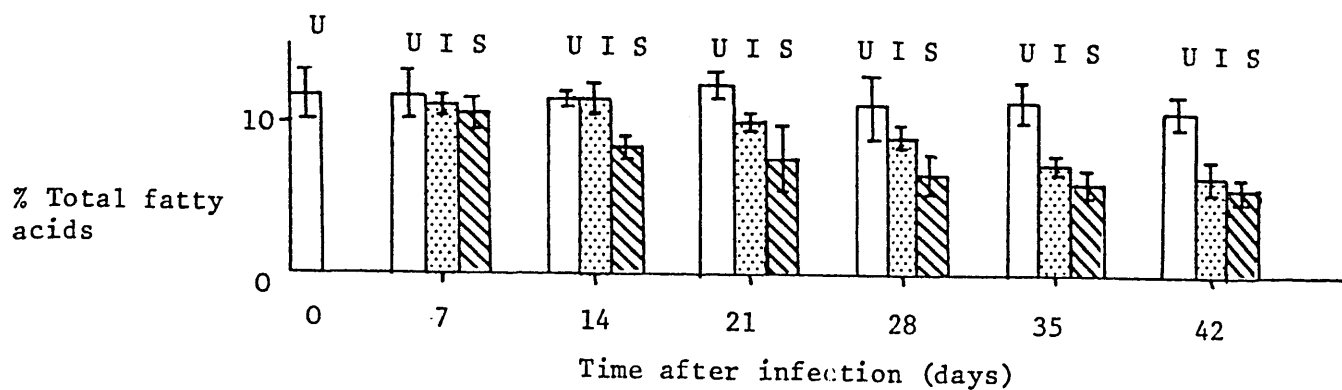
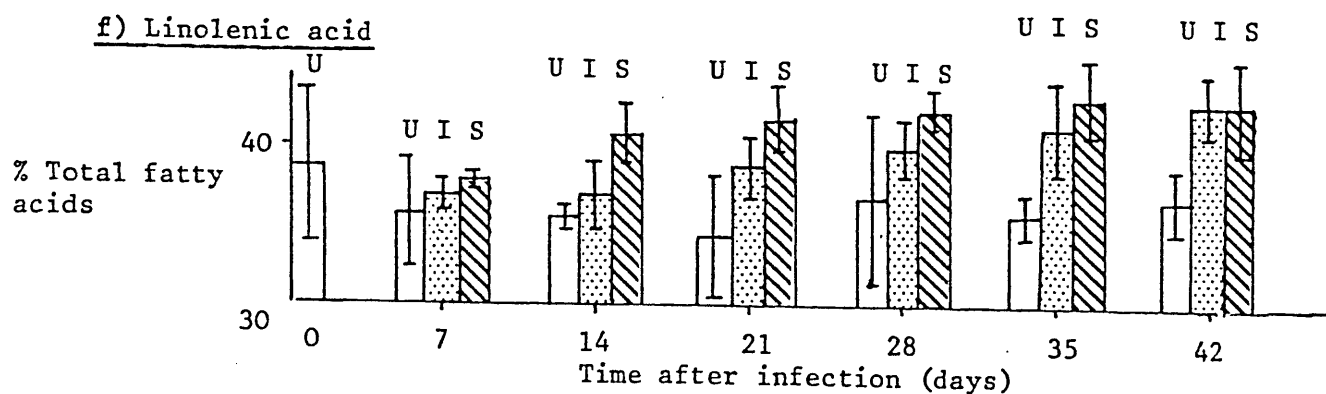


Figure 74 (cont.)

e) Linoleic acid



f) Linolenic acid



U Uninfected Plants
 I Infected Plants
 S Plants grown in the dark

I 95% Confidence Limits

4.5.3. Individual Neutral Lipids

Figure 75 shows that the neutral lipids of epidermal strips from plants grown in the dark show similar changes in composition to the infected plants. The only difference being that the changes become statistically significant ($p = 0.05$) earlier in the plants grown in the dark than in the infected plants. These changes consist of a decrease in the amount of sterol ester and an increase in the amount of triglyceride present. The increase becomes statistically significant ($p = 0.05$) in infected plants and plants grown in the dark after 42 days for triglycerides, after 28 days for fatty acids from plants grown in the dark and 42 days for infected plants and after 35 days for sterol esters from plants grown in the dark and 42 days for infected plants.

No significant changes were found in the fatty acids from neutral lipids either from the total fraction or from the individual neutral lipids. The result agreed with those shown in Figure 65 and Tables 22 and 23.

4.5.4. Appearance of the plants grown in the dark

By the 7th day there was little visible change in the plants grown in the dark, however by the 14th day the leaves were etiolated. By the 28th day after growth in the dark the leaves began to fall from the plant. During all this time the stem remained healthy, however by the 35th day it was showing signs of drying out and by the 42nd day the stem was almost totally dry.

Figure 75: The effect of infection by E. pisi and growth in the dark on the neutral lipid composition in epidermal cells from the pea cultivar Onward

Figure 75

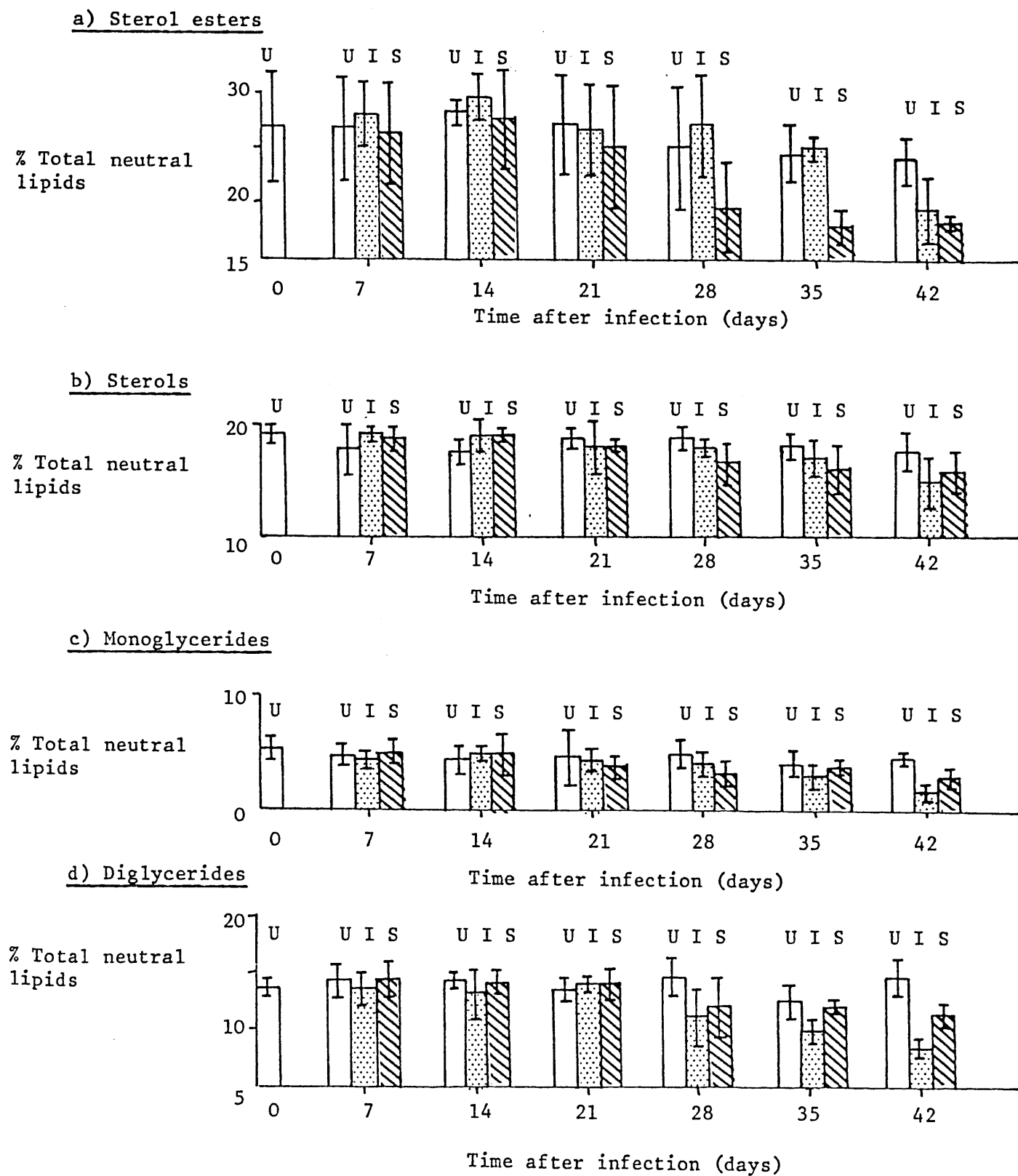
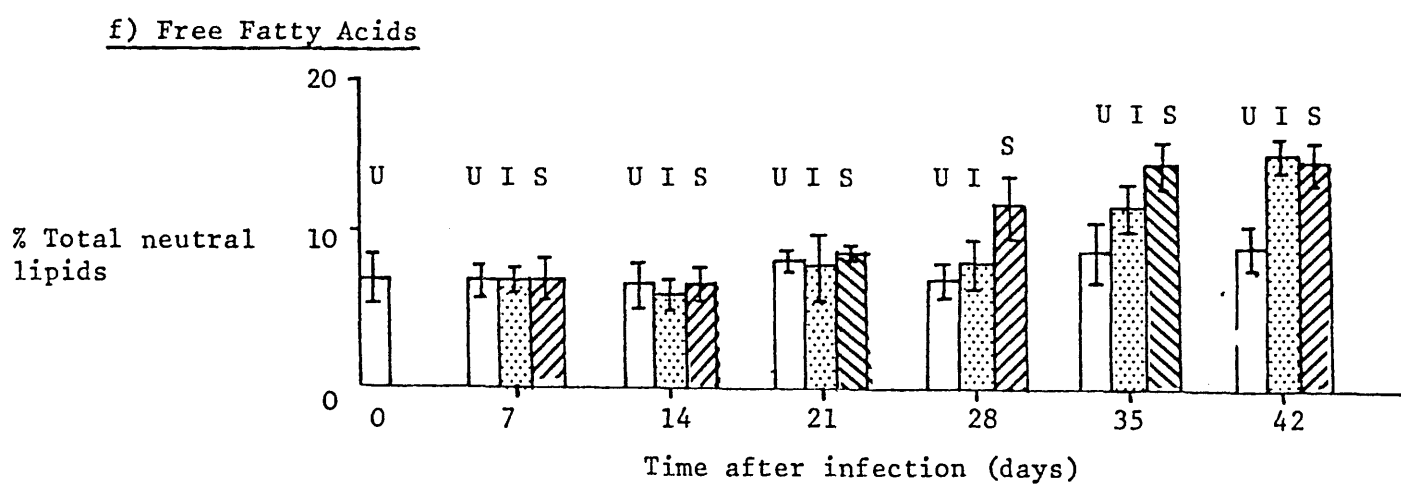
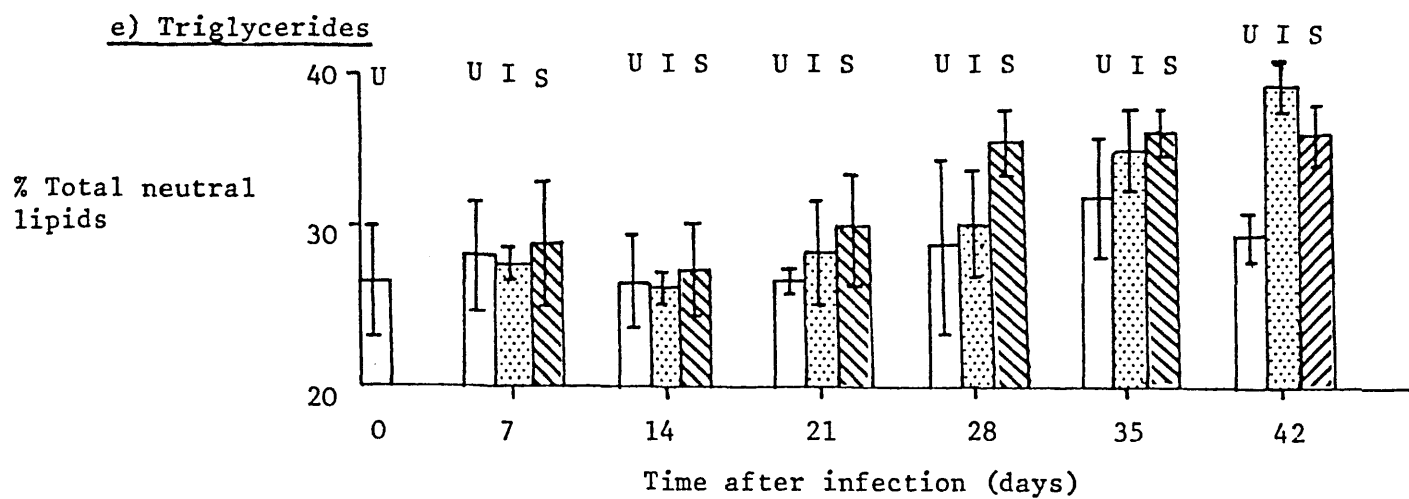


Figure 75 (cont.)



U Uninfected Plants
 I Infected Plants
 S Plants grown in the dark

| 95% Confidence Limits

Figure 76: The effect of E. pisi and growth in the dark on the neutral lipid fatty acid composition in epidermal cells from the pea cultivar Onward

Figure 76

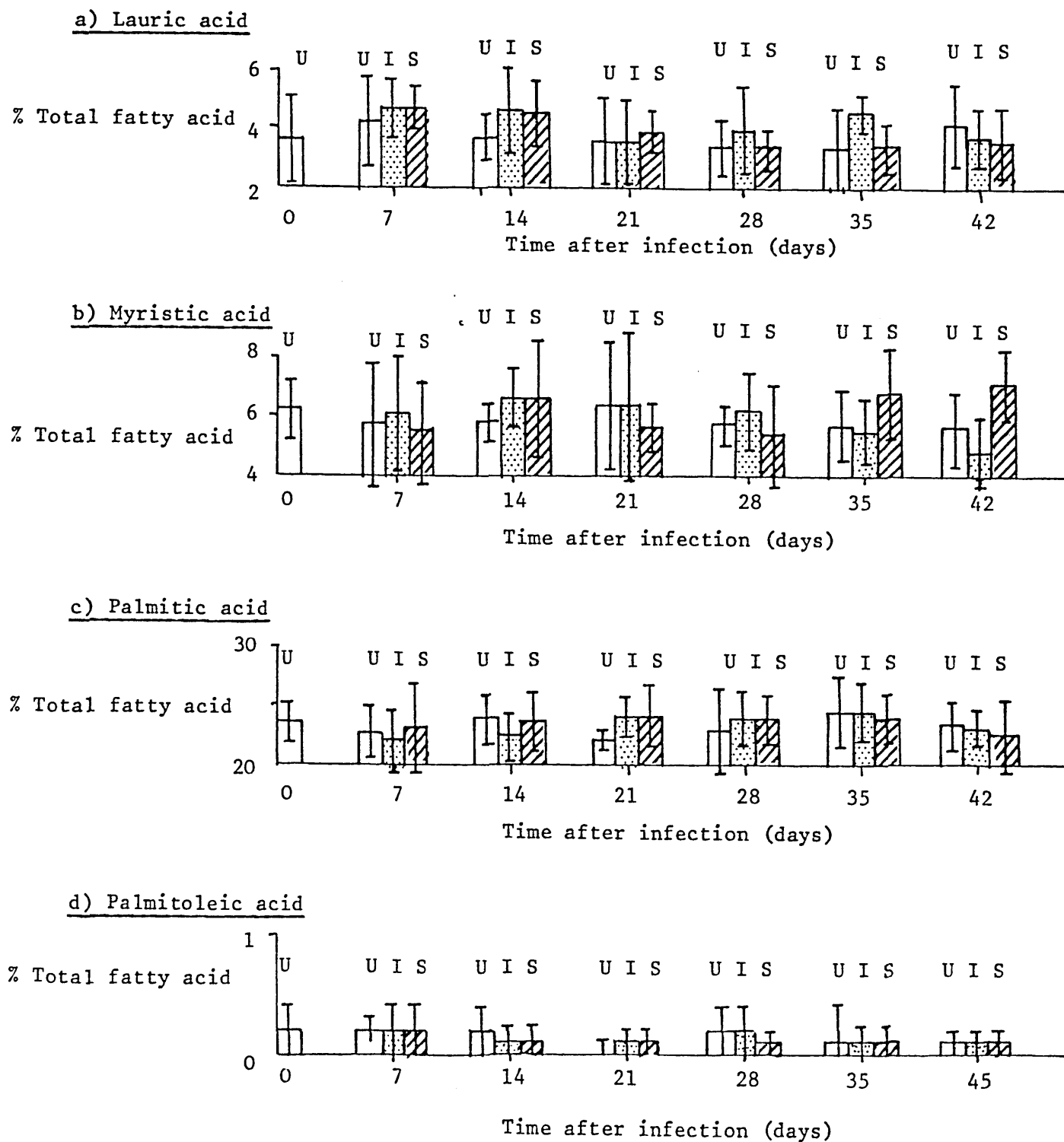
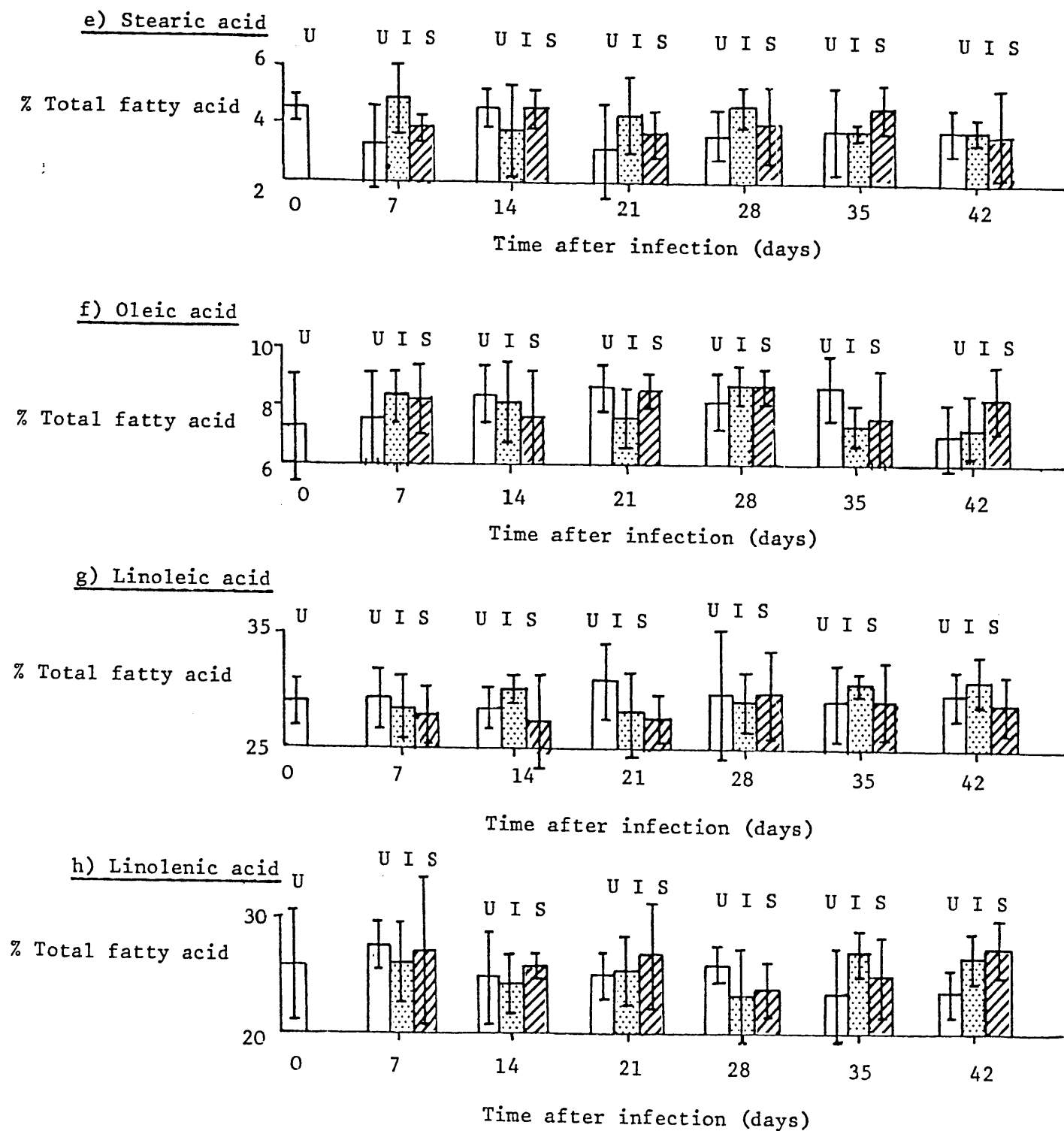


Figure 76 (cont.)



U Uninfected Plants
 I Infected Plants
 S Plants grown in the dark

┌ 95% Confidence Limits

4.5.5. Protein Nitrogen content in epidermal cells

The amount of protein nitrogen during the 42 day infection period was assessed as a measure of the extent of senescence. The method used is described in Section 3.15. The results expressed as a percentage of the control at day 0. It can be seen that there is no significant change with time in the protein nitrogen content in uninfected plants and infected plants. There was however a significant reduction in the amount of protein bound nitrogen in plants kept in the dark (Figure 78).

4.5.6. Amino acid nitrogen content in epidermal cells

The amount of amino acid nitrogen was assessed during the 42 day infection period again as a measure of the extent of senescence in the epidermal tissue. The method used is described in Section 3.14. The results expressed as a percentage of the control at day 0, as shown in Figure 77. It can be seen that there is no significant change in the amino acid nitrogen content of healthy plants during this period whether they are grown in the light or in the dark. There is however a significant increase in the amount of amino acid nitrogen present in infected plants, this difference becomes significant ($p = 0.05\%$) from the 28th day after inoculation.

4.5.7. Chlorophyll content in epidermal cells

A further way of assessing the extent of senescence was to assess the chlorophyll content of epidermal cells. The method used is described in Section 3.13. The results expressed as a percentage of the control at day 0 are shown in Figure 79. There was a significant decrease ($p = 0.05\%$) in the amount of chlorophyll in the plants grown in the dark compared with both the uninfected and infected plants grown in the light. This decrease in chlorophyll content became significant ($p = 0.05\%$) after 14 days and increased throughout the experiment. The infected plants showed a decrease in the level of chlorophyll from the 35th day after infection onwards without ever having as great a reduction as was found in the dark grown plants.

Figure 77: The effect of infection by E. pisi and growth in the dark on the amino acid bound nitrogen in the epidermal cells from the cultivar Onward

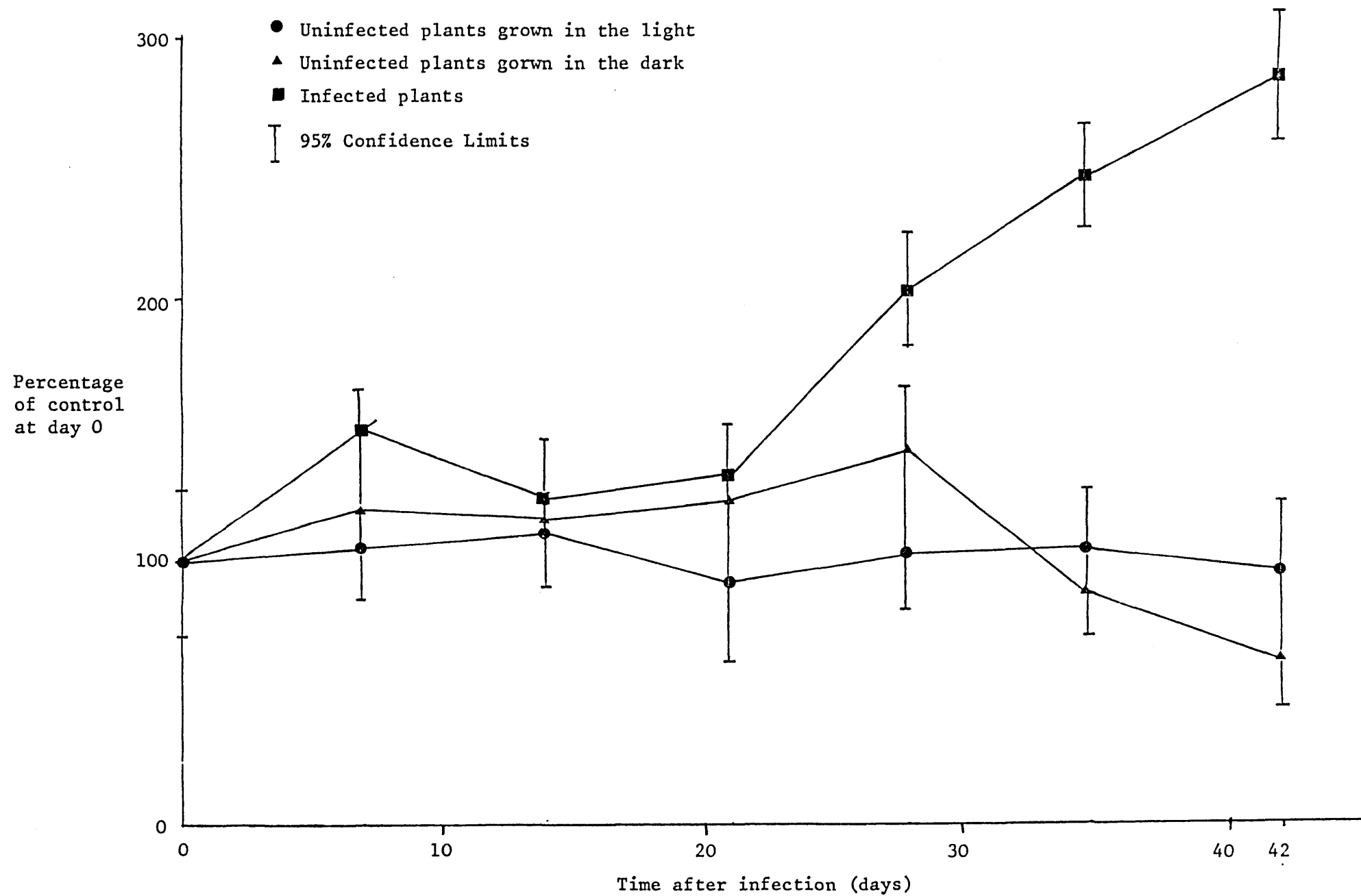


Figure 78: Protein nitrogen in epidermal cells of healthy, dark incubated, and powdery mildew infected peas of the cultivar Onward

Figure 78

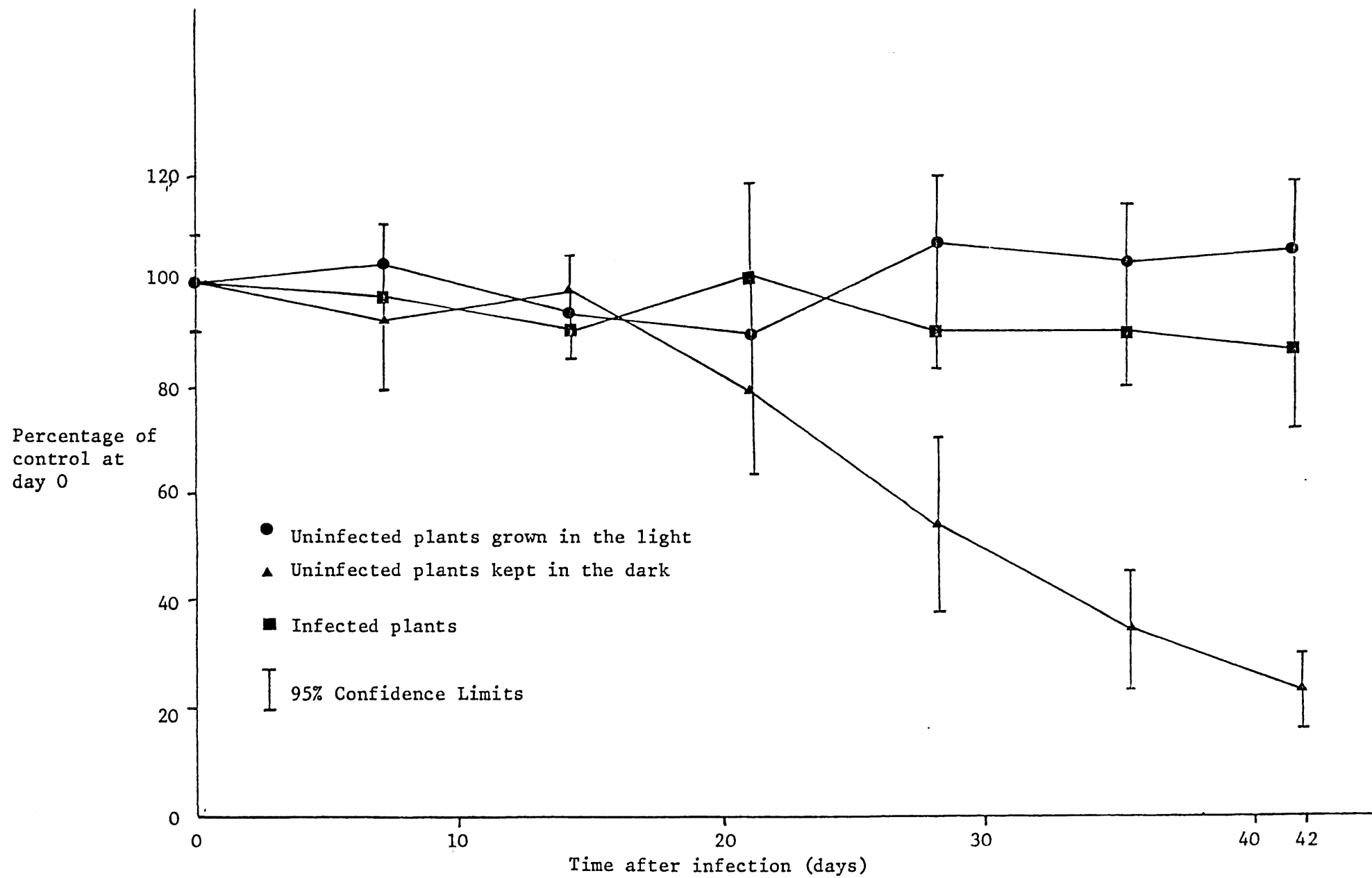
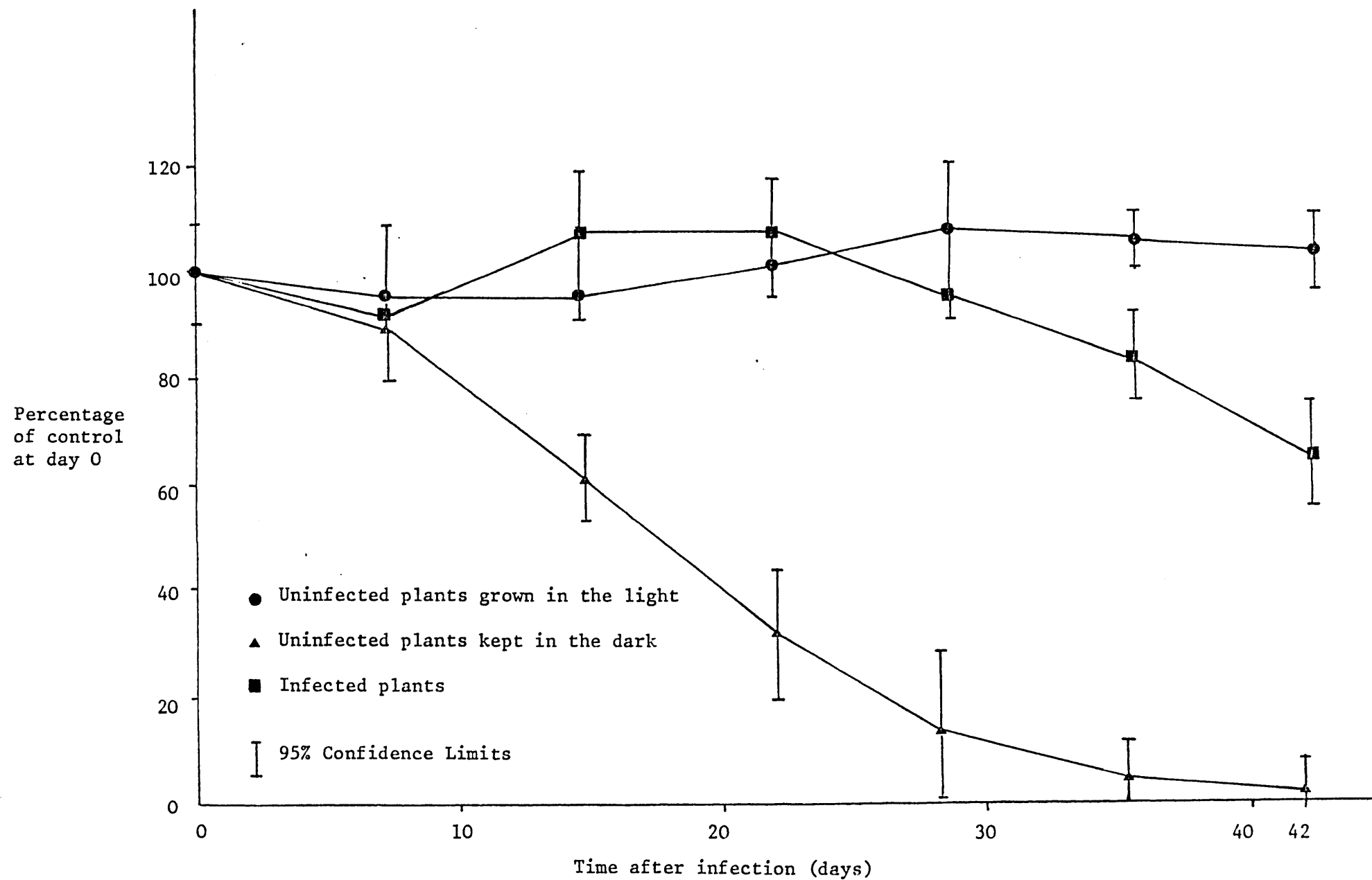


Figure 79: The effect by E. pisi and growth in the dark on the chlorophyll content in epidermal cells from the cultivar Onward

Figure 79



4.6. Isolation and lipid analysis of haustoria and membrane fractions

4.6.1. Isolation and analysis of haustorial complexes

This experiment was performed to test the lipid composition of the haustorial complexes in order to ascertain whether it was the presence of fungal lipids in the lipid extracts that was responsible for the observed increase in unsaturation in lipid extracts from infected stem epidermal cells. Haustorial complexes were isolated from leaf tissue following the method of Manners (1979). The resulting complexes were then analysed for lipids using the method described in Section 3.12. The polar lipid content of the isolated complex fractions is shown in Table 26, the fatty acids of the polar lipid fraction in Table 27, and the neutral lipids in Table 28.

Unfortunately due to contamination of the haustorial complexes with other cellular components the figures quoted are unlikely to be a true reflection of the lipid content of the haustorial complexes themselves.

The extent of the impurities in the haustorial complex fraction was assessed by measuring the proportion of a microscopic field of view occupied by the non haustorial complex components when spread on a haemocytometer stage. The result from 10 replicate observations was that the haustoria comprised only $23.6 \pm 5.7\%$ of the total fraction.

In order to calculate the contribution that the contaminants made to the lipid analysis from the haustorial complexes a second series of experiments was undertaken where the procedure for haustorial isolation was followed using uninfected leaves. The results from these experiments are shown in Tables 29, 30 and 31. From these results and those of the original isolation it was possible to calculate the likely lipid composition of the haustorial complexes themselves (Table 32, 33, 34).

Table 26: Composition of haustorial complex

Polar Lipid	% Composition
PG	10.1 $\pm$ 0.9%
PC	17.2 $\pm$ 1.9%
PE	13.4 $\pm$ 0.7%
PI	3.3 $\pm$ 0.2%
PS	2.0 $\pm$ 0.3%
PI	1.2 $\pm$ 0.2%
MGDG	30.0 $\pm$ 2.1%
DGDG	21.8 $\pm$ 1.3%

Table 27: Fatty acid composition of polar lipids from the haustorial complex preparation

Fatty Acid	% Composition
Myristic acid	0.8 $\pm$ 0.2%
Palmitic acid	10.4 $\pm$ 0.7%
Palmitoleic acid	2.7 $\pm$ 0.2%
Stearic acid	3.7 $\pm$ 1.1%
Oleic acid	5.7 $\pm$ 0.6%
Linoleic acid	17.2 $\pm$ 1.6%
Linolenic acid	58.7 $\pm$ 3.9%
Arachidic acid	1.6 $\pm$ 0.4%
Arachidonic acid	2.6 $\pm$ 0.4%

All figures represent the means of 4 replicates quoted with their 95% confidence limits

Table 28: The composition of neutral lipid from haustorial complex fractions using the method of Manners (1979)

Neutral Lipid	% Composition
Sterol esters	22.0 $\pm$ 1.3%
Sterol	22.4 $\pm$ 0.9%
Monoglycerides	7.7 $\pm$ 0.4%
Diglycerides	13.1 $\pm$ 1.4%
Triglycerides	25.1 $\pm$ 2.6%
Free fatty acids	9.4 $\pm$ 3.1%

All figures represent the means of 4 replicates quoted with their 95% confidence limits

Table 29: Polar lipid composition of the haustorial complex fraction prepared from healthy plants

Polar Lipid	% Composition
PG	39.7 $\pm$ 1.7%
PC	13.4 $\pm$ 1.3%
PE	7.1 $\pm$ 0.4%
PI	1.1 $\pm$ 0.2%
PS	0.9 $\pm$ 0.2%
PA	0.5 $\pm$ 0.3%
MGDG	39.7 $\pm$ 1.7%
DGDG	26.0 $\pm$ 2.1%

All figures represent the means of 4 replicates quoted with their 95% confidence limits

Table 30: The fatty acid composition of the polar lipids isolated from the haustorial complex fraction prepared from healthy plants

Fatty Acid	% Composition
Palmitic acid	12.4 $\pm$ 1.4%
Palmitoleic acid	2.3 $\pm$ 0.1%
Stearic acid	2.3 $\pm$ 0.7%
Oleic acid	1.5 $\pm$ 0.4%
Linoleic acid	12.8 $\pm$ 1.1%
Linolenic acid	68.7 $\pm$ 2.8%

All figures represent the means of 4 replicates quoted with their 95% confidence limits

Table 31: The composition of neutral lipids from the haustorial complex fractions taken from uninfected leaves using the method of Manners (1979)

Neutral Lipid	% Composition
Sterol esters	24.3 $\pm$ 0.8%
Sterol	21.5 $\pm$ 0.6%
Monoglycerides	8.7 $\pm$ 1.3%
Diglycerides	16.0 $\pm$ 1.6%
Triglycerides	20.8 $\pm$ 2.2%
Free fatty acids	8.9 $\pm$ 0.9%

All figures represent the means of 4 replicates quoted with their 95% confidence limits

Table 32: The estimated polar lipid composition of haustorial complexes

Polar Lipid	% Composition
PG	6.3%
PC	29.4%
PE	33.6%
PI	10.5%
PS	5.5%
PA	3.4%
MGDG	5.0%
DGDG	6.3%

Table 33: The estimated polar lipid-bound fatty acid composition of haustorial complexes

Fatty Acid	% Composition
Myristic acid	1.8%
Palmitic acid	4.2%
Palmitoleic acid	3.8%
Stearic acid	8.0%
Oleic acid	19.3%
Linoleic acid	31.9%
Linolenic acid	26.3%
Arachidic acid	1.7%
Arachidonic acid	3.6%

Figures calculated as follows:-

$$\frac{\text{Haustorial complex from infected cells}}{\text{Haustorial complex from uninfected cells}} \times \frac{76.4}{100} \times \frac{100}{23.6}$$

Table 34: The estimated neutral lipid composition of haustorial complexes

Neutral Lipid	% Composition
Sterol esters	6.3%
Sterol	25.2%
Monoglycerides	3.8%
Diglycerides	5.2%
Triglycerides	38.6%
Free fatty acids	10.9%

When assessing the relevance of these results it is important to bear in mind that the percentage of the impurities was taken as a percentage of the area and no allowance was made, or could possibly be made, for the relative lipid content of the individual impurities. This means that the accuracy of these estimations is open to question.

4.6.2. Isolation and lipid analysis of membrane fractions from infected plants

This series of experiments was performed in order to determine whether the changes observed in lipid composition in the earlier experiment were a general occurrence or confined to specific membranes. It was not possible to use epidermal strips for the isolation of the membrane fractions due to the need to collect a large amount of initial material. As stem epidermis was used in the previous experiments it was decided to use stems for the lipid extractions.

In order to determine a suitable time in the infection period when lipid changes could be observed in lipid extracts from stems a series of experiments were conducted. The results from these experiments are shown in Table 35. There was no significant change with time in the amount of total lipid, polar lipids and neutral lipids in plants, nor were there differences between infected and uninfected plants.

There were no changes in the levels of the individual polar lipid classes during the 42 days of this experiment (Table 35). When the total polar lipid bound fatty acids were assessed the results for uninfected plants and infected plants over the 42 day period confirmed the results in the previous experiment with epidermal tissue although the changes were seen later and were not as great. This pattern was consistently found for the fatty acids bound to the individual polar lipids, this is illustrated in the table by using PC as a typical example but the pattern was similar for PE, PI, PS and PA.

The results for MGDG and DGDG bound fatty acids in uninfected plants, and infected plants showed no significant change throughout the 42 day infection period. The results were the same as those illustrated in Figures 54, 55).

Table 35: The effect of infection by E. pisi on the lipid composition in the stem of the pea cultivar Onward

a) Total Lipids

Time after Inoculation (Days)	Uninfected Plants (μg lipid g^{-1} dry weight)	Infected Plants (μg lipid g^{-1} dry weight)
0	61.2 $\pm$ 3.1	-
28	60.5 $\pm$ 2.2	61.9 $\pm$ 3.3
35	64.2 $\pm$ 2.7	61.3 $\pm$ 2.5
42	63.2 $\pm$ 3.2	62.1 $\pm$ 4.5

b) Polar Lipids

Time after Inoculation (Days)	Uninfected Plants (μg lipid g^{-1} dry weight)	Infected Plants (μg lipid g^{-1} dry weight)
0	49.2 $\pm$ 3.1	-
28	47.8 $\pm$ 1.8	50.5 $\pm$ 2.5
35	50.5 $\pm$ 2.9	48.3 $\pm$ 2.9
42	48.5 $\pm$ 1.9	52.5 $\pm$ 2.5

c) Neutral Lipids

Time after Inoculation (Days)	Uninfected Plants (μg lipid g^{-1} dry weight)	Infected Plants (μg lipid g^{-1} dry weight)
0	13.1 $\pm$ 2.9	-
28	12.7 $\pm$ 3.0	11.7 $\pm$ 4.5
35	12.6 $\pm$ 2.3	12.9 $\pm$ 2.3
42	14.7 $\pm$ 3.1	11.2 $\pm$ 4.9

All figures represent the means of 4 replicates quoted with their 95% confidence limits

d) Individual Polar Lipids

Polar Lipids	Days after infection						
	Uninfected Plants			Infected Plants			
	0	28	% Composition 35	42	28	% Composition 35	42
PC	45.7 ± 3.2	45.8 ± 3.0	48.7 ± 2.1	46.1 ± 1.6	48.8 ± 4.2	47.4 ± 3.3	49.3 ± 5.2
PG	6.9 ± 1.2	7.2 ± 0.3	7.7 ± 0.5	6.9 ± 0.9	6.9 ± 1.0	7.3 ± 1.0	6.8 ± 0.9
PE	23.4 ± 2.1	26.8 ± 0.9	22.1 ± 1.1	23.9 ± 1.2	23.4 ± 0.6	24.9 ± 2.1	23.9 ± 2.3
PI	8.8 ± 0.7	7.9 ± 0.7	9.0 ± 1.0	8.3 ± 0.3	8.2 ± 0.6	7.7 ± 0.9	8.7 ± 1.4
PS	2.8 ± 0.3	2.4 ± 0.4	2.1 ± 0.5	2.4 ± 0.2	2.6 ± 0.3	2.1 ± 0.7	3.1 ± 0.5
PA	0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.4	0.2 ± 0.2	0.3 ± 0.5	0.2 ± 0.1	0.2 ± 0.2
MGDG	6.8 ± 0.7	6.5 ± 1.4	5.9 ± 1.4	5.9 ± 0.7	6.2 ± 1.6	6.3 ± 1.4	4.7 ± 0.8
DGDG	4.9 ± 1.3	5.3 ± 0.7	4.4 ± 1.4	5.9 ± 1.2	3.9 ± 1.2	4.2 ± 2.0	5.3 ± 1.5

e) Total Polar Lipid bound fatty acids

Polar Lipids	Days after infection						
	Uninfected Plants			Infected Plants			
	0	28	% Composition 35	42	28	% Composition 35	42
Palmitic acid	20.4 ± 1.0	19.8 ± 1.7	22.4 ± 2.0	21.0 ± 1.5	22.6 ± 2.0	21.6 ± 0.7	21.8 ± 1.3
Palmitoleic acid	1.5 ± 0.2	1.9 ± 0.6	1.2 ± 0.8	1.5 ± 1.4	1.3 ± 0.1	1.6 ± 0.4	1.4 ± 0.5
Stearic acid	4.5 ± 1.0	4.1 ± 0.6	3.9 ± 0.8	4.0 ± 4.5	4.7 ± 0.5	3.7 ± 0.3	3.9 ± 0.5
Oleic acid	2.4 ± 0.9	2.8 ± 1.0	3.4 ± 0.4	3.2 ± 0.8	2.5 ± 0.7	1.9 ± 0.3	2.1 ± 0.7
Linoleic acid	34.3 ± 1.0	35.6 ± 1.7	32.9 ± 1.1	34.0 ± 2.2	35.5 ± 0.3	29.4 ± 2.7	29.5 ± 3.3
Linolenic acid	36.2 ± 1.1	35.8 ± 2.6	36.2 ± 3.2	32.3 ± 2.4	34.9 ± 2.5	41.7 ± 3.3	41.5 ± 2.9

f) PC bound fatty acids

Fatty acids	Days after infection						
	Uninfected Plants			Infected Plants			
	0	28	% Composition 35	42	28	% Composition 35	42
Palmitic acid	22.1 ± 2.9	22.0 ± 3.3	21.9 ± 2.9	22.1 ± 1.5	23.7 ± 4.1	23.3 ± 2.0	23.0 ± 1.2
Stearic acid	5.2 ± 0.3	4.6 ± 0.9	4.8 ± 0.8	4.4 ± 0.6	4.1 ± 0.6	4.1 ± 0.9	4.3 ± 0.9
Oleic acid	2.4 ± 1.1	2.0 ± 0.3	2.0 ± 1.0	2.3 ± 0.8	1.8 ± 1.1	1.8 ± 1.1	1.6 ± 0.8
Linoleic acid	41.2 ± 1.3	40.2 ± 1.8	38.0 ± 1.2	42.6 ± 3.4	34.1 ± 5.8	33.2 ± 3.7	
Linolenic acid	41.2 ± 1.3	40.2 ± 1.8	38.0 ± 1.2	38.4 ± 2.8	42.6 ± 3.4	34.1 ± 5.8	33.2 ± 3.7
Linolenic acid	29.0 ± 2.8	31.4 ± 4.7	33.2 ± 3.1	32.7 ± 4.6	27.1 ± 1.9	36.6 ± 5.0	37.6 ± 2.8

g) PE bound fatty acids

Fatty acids	Days after infection							
	Uninfected Plants				Infected Plants			
	0	28	35	42	28	35	42	
Palmitic acid	26.6 ± 2.0	25.1 ± 2.0	24.5 ± 1.8	25.4 ± 2.1	26.9 ± 3.8	26.0 ± 1.8	25.4 ± 1.6	
Palmitoleic acid	-	-	-	-	-	-	-	
Stearic acid	3.6 ± 0.9	3.0 ± 1.1	4.2 ± 0.9	4.0 ± 1.1	4.2 ± 1.3	4.1 ± 1.1	4.2 ± 0.5	
Oleic acid	2.1 ± 0.3	2.0 ± 1.1	1.4 ± 0.4	1.4 ± 1.3	1.9 ± 0.7	1.8 ± 1.0	1.8 ± 0.8	
Linoleic acid	41.9 ± 1.6	41.9 ± 1.8	40.3 ± 0.6	39.6 ± 1.4	40.4 ± 2.3	36.9 ± 1.7	32.7 ± 2.9	
Linolenic acid	27.8 ± 2.7	28.0 ± 4.2	29.5 ± 3.0	29.6 ± 1.9	27.6 ± 3.8	31.2 ± 2.9	35.9 ± 1.6	

h) PG bound fatty acids

Fatty Acids	Days after infection							
	Uninfected Plants				Infected Plants			
	0	28	35	42	28	35	42	
Palmitic acid	24.3 ± 1.8	25.5 ± 2.9	24.3 ± 3.7	24.0 ± 3.1	23.6 ± 3.1	25.4 ± 1.2	25.9 ± 1.9	
Palmitoleic acid	22.3 ± 2.5	21.2 ± 2.2	20.6 ± 1.7	20.4 ± 0.9	21.0 ± 1.4	21.6 ± 1.8	21.2 ± 2.4	
Stearic acid	2.1 ± 0.2	1.9 ± 0.6	1.2 ± 0.7	2.2 ± 0.9	2.5 ± 0.6	1.7 ± 0.3	1.6 ± 0.6	
Oleic acid	4.3 ± 0.5	4.5 ± 0.9	3.9 ± 0.5	2.9 ± 1.3	2.3 ± 0.7	3.9 ± 0.2	3.1 ± 1.1	
Linoleic acid	12.9 ± 1.2	12.3 ± 1.0	11.6 ± 0.7	12.4 ± 0.8	13.4 ± 1.1	13.6 ± 2.3	16.5 ± 5.7	
Linolenic acid	34.0 ± 1.6	34.6 ± 1.5	38.6 ± 4.3	37.6 ± 3.7	37.2 ± 1.4	33.9 ± 2.7	31.6 ± 2.1	

k) PA bound fatty acids

Fatty Acids	Days after infection							
	Uninfected Plants				Infected Plants			
	0	28	35	42	28	35	42	
Palmitic acid	25.4 ± 1.4	27.51 ± 1.7	25.6 ± 1.9	25.9 ± 1.3	27.0 ± 0.9	26.9 ± 6.9	25.5 ± 1.9	
Stearic acid	3.9 ± 0.1	4.3 ± 1.7	4.3 ± 0.7	3.4 ± 0.4	3.5 ± 0.5	4.2 ± 1.0	4.0 ± 1.0	
Oleic acid	1.4 ± 0.6	2.3 ± 0.5	1.7 ± 0.6	0.6 ± 0.3	2.0 ± 0.3	1.3 ± 0.4	1.8 ± 0.2	
Linoleic acid	47.7 ± 1.5	40.0 ± 1.0	40.5 ± 1.5	41.7 ± 3.4	41.6 ± 1.1	34.3 ± 3.1	30.9 ± 2.0	
Linolenic acid	27.0 ± 1.3	25.8 ± 3.1	27.8 ± 3.9	28.3 ± 5.1	25.9 ± 1.8	33.2 ± 2.6	37.7 ± 2.3	

l) MGDG bound fatty acid

Fatty Acids	Days after infection							
	Uninfected Plants				Infected Plants			
	0	28	35	42	28	35	42	
Palmitic acid	3.5 ± 0.9	2.6 ± 1.4	3.9 ± 0.9	3.9 ± 0.4	3.8 ± 1.4	3.1 ± 1.3	3.6 ± 1.6	
Stearic acid	0.9 ± 0.4	0.9 ± 0.2	1.0 ± 0.6	1.2 ± 0.6	1.2 ± 0.6	0.8 ± 0.3	1.3 ± 0.4	
Oleic acid	1.4 ± 0.6	1.4 ± 0.8	1.7 ± 0.8	1.2 ± 0.5	1.0 ± 0.8	0.8 ± 0.9	1.2 ± 1.0	
Linoleic acid	3.4 ± 0.7	3.5 ± 1.2	3.1 ± 0.3	2.4 ± 0.6	3.5 ± 0.8	3.8 ± 0.9	4.1 ± 0.3	
Linolenic acid	90.7 ± 0.8	91.5 ± 1.7	90.3 ± 1.2	91.0 ± 0.8	90.2 ± 2.1	91.4 ± 0.4	89.8 ± 1.4	

m) DGDG bound fatty acids

Fatty Acids	Days after infection						
	Uninfected Plants			Infected Plants			
	0	28	% Composition 35	42	28	% Composition 35	42
Palmitic acid	10.6 ± 1.5	9.5 ± 3.0	10.0 ± 1.2	11.6 ± 2.5	10.7 ± 1.0	10.8 ± 1.4	11.2 ± 0.9
Stearic acid	2.0 ± 0.2	2.1 ± 0.3	2.5 ± 1.1	2.4 ± 1.2	1.7 ± 1.0	2.6 ± 0.8	2.1 ± 1.0
Oleic acid	0.9 ± 0.1	1.4 ± 0.8	1.1 ± 0.2	1.3 ± 1.0	1.2 ± 0.6	1.1 ± 0.6	1.1 ± 0.6
Linoleic acid	3.2 ± 0.2	3.3 ± 0.8	2.8 ± 0.5	3.3 ± 0.5	3.2 ± 1.1	3.0 ± 1.6	2.8 ± 1.2
Linolenic acid	83.4 ± 1.4	83.7 ± 2.0	83.6 ± 1.6	81.7 ± 2.7	83.2 ± 3.0	82.4 ± 1.7	82.7 ± 2.6

n) Individual neutral lipids

Fatty Acids	Days after infection						
	Uninfected Plants			Infected Plants			
	0	28	% Composition 35	42	28	% Composition 35	42
Sterol esters	24.4 ± 0.8	23.6 ± 1.3	21.6 ± 2.6	21.6 ± 1.2	22.4 ± 1.0	20.3 ± 4.2	19.6 ± 1.1
Sterols	21.6 ± 0.5	20.5 ± 1.7	18.9 ± 0.6	18.8 ± 1.1	21.4 ± 0.4	18.6 ± 3.4	17.0 ± 1.8
Monoglycerides	8.6 ± 1.2	8.9 ± 1.3	7.2 ± 0.5	8.1 ± 1.0	8.5 ± 1.0	7.2 ± 1.4	6.6 ± 1.0
Diglycerides	16.0 ± 1.7	14.8 ± 0.8	13.4 ± 0.6	14.3 ± 1.3	14.3 ± 0.5	11.8 ± 3.0	10.3 ± 2.0
Triglycerides	20.5 ± 2.5	22.0 ± 1.6	25.3 ± 1.7	23.3 ± 2.3	23.1 ± 0.6	26.9 ± 3.4	28.5 ± 1.9
Free fatty acids	8.8 ± 1.9	10.2 ± 0.6	13.6 ± 1.0	13.9 ± 4.1	10.1 ± 0.4	15.2 ± 2.2	17.9 ± 1.8

All figures are the means of four replicates quoted with their 95% confidence limits

o) Total neutral lipid fatty acids

Fatty Acids	Days after infection						
	Uninfected Plants			Infected Plants			
	0	28	% Composition 35	42	28	% Composition 35	42
Lauric acid	3.3 ± 1.1	3.2 ± 0.2	2.6 ± 0.6	3.0 ± 1.2	3.0 ± 0.5	2.7 ± 0.8	2.8 ± 0.5
Myristic acid	7.3 ± 0.6	7.7 ± 1.1	7.8 ± 0.9	6.9 ± 1.0	7.7 ± 0.5	7.1 ± 0.4	6.6 ± 0.8
Palmitic acid	22.9 ± 2.0	22.8 ± 1.6	21.2 ± 4.8	20.3 ± 2.6	23.1 ± 1.2	21.7 ± 0.8	22.7 ± 1.7
Stearic acid	2.5 ± 0.4	3.1 ± 0.6	2.8 ± 1.2	2.8 ± 1.3	3.2 ± 0.5	2.5 ± 0.5	2.4 ± 0.6
Oleic acid	8.9 ± 1.1	9.2 ± 0.8	8.4 ± 0.6	8.5 ± 1.3	8.4 ± 0.7	7.2 ± 1.0	6.7 ± 0.9
Linoleic acid	29.8 ± 2.1	28.9 ± 4.3	30.6 ± 1.4	31.5 ± 1.7	30.2 ± 1.0	32.1 ± 1.1	31.7 ± 1.9
Linolenic acid	25.2 ± 3.2	25.0 ± 1.4	26.5 ± 1.7	26.9 ± 2.4	24.2 ± 0.8	26.6 ± 1.5	27.0 ± 2.9

All figures represent the means of 4 replicates quoted with their 95% confidence limits

The results for PG bound fatty acids are shown in table 35 once again the only changes were found in linoleic acid and linolenic acid.

Table 21 shows the changes in neutral lipid composition in stem as a result of infection. These changes consist of a decrease in the amount of sterol esters, sterols, monoglycerides and diglycerides and an increase in the proportion of triglycerides and free fatty acids. ^{epidermis}

No significant changes were found in the fatty acids from neutral lipids either from the total fraction or from the individual neutral lipids. The results agreed with those shown in Figure 65.

As it was possible to obtain significant increases in the level of unsaturation in some polar lipid bound fatty acids in infected stems 42 days after infection it was decided to use these stems in experiments looking at membrane lipids.

4.6.2.1. Isolation of membrane fractions

The method used for isolating membrane fractions from pea stems was that described in Section 3.19. Two different types of tissue were studied, uninfected stems and stems that had been infected by E. pisi for 42 days. In order to determine the nature of the membranes in the membrane enriched fractions the following enzyme assays were used; cytochrome C oxidase to indicate the presence of mitochondria, K⁺ stimulated ATPase at pH 6.5 to indicate the presence of plasma membranes and K⁺ stimulated ATPase at pH 9 to indicate the presence of mitochondria.

The results of these assays on the different fractions are shown in Table 36. As can be seen all the fractions contained some activity of each of the enzymes. However, the majority of the cytochrome C oxidase activity was in the 1000-8000g fraction. the highest activity of K⁺ stimulated ATPase at pH 9 was recorded in the 1000-8000g fraction. Most of the K⁺ stimulated ATPase at pH 6.5 was in the fractions sedimenting between 1000g and 40,000g. The Mg²⁺ stimulated ATPase activity at both pH's was fairly evenly distributed through the different pellets.

The one noticeable effect from this experiment was the generalised decrease in enzyme activity by infection.

The polar lipid composition of each of these fractions was then analysed and the results are shown in Table 37. there are considerable differences in lipid composition between fractions but there are no significant differences between infected and uninfected plants.

When the total polar lipid bound fatty acids were analysed significant differences were apparent in fatty acid composition between the different fractions in both uninfected and infected plants. There was also a significant increase ($p = 0.05\%$) in the level of fatty acid unsaturation in all the fractions caused by an increase in the proportion of linolenic acid and a decrease in the proportion of linoleic acid (Table 38).

Table 36: Enzyme assays in various membrane fractions from uninfected and infected plants

a) Mg^{2+} stimulated ATPase

Fraction (g) sedimenting a - g	Specific Activity (μ moles pi/ g protein/lhr)			
	Uninfected Plants		Infected Plants	
	pH 6.5	pH 9	pH 6.5	pH 9
1,000	6.5	4.3	5.3	2.1
1,000 - 8,000	7.4	12.5	6.2	9.7
8,000 - 13,000	8.3	8.0	6.4	6.9
13,000 - 40,000	11.6	6.1	11.1	5.1
40,000 - 80,000	10.1	5.2	8.3	4.7

b) K^{+} stimulated ATPase

Fraction (g) sedimenting a - g	Specific Activity (μ moles pi/ g protein/lhr)			
	Uninfected Plants		Infected Plants	
	pH 6.5	pH 9	pH 6.5	pH 9
1,000	1.5	2.5	0.9	2.1
1,000 - 8,000	3.4	8.7	1.9	6.1
8,000 - 13,000	4.4	5.0	3.3	4.1
13,000 - 40,000	3.1	2.5	3.0	2.2
40,000 - 80,000	1.1	2.0	0.4	0.9

c) Cytochrome Oxidase

Fraction (g)	Specific Activity (μ moles pi/ g protein/lhr)	
	Uninfected Plants	Infected Plants
1,000	0.2	0.1
1,000 - 8,000	1.4	0.9
8,000 - 13,000	0.5	0.3
13,000 - 40,000	0.1	0.1
40,000 - 80,000	0.1	0.0

Table 37: The individual polar lipid composition of different membrane fractions from the stems of infected and uninfected pea plants

Polar Lipids	% Composition									
	Uninfected Plants Fraction					Infected Plants Fraction				
	1000	1000	8000	13000	40000	1000	1000	8000	13000	40000
		8000	13000	40000	80000		8000	13000	40000	80000
PC	40.4 ± 3.7	45.4 ± 3.4	49.3 ± 5.9	51.3 ± 6.2	47.3 ± 4.4	44.8 ± 4.1	46.1 ± 4.7	40.8 ± 3.7	54.5 ± 1.9	50.6 ± 5.1
PG	6.3 ± 0.8	8.3 ± 1.2	7.4 ± 1.6	8.3 ± 1.1	1.8 ± 0.4	5.9 ± 0.1	6.9 ± 1.7	8.2 ± 0.4	7.6 ± 0.7	3.2 ± 0.3
PE	29.7 ± 2.1	39.6 ± 3.1	18.9 ± 4.4	24.6 ± 2.8	24.1 ± 2.9	30.1 ± 4.1	36.8 ± 3.1	23.4 ± 3.3	25.6 ± 4.1	25.6 ± 3.2
PI	10.4 ± 1.7	7.2 ± 1.7	6.4 ± 1.2	7.1 ± 1.4	21.2 ± 3.4	9.6 ± 1.0	7.4 ± 0.8	8.6 ± 0.9	6.4 ± 2.6	17.1 ± 2.3
PS	2.1 ± 0.1	1.3 ± 0.4	2.3 ± 0.9	2.3 ± 0.9	3.0 ± 0.7	2.4 ± 0.9	1.7 ± 0.9	1.4 ± 1.0	2.7 ± 0.3	2.9 ± 0.7
PA	1.7 ± 0.7	-	-	-	1.6 ± 0.6	0.6 ± 0.3	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	-
MGDG	2.1 ± 0.7	-	10.1 ± 0.7	4.3 ± 0.4	-	3.9 ± 0.4	0.7 ± 0.1	9.1 ± 1.7	0.9 ± 0.4	0.4 ± 0.1
DGDG	3.7 ± 0.3	-	5.6 ± 0.5	2.3 ± 1.1	-	2.7 ± 0.9	0.3 ± 0.2	8.3 ± 0.9	1.7 ± 1.0	0.2 ± 0.1

All the figures are the means of 4 replicates quoted with the 95% confidence limits

Table 38: The polar lipid bound fatty acid composition of different membrane fractions from stems of infected and uninfected pea plants

Polar Lipids	% Composition									
	Uninfected Plants Fraction					Infected Plants Fraction				
	1000	1000	8000	13000	40000	1000	1000	8000	13000	40000
		8000	13000	40000	80000		8000	13000	40000	80000
Palmitic acid	21.3 ± 1.4	19.9 ± 1.4	21.7 ± 2.1	23.4 ± 1.1	22.4 ± 0.9	21.9 ± 2.3	22.7 ± 1.4	19.7 ± 1.9	20.4 ± 2.9	22.7 ± 3.1
Palmitoleic acid	0.9 ± 0.1	0.1 ± 0.2	1.9 ± 0.4	2.1 ± 0.9	0.4 ± 0.1	1.3 ± 0.4	0.7 ± 0.1	2.1 ± 0.9	2.3 ± 0.4	1.1 ± 0.2
Stearic acid	4.7 ± 0.9	4.8 ± 0.5	4.2 ± 1.1	3.7 ± 0.2	3.9 ± 0.4	3.8 ± 0.5	4.2 ± 0.6	3.4 ± 0.3	6.7 ± 0.7	4.8 ± 0.8
Oleic acid	2.4 ± 1.1	3.1 ± 0.3	2.3 ± 1.4	2.6 ± 0.8	2.1 ± 0.9	2.1 ± 0.4	2.3 ± 0.9	2.7 ± 1.7	2.5 ± 0.4	2.4 ± 0.9
Linoleic acid	35.2 ± 3.4	33.7 ± 0.7	34.6 ± 2.4	34.3 ± 1.7	35.2 ± 4.1	29.9 ± 2.6	31.2 ± 1.7	28.9 ± 2.1	32.4 ± 1.1	28.0 ± 3.7
Linolenic acid	35.5 ± 3.6	36.4 ± 3.8	35.3 ± 2.7	33.9 ± 4.1	36.2 ± 3.1	41.5 ± 3.9	38.9 ± 4.2	43.2 ± 2.6	37.7 ± 3.1	41.1 ± 2.6

All the figures are the means of 4 replicates quoted with their 95% confidence limits

5. Discussion

The aim of this project was to examine the host: parasite interface in a plant disease caused by a biotrophic fungal parasite, in this case powdery mildew of peas caused by Erysiphe pisi. As mentioned previously one of the most important characteristics of biotrophic pathogenesis is the redirection of metabolites from the host to the fungus. Gay and Manners (1981) proposed a model for the mechanism of assimilate transport as follows, entry into the epidermal cells is presumed to be almost exclusively by transmembrane import and the high ATPase activity of the plasmalemma suggests that the epidermal cells scavenge solutes from the apoplast. The ATPase activity of the haustorial plasmalemma is assumed simultaneously to deplete the solutes in the extrahaustorial matrix and thus a high concentration gradient is maintained across the extrahaustorial membrane thus constituting a transport system. Therefore for solutes to enter the fungal cytoplasm they have to pass through the epidermal plasmalemma, the extra-haustorial membrane and the haustorial plasmalemma. A study of the composition of these membranes would help elucidate how membrane structure is involved in this transport system.

There are two main components of membranes that are involved in solute transport and these are proteins and lipids as discussed in Section 2.5. According to the model of Singer and Nicholson (1972) phospholipid and protein both contribute to the structure of the membrane such that a cytoplasmic solute may be prevented from diffusing through a membrane by phospholipids in one region or by protein molecules in another. It follows that the modification of either component might enhance membrane permeability, effectively there being two channels in parallel.

In this study the composition of membrane lipids were studied in order to see how disease affects the membrane lipids and how this could be related to changes in permeability. As mentioned in Section 2 the permeability of membranes depends to a considerable extent on the nature of the polar lipid head groups and the interaction between the membrane lipids and proteins.

To obtain a clear understanding of the lipid composition it would have been preferable to separate the individual membrane fractions prior to analysis of the different membrane lipid components. This presents certain practical problems, firstly there are difficulties in obtaining pure preparations of the individual membranes. Secondly, there are no marker enzymes for the three membranes and it would be necessary to carry out a search for suitable markers before conducting the experiments and there is no guarantee that such enzymes could be found. Finally in order to make a detailed analysis it would be necessary to collect large amounts of these membranes as the techniques of analysis although sensitive would not detect extremely small amounts. At present the best approximation is to analyse haustorial complexes, which can be harvested in some quantity from infected leaves (Manners, 1979), but as is discussed later not in high purity.

To study the effects of disease on lipid composition in plants previous workers have either used leaf halves (Hoppe and Heitefuss, 1974), leaf discs taken from around the pustules (Lösel and Lewis, 1974) or roots taken from uninfected tissue adjacent to an infected area (Sedrina et al, 1981). All these methods, although suitable in their own context at the time suffer from the same disadvantages as an experimental system. They all involve looking at whole ale lipid changes without being able to separate the different membrane components and without separating uninfected from infected cells. It is therefore only possible to draw fairly general conclusions about the types of changes occurring. In this study 3 different types of tissue were used for lipid analysis, leaves, epidermal strips and isolated membranes. The leaves were used to give an indication whether any general changes in lipid composition were occurring in infected organs. The use of epidermal strips allowed analysis to be extended to the cellular level and the use of isolated membranes permitted a preliminary examination of changes at an even higher level of resolution.

Before it was possible to analyse the lipid contents of these tissues it was first necessary to develop a quantitative extraction and analytical procedure for lipids from pea leaves and epidermal strips as well as for fungal lipids and haustorial complexes.

5.1. Method Development

Considerable effort was invested in optimizing the conditions for the extraction, purification and analysis of the lipid samples to obtain the most appropriate methods. This was an extremely important part of the project as the validity of all later analyses depended on the accuracy of the procedure.

The results of these experiments are recorded in Section 4.1 and due to the nature of the work discussion is included at that stage. Reiteration of the arguments would be inappropriate here. The success of the optimization can be seen from the results in Section 4.1.9 where it was shown that by using the optimized extraction procedures and analytical methods a lipid recovery of 80% could be obtained without bias towards any one lipid class or group of fatty acids provided that appropriate precautions were taken to prevent fatty acid oxidation.

The method derived from this work and used in the experimental system is summarized in Figure 7.

5.2. Lipids in infected and uninfected pea leaves

In order to determine whether infection causes any general changes in leaf lipid composition or in the constituent fatty acids four different pea cultivars were studied over a 24 day period following infection with E. pisi and compared with uninfected plants over the same period. Four different cultivars were chosen to test whether any changes which may occur were consistent between cultivars.

Hoppe and Heitefuss (1974) in their series of experiments with Phaseolus vulgaris infected by Uromyces phaseoli used leaf discs taken from uninfected leaves or from areas surrounding the pustules in infected leaves as did Lösel and Lewis (1974). This method for obtaining material for analysis was ideal for the host: parasite systems under study, namely rust infections where, in the early stages of infection, it is possible to isolate individual pustules and study lipid changes around these. In powdery mildew infections it is possible to do this at low levels of infection during the early stages but as the infection develops the whole

leaf becomes covered by a mycelial mat which makes the isolation of individual pustules impossible. So in these initial experiments whole leaves were used as the experimental system and changes in lipid over an extended period were monitored.

The results show that there were no significant changes in the total amount of polar and neutral lipid, no alteration in the composition of the lipid classes nor in the composition of the lipid-bound fatty acids in the leaves of any of the four cultivars tested during the course of a 24 day infection period. Furthermore there was considerable uniformity amongst the cultivars in their lipid composition. The results present new data on the lipid composition of pea leaves and also show that regardless of infection there was no change in the leaf lipid composition from the fourth week after germination until the eighth week provided that growth conditions remained constant.

These results reveal that despite a high level of infection corresponding to 2-4 haustoria in each epidermal cell, the lipid composition of the leaves remains constant. This must reflect the high level of cellular compatibility between the parasite and its host. Although the parasite is able to grow on the leaf surface it causes no gross structural changes to the lipid composition of its host. This is of significant ecological advantage to the parasite as it enables it to obtain nutrients from the host for a period long enough for it to sporulate and complete its life cycle. It is precisely this capacity to infect host cells while causing minimal damage which makes Erysiphe pisi an extremely efficient parasite.

5.3. Lipid metabolism in infected and uninfected pea epidermal cells

5.3.1. The suitability of epidermal strips as an experimental system

To conduct experiments involving epidermal cells it is essential to remove epidermes without damaging the integrity of the cells and with minimal contamination from other cell types. The viability of cells stripped from the leaves of 6 different pea cultivars was only 10.20% and this deteriorated to 5-10% in plants that had been infected by E. pisi for

21 days. For stem epidermal cells the respective figures were 30-40% for uninfected plants and 10-15% for infected plants. These results show that either the cells suffered mechanical damage during stripping or that even in intact tissue many epidermal cells may be senescent and that this increases during infection.

To test this the stems and leaves were plasmolysed for 2 hours in 0.5M mannitol prior to peeling. This treatment enhanced cell viability considerably suggesting that mechanical damage rather than inherent senescence was the cause. Plasmolysis improved the viability of the stripped cells to 50-60% for uninfected leaves and 85-90% for uninfected stems, with the exception of the cultivar, Pilot which had a viability of only 36.3% in stem cells. When strips from plants that had been infected for 21 days were tested values were lower (9-11% for leaf cells and 60-75% for stem cells), cv. Pilot again giving an anomalously low figure (27.5%).

A viability of 60-75% for stem epidermal cells was considered acceptable, and this tissue was used routinely for lipid analysis.

The capacity for plasmolysis and the ability to accumulate vital stains are probably the most realistic measure of viability in the present context. They do not indicate a total absence of cellular damage, but they do indicate an intact plasmalemma and/or tonoplast capable of carrying out at least some of the normal functions. The reasons for impaired viability in leaves of cv. Pilot are not known but a greater degree of damage during the stripping process may be to blame. It is relevant that mildew infected epidermes are noticeably more difficult to remove from both leaves and stems probably because of wall modification during parasite development.

Contamination of epidermal strips by non epidermal cells was greater for leaves (25-35%) than for stems (1-6%) and this contamination increased upon infection to 45-55% and 8-10% respectively, although the increase for stems was not statistically significant. The purity of the stem epidermal preparations was a further reason for the choice of this tissue in preference to leaves.

5.3.2. The influence of infection by *E. pisi* on the lipid composition in epidermal strips of pea, cv. Onward

For all these experiments epidermal strips were taken from stems which had

been plasmolysed in 0.5M mannitol for two hours prior to peeling. The experiments were carried out over an infection period of 42 days in order to follow any changes through to the latter stages of disease.

The amount of lipid was expressed per gram dry weight; this value was obtained by collecting lg fresh weight samples of epidermal tissue and using a conversion factor to obtain the result in g dry weight. This conversion factor was calculated by measuring the dry weight: fresh weight ratio at various times after infection. The ratio was not affected by infection and the same conversion factor was used throughout. Lösel and Lewis (1974) calculated their lipid values per unit area which has the advantage that the results are not affected by changes in the amounts of other cellular material provided that there is no significant cell enlargement. However while agreeing that a unit area basis is a more accurate assessment method for this type of experiment, in practice it was not possible to calculate a total area for the epidermal strips, and cells counts, another possible method, were similarly unsuitable for practical reasons. Consequently the data reported here may be a less accurate representation of changes per unit cell than had another accounting method been adopted, but in practice since the fresh weight: dry weight ratio did not alter significantly after infection or with age of the plant such inaccuracies are probably negligible. Further it is unlikely that an inadequate method of expression could have masked a threefold change in lipid concentration as found by Lösel and Lewis (1974). Therefore for the purpose of the discussion the results of different studies even when reported on a different basis will be considered as directly comparable. Whatever method of assessment is used any changes in the proportions of the individual lipids will still be valid.

The most notable feature of the results from the series of experiments was the consistent lack of general changes in lipid composition in

uninfected plants during this period and the fact that only a few changes occurred to the lipids in infected plants. There was no decrease in the total amount of lipid nor did the ratio between polar and neutral lipids change during this period for both infected and uninfected plants.

The only significant changes in the composition of the polar lipid fraction from diseased plants was the decrease in the amount of the galactolipids, MGDG and DGDG along with a trend towards a reduction in PG. All three of these lipids were relatively minor components comprising 0.8%, 0.4% and 5.4% respectively of the polar lipid fraction even in uninfected plants. As all of these lipids are associated with chloroplasts, which are not usually found in the epidermal cells, it is likely that this decrease was indicative of a general disease-induced breakdown of chloroplasts, possibly in the non-epidermal contaminant cells. This loss did not become statistically significant until the 35th day after infection which would explain why it was not found in the experiment using whole leaves which was terminated at 24 days.

Despite this loss of chloroplast-associated lipids the general conclusion is of limited, if any changes, in the polar lipids during infection.

The most significant differences in the composition of the lipids following infection in these experiments were found for the lipid-bound fatty acids where an increase in the level of unsaturation was detected. This was mainly caused by a dramatic decrease in the level of linoleic acid and a concomitant increase in linolenic acid. This change in the lipid-bound fatty acids became apparent at about 10 days after infection and increased throughout the infection period.

Analysis of the lipid-bound fatty acids from the individual lipids revealed that this change in fatty acid composition occurs in all phospholipids, although for PG the change was smaller and later, but it was not seen for either of the two galactolipids. The divergence in the data for healthy and infected plants was generally apparent from an early stage but statistical significance at the 5% level was not achieved until 10 days (PA), 14 days (PE and PS), 17 days (PC and PI) or 35 days for PG

after infection.

These results are extremely important in relation to the permeability properties of the membrane since this is governed to a large extent by the nature of the hydrocarbon chains of the lipids. Using black film studies it has been shown that an increase in the degree of unsaturation of the acyl chains of the lipids increases their permeability to water (Fettiplace, 1978) and to non-electrolytes such as urea, glycerol and erythritol (Lippe, 1969). De Gier^{et al} (1968) found that the permeability of liposomes constructed from synthetic PC to non-electrolytes was enhanced by the presence of double bonds in the acyl chains. These results together with other studies performed with natural systems such as Mycoplasma laidlawii B (McElhargy, et al., 1970) indicates that the permeability of membranes towards water soluble non-electrolytes may depend at least in part on the degree of unsaturation and the chain length of fatty acids in the membrane lipids. The results in this experiment therefore suggest that the changes in fatty acids in the stem epidermal cells of pea plants during infection by E. pisi may make the membranes of the plants more permeable towards non-electrolytes. The possibility that the changes just discussed did not result from the presence in samples of fungal lipids was eliminated by appropriate control experiments, the results of which are discussed later.

There were time-dependent changes in the composition of the neutral lipids even in uninfected plants which showed a decrease in the amount of diglyceride and an increase in the amount of triglyceride. In infected plants there were decreases in the amounts of sterol esters, monoglycerides and diglycerides and an increase in the amount of triglycerides and free fatty acids.

These changes in neutral lipids are of interest because, when haustoria form in epidermal cells of pea plants, osmiophilic spherical structures can be observed on the extrahaustorial and other host cell membranes. When Manners (1979) analysed the lipids from haustoria he found that 50% consisted of triglycerides and he postulated that these could be accounted for by the osmiophilic spherical structures.

There was no change in the total amount of neutral lipid, merely changes in the percentage composition between the different neutral lipid classes which could be explained by the presence of triglycerides and free fatty acids in the osmophilic structures just described. Ideally confirmation for this should be sought by isolating and analysing the droplets/granules themselves.

Again it is unclear whether these changes result from the presence of fungal lipids within the epidermal cells, whether they are caused by the accumulation of lipids for the parasite to assimilate or whether they are the result of the host's response to the pathogen. Further studies are needed on the nature of fungal neutral lipids and on the effect of senescence on the host neutral lipids and these are reported in a later section.

In contrast with the results for polar lipid fatty acids, the neutral lipid fatty acids showed no change during the course of the infection period, even the composition of the free fatty acid fraction, which may have been expected to change as a result of infection-induced lipid breakdown, showed no change. The possible significance of this is discussed further after a consideration of the fungal lipids.

The conclusion from these series of experiments was that in contrast to whole leaves the lipids in the epidermal cells did show some alteration as a result of infection notably an increase in the unsaturation of fatty acids. This shows that local changes in lipid composition do take place which may result in changes in membrane permeability. These experiments do not show whether these changes were general or confined to a specific membrane, nor do they exclude the presence of fungal material as a possible cause.

5.4. Isolation and lipid analysis of membrane fractions from infected plants

The reason for conducting these experiments was to determine whether the changes observed in the epidermal strips was a general change or whether it was confined to particular membranes.

In order to obtain a large enough sample for analysis whole stem tissue was used, preliminary experiments having shown that the increase in lipid unsaturation following infection could also be detected in tissues deeper than the epidermis.

In whole stems this change occurred later, becoming statistically significant ($P = 0.05\%$) 35 days after infection compared with 10 days after infection in epidermal cells, and the magnitude of the changes was less.

Subcellular fractions were isolated from stems using Cytochrome C oxidase as a mitochondrial and K stimulated ATPase at pH 6.5 as a plasma membrane marker. All of the fractions contained some of each marker enzyme but Cytochrome C oxidase was present mainly in the 1,000g-8,000g fraction and K stimulated ATPase activity was found mainly in the 8,000g-40,000g fraction.

For polar lipids there were considerable differences in composition between fractions but there were no differences between infected and uninfected plants. The difference between the fractions undoubtedly reflects the presence of different organelles, for instance the lack of MGDG and DGDG and the low levels of PG in the 40,000g-80,000g fraction correlates with the absence of chloroplasts. In contrast the relatively high levels of these lipids in the 8,000g-13,000g fraction reflects the relative abundance of this organelle. These results show that the increased level of unsaturation is common to all fractions and is thus not confined to particular membranes.

This result further suggests that the changes observed are not a reflection of any specific pathogen-inspired manipulation of host membrane composition but are rather a generalised response of the plant to

infection.

5.5. Lipid composition of *E. pisi* mycelium

There were two possible ways for testing the hypothesis that the presence of fungal lipids in the lipid extract was responsible for the observed changes in composition. Firstly by analysing the lipid composition of *E. pisi* mycelium and secondly by analysing haustorial complexes isolated from infected cells. If a pure sample could be obtained the second approach would be more accurate as it is the haustoria which contribute lipids to the plant extract. However, there were many difficulties encountered in obtaining a pure sample of haustorial complexes and these are discussed later in this section. Accordingly the first approach was adopted and the percentage composition of polar lipids by class for mycelium and uninfected and infected epidermes is presented in Table 39. Mycelium contains higher amounts of MGDG, DGDG, PA and PG, lower amounts of PC and PI and similar amounts of PE and PS to the epidermal strip extracts. Assuming that these mycelial lipid extracts are a fair reflection of the lipid composition of the haustoria then this is an important result. If fungal lipids within the host cells are causing the increased fatty acid unsaturation as described in Section 4.3 then there should also be a change in the composition of the polar lipids. As there is no such change then it is unlikely that contamination by fungal lipids is the cause of the observed changes.

This is confirmed by the composition of the overall polar lipid-bound fatty acids. In mycelium there is a consistent fatty acid composition which does not change throughout the duration of the experiment. When compared with the fatty acids from epidermal strips some distinct differences are revealed certain fatty acids are present in mycelium which are absent from epidermal strips, notably myristic acid, arachidic acid, arachidonic acid and lignoceric acid.

Table 39: The composition of polar lipids in mycelium and epidermal strips

Lipid Class	Mycelial Lipids	Uninfected Epidermal Strips	Infected (42 days) Epidermal Strips
PC	22.1 $\pm$ 1.6%	46.0 $\pm$ 4.9%	42.0 $\pm$ 3.2%
PE	29.4 $\pm$ 1.9%	25.5 $\pm$ 5.3%	27.8 $\pm$ 3.2%
PG	11.3 $\pm$ 0.6%	6.2 $\pm$ 2.3%	4.2 $\pm$ 2.0%
PI	5.8 $\pm$ 0.8%	14.7 $\pm$ 2.3%	16.9 $\pm$ 1.8%
PS	4.9 $\pm$ 0.8%	3.7 $\pm$ 2.3%	4.1 $\pm$ 1.1%
PA	3.4 $\pm$ 0.8%	0.7 $\pm$ 0.7%	1.0 $\pm$ 0.9%
MGDG	11.5 $\pm$ 1.3%	2.0 $\pm$ 1.1%	0.1 $\pm$ 0.1%
DGDG	11.4 $\pm$ 0.8%	1.0 $\pm$ 1.1%	0.1 $\pm$ 0.1%

The figures are the means of four replicates and are quoted with 95% confidence limits.

These fatty acids are found in neither the lipid extracts from uninfected epidermal strips nor the lipid extracts from infected epidermal strips. The absence of these fatty acids even in trace amounts from infected epidermis is further evidence that mycelial lipids make no significant contribution to the composition of infected plant extracts. Consequently the increased unsaturation observed following infection cannot be due to fungal lipid contamination. The results for the individual polar lipid-bound fatty acids confirm this (table 40).

For the neutral lipids (table 41) however some interesting comparisons emerge. Once again the neutral lipid composition of the mycelial extracts does not change during infection. However there are some differences between healthy and infected plants. These include a decrease in the level of sterol esters and an increase in triglycerides and free fatty acids. The changes in the triglycerides and the sterol esters could be caused by the presence of fungal lipids but the increase in free fatty acids along with the lack of changes in the amount of sterol present suggests that this is unlikely.

The results for the neutral lipid-bound fatty acids further support this conclusion (table 42). There are no changes in the overall neutral lipid-bound fatty acids in epidermal strips or mycelium during infection. If fungal lipids were contributing to the total neutral lipid in the extracts there would be a decrease in the amounts of palmitic acid, oleic acid and linoleic acid and an increase in linolenic acid and trace amounts

of arachidic acid, arachidonic acid and lignoceric acid. As this does not occur then it is unlikely that the fungal lipids are contributing to the composition of the extracts.

Table 40: The composition of polar lipid-bound fatty acids in mycelium and epidermal strips

Fatty Acid	Mycelial extracts	Uninfected Epidermal Strips	Infected (42 days) Epidermal Strips
Palmitic acid	13.1 $\pm$ 1.4%	21.8 $\pm$ 3.4%	22.0 $\pm$ 2.5%
Palmitoleic acid	3.3 $\pm$ 0.6%	0.5 $\pm$ 0.5%	0.9 $\pm$ 0.7%
Stearic acid	7.1 $\pm$ 0.8%	6.9 $\pm$ 1.4%	6.8 $\pm$ 0.8%
Oleic acid	12.3 $\pm$ 1.0%	4.2 $\pm$ 1.1%	3.7 $\pm$ 1.0%
Linoleic acid	25.7 $\pm$ 1.4%	40.5 $\pm$ 3.3%	13.2 $\pm$ 4.3%
Linolenic acid	21.0 $\pm$ 2.8%	28.1 $\pm$ 2.6%	53.3 $\pm$ 2.5%
Arachidic acid	4.0 $\pm$ 1.3%	-	-
Arachidonic acid	8.7 $\pm$ 0.7%	-	-
Lignoceric acid	1.6 $\pm$ 0.6%	-	-

The figures are the means of four replicates and are quoted with 95% confidence limits

Table 41: The composition of neutral lipids in mycelium and epidermal strips

Neutral Lipid	Mycelial Extracts	Uninfected Epidermal Strips	Infected (42 days) Epidermal Strips
Sterol Esters	14.6 $\pm$ 1.6%	26.7 $\pm$ 3.3%	16.0 $\pm$ 2.0%
Sterols	28.0 $\pm$ 2.7%	15.5 $\pm$ 2.3%	15.1 $\pm$ 1.4%
Monoglycerides	3.0 $\pm$ 1.1%	2.1 $\pm$ 1.0%	1.5 $\pm$ 0.9%
Diglycerides	5.4 $\pm$ 1.7%	11.2 $\pm$ 2.5%	7.9 $\pm$ 1.8%
Triglycerides	40.5 $\pm$ 4.1%	33.5 $\pm$ 4.6%	43.4 $\pm$ 1.3%
Free Fatty Acids	8.3 $\pm$ 1.2%	10.0 $\pm$ 1.4%	16.1 $\pm$ 1.3%

The figures are the means of four replicates and are quoted with 95% confidence limits

Table 42: The composition of neutral lipid bound fatty acids in mycelium and epidermal strips

Fatty Acid	Mycelial Extracts	Uninfected Epidermal Strips	Infected (42 days) Epidermal Strips
Lauric acid	-	4.0 ± 5.7%	4.1 ± 1.0%
Myristic acid	3.4 ± 1.7%	5.7 ± 1.2%	6.2 ± 1.0%
Palmitic acid	13.1 ± 1.8%	24.3 ± 2.8%	22.7 ± 1.6%
Palmitoleic acid	0.4 ± 0.7%	0.2 ± 0.2%	0.1 ± 0.1%
Stearic acid	4.0 ± 0.5%	4.6 ± 0.8%	4.5 ± 0.7%
Oleic acid	3.0 ± 1.6%	8.4 ± 1.6%	7.5 ± 0.7%
Linoleic acid	22.5 ± 2.5%	27.8 ± 2.8%	33.2 ± 2.4%
Linolenic acid	38.5 ± 2.2%	23.4 ± 4.3%	24.7 ± 1.2%
Arachidic acid	3.2 ± 1.2%	-	-
Arachidonic acid	9.5 ± 1.2%	-	-
Lignoceric acid	2.1 ± 0.8%	-	-

The figures are the means of four replicates and are quoted with 95% confidence limits

Possible interpretations of these results are difficult to make, it could be that during senescence of the host changes in neutral lipid fatty acids do not occur or finally that the parasite assimilates neutral lipids of host origin in the same conformation as they are present in the host. A radiolabelled chase study following neutral lipids would reveal whether neutral lipids are assimilated by the parasite in the same composition as they are found in the host, however, such a study was not made in this series of experiments.

As already stated the validity of these conclusions depends upon a degree of similarity between haustorial and mycelial lipids. It is possible, due to their unique function that the composition of haustorial lipids is entirely different from that of mycelial lipids, however due to the difficulty of obtaining a pure sample of haustorial complexes it was not possible to get an accurate idea of their lipid composition (Section 4.6 and 5.4.1) and these results for mycelial lipids are the best approximation available.

5.5.1. Isolation and lipid analysis of haustorial complexes

The analysis of haustorial complexes was hampered by contamination. Impurities in the extract were such that haustoria comprised only $23.6 \pm 5.7\%$ of the total fraction. In an attempt to by-pass this difficulty uninfected leaves were fractionated in the same way as infected leaves and an estimate of the lipid composition of the haustorial complexes was derived by difference.

Whether or not this estimation provides a fair reflection of the lipid composition of the haustorial complexes, some firm observations are still possible. Firstly the haustorial complexes contain myristic acid, arachidic acid and arachidonic acid which are all found in the mycelial lipids but are not found in sufficient quantities to be detected in lipid extracts from epidermal cells of infected plants. This strengthens the conclusion that fungal lipids are absent from leaf extracts.

The lipid composition of haustorial complexes differs from infected and uninfected epidermal strips, in a number of ways (table 43). Haustorial complexes contain greater amounts of MGDG, DGDG, PA and PE, lesser amounts of PC and PI and similar amounts of PG and PS. Assuming that these estimations are a fair reflection of the actual lipid composition of the haustorial complexes this is an important result as it shows that the haustoria cannot be causing changes in lipid composition in infected plants.

Likewise with the polar lipid-bound fatty acids there are a number of differences between haustorial complexes and the infected epidermal strips. Haustorial complexes contain greater amounts of palmitoleic acid, stearic acid, oleic acid and linoleic acid and lower amounts of linolenic acid and palmitic acid than infected epidermal strips. In addition they contain several fatty acids; myristic acid, arachidic acid and arachidonic acid. In addition to this the degree of fatty acid unsaturation of the haustorial complexes is similar to that found in uninfected epidermal cells and therefore the presence of fungal material in the infected epidermal cells cannot be contributing to the increase in unsaturation.

The results from these experiments thus agree with the results from the mycelial lipid extracts in that it is unlikely that the presence of haustorial complexes in the epidermal cells is contributing to the changes in lipid composition observed in infected epidermal cells.

When the lipids from the haustorial complex and mycelium are compared some interesting differences emerge. Firstly the mycelium contains much higher amounts of the galactosyl diglycerides and correspondingly lower amounts of the major polar lipids PC and PE, as well as higher amounts of PG and lower amounts of PI. The polar lipid-bound fatty acids patterns for haustorial complexes and mycelium are very similar except that the latter contains a higher proportion of palmitic, arachidic and arachidonic acids and thus a correspondingly lower proportion of the other fatty acids. The mycelial lipid extract also contains lignoceric acid which is not present in haustorial complexes. The neutral lipids are fairly similar (table 45), mycelium contains a higher proportion of sterol esters but the proportion of the other neutral lipids is very similar.

These results may have some significance as to the permeabilities of the various membranes. Haustoria are specialised and differentiated structures whose purpose is to obtain nutrients from the invaded host. Therefore the haustorial membranes would be expected to have a composition adapted for this function and thus differences between mycelial and haustorial lipids would be expected. Interpretation is complicated by the inherent inaccuracy of the experimental protocol and by the fact that the lipids in the membranes of interest, haustorial plasmalemma and the extrahaustorial membrane, will be diluted by the lipids in the general cytoplasmic membranes of the haustoria. It is noteworthy that within the limits of the experimental system employed differences between the mycelial lipids and the haustorial lipids were found.

It is likely that the membrane lipids in haustoria are adapted for their specialised role, but until individual membranes can be isolated in a reasonable degree of purity further information on their lipid and protein content will not be forthcoming. In the short term a superficial approach to the study of the extra-haustorial membrane is likely to be most fruitful.

Table 43: The composition of polar lipids in haustorial complexes, epidermal strips and mycelium

Lipid	Hautorial Complex Estimation	Mycelial Extracts	Uninfected Epidermal Strips	Infected (42 days) Epidermal Strips
PC	29.4%	22.1 ± 1.6%	46.0 ± 4.9%	42.0 ± 3.2%
PE	33.6%	29.4 ± 1.9%	25.5 ± 5.3%	27.8 ± 3.2%
PG	6.3%	11.3 ± 0.6%	6.2 ± 2.3%	4.2 ± 2.0%
PI	10.5%	5.8 ± 0.6%	14.7 ± 2.3%	16.9 ± 1.8%
PS	5.5%	4.9 ± 0.8%	3.7 ± 2.3%	4.1 ± 1.1%
PA	3.4%	3.4 ± 0.8%	0.7 ± 0.7%	1.0 ± 0.9%
MCDG	5.0%	11.5 ± 1.3%	2.0 ± 1.1%	0.1 ± 0.1%
DGDG	3.4%	11.4 ± 0.8%	1.0 ± 1.1%	0.1 ± 0.1%

Table 44: The composition of the polar lipid-bound fatty acid fraction from haustorial complexes, epidermal strips and mycelium

Fatty Acid	Hautorial Complex Estimation	Mycelial Extracts	Uninfected Epidermal Strips	Infected (42 days) Epidermal Strips
Myristic Acid	1.8%	3.1 ± 1.7%	-	-
Palmitic Acid	4.2%	13.1 ± 1.4%	21.8 ± 3.4%	22.0 ± 2.5%
Palmitoleic Acid	3.8%	3.3 ± 0.6%	0.5 ± 0.5%	0.9 ± 0.7%
Stearic Acid	8.0%	7.1 ± 0.8%	6.9 ± 1.4%	6.8 ± 0.8%
Oleic Acid	19.3%	12.3 ± 1.0%	4.2 ± 1.1%	3.7 ± 1.0%
Linoleic Acid	31.9%	25.7 ± 1.4%	40.5 ± 3.3%	13.2 ± 4.3%
Linolenic Acid	26.3%	21.0 ± 2.8%	28.1 ± 2.6%	53.3 ± 2.5%
Arachidic Acid	1.7%	4.0 ± 1.3%	-	-
Arachidonic Acid	3.6%	8.7 ± 0.7%	-	-
Lignoceric Acid	-	1.6 ± 0.6%	-	-

Table 45: The composition of the neutral lipid component of haustorial complexes, epidermal strips and mycelium

Neutral Lipid	Hautorial Complex Estimation	Mycelial Extracts	Uninfected Epidermal Strips	Infected (42 days) Epidermal Strips
Sterol esters	6.3%	14.6 ± 1.6%	26.7 ± 3.3%	16.0 ± 2.0%
Sterols	25.2%	28.0 ± 2.7%	15.5 ± 2.3%	15.1 ± 1.4%
Monoglycerides	3.8%	3.0 ± 1.1%	2.1 ± 1.0%	1.5 ± 0.9%
Diglycerides	5.2%	5.4 ± 1.7%	11.2 ± 2.5%	7.9 ± 1.8%
Triglycerides	38.6%	40.5 ± 4.1%	33.5 ± 4.6%	43.1 ± 1.3%
Free Fatty Acids	10.9%	8.3 ± 1.2%	10.0 ± 1.4%	16.1 ± 1.3%

The figures are the means of four replicates and are expressed with 95% confidence limits

5.6. Comparisons with other host parasite systems

Hoppe and Heitefuss (1974b) found that in leaf discs taken from Phaseolus vulgaris infected by Uromyces phaseoli there was a significant increase in the level of PS and PA and a decrease in the level of MGDG, DGDG and PG which began 4 days after infection and an increase in the unsaturation of fatty acids bound to PC, PE and PG in infected leaf halves between 4 and 8 days after inoculation which was more pronounced in PE. These differences were found in the region around the pustule and were not observed in the surrounding mycelium-free tissue. With the exception of the increase in the level of PS and PA these results are very similar to those found in the epidermal strips in this series of experiments.

The fact that no changes were observed in the mycelium free tissue in the leaves of Phaseolus vulgaris is paralleled in these experiments by the lack of equivalent changes in infected whole leaves. This shows that any effect of the pathogen on its host is confined to the immediate vicinity of the infection in both diseases.

Lösel and Lewis (1974) found that infection of Tussilago farfara by Puccinia poarum caused a general increase in the amount of lipid in leaves. Mature aecial pustules had more than 3 times the total lipid content of healthy parts of diseased plants. These authors found an increase in the amount of PC and PE corresponding to this increase and a significant increase in the level of PS, while all of the chloroplast lipids showed a decrease. This decrease in the level of chloroplast lipids is a consistent feature of rust diseases. Caution must be exercised when comparing the results from different syndromes. Firstly all three sets of experiments involved different hosts and pathogens, although the work of Hoppe and Heitefuss (1974) and Lösel and Lewis (1974) both involve rust diseases. Secondly this study specifically utilized epidermal strips whereas the two previous studies involved leaf segments. However despite this there are some interesting parallels between these studies.

The most important disparity between the present results and those of Lösel and Lewis (1974) was that in the present study there was no increase in the total amount of lipid upon infection. One possible explanation is

the difference in sampling methods, as discussed in Section 5.3.1 however it is unlikely that this would be responsible for masking a threefold change in lipid concentration as found by Lösel and Lewis (1974). More likely explanations are the proliferation of fungal membraneous tissues within the host and the frequency of lipid inclusion bodies associated with this rust infection. Whichever explanation is validated there is a distinct difference between the two diseases in this respect.

As far as neutral lipids are concerned in the present study only small changes were observed between the different classes in epidermal strips namely an increase in the amount of triglycerides and free fatty acids and a corresponding decrease in sterol esters, sterols and diglycerides. This change was thought to be caused by the presense of triglycerides and free fatty acids in osmiophilic structures but this has not been proved.

Hoppe and Heitefuss (1975b) separated out the sterol fraction into its constituents and found that two unknown additional sterols were present in infected bean leaves. These sterols are characteristic of germinated uredospores and it was thought that their presense in the extracts was due to the presense of fungal lipids. The presenCe of unusual sterols was not tested for in the present study.

Sadrina et al (1981) showed that the content of phospholipids and neutral lipids per unit membrane protein nitrogen increased following infection of roots by Rhizoctonia solani. They also found an increase in fatty acid saturation caused by infection in mitochondrial membranes which was paralleled by a decrease in the activity of mitochondrial enzymes. This is the opposite of what was found in the present experiments where the degree of unsaturation was increased by infection probably paralleled by an increase in the activity of the membrane enzymes, as found by Kotyk (1977).

As so few studies on diseased induced changes in lipid metabolism have been conducted it is difficult to make any generalisations. However if you just look at the biotrophic diseases, there does seem to be some consistency such as the loss of chloroplast lipids and the increase in unsaturation leading to increased membrane permeability.

The case of Rhizoctoria solani is interesting as it is a necrotrophic pathogen and the changes in the lipid composition are very different to those found in biotrophic diseases, which suggests that different and more dramatic changes occur to the lipids in the cells of plants infected by necrotrophic pathogens. However no general conclusions can be made as there is so little information is available on lipid metabolism in necrotrophic diseases.

5.7. Lipid composition in infected and uninfected pea plants (cv Onward) and plants kept in the dark

This study was undertaken to ascertain whether the changes in lipid composition found in diseased plants were caused by premature senescence. El Hussein (1977) has shown that in wheat plants prematurely senesced by darkening there is a significant increase in the level of fatty acid unsaturation in PC, PE and PI. Since similar changes were observed in the present study it was decided to examine the effect of senescence on the composition of pea epidermal lipids. Plants kept in the dark exhibited little change in the relative proportions of polar and neutral lipids and the percentage composition of the polar lipids was also unaltered. On the other hand the degree of unsaturation of the polar lipid-bound fatty acids increased substantially as a result of a substitution of linoleic by linolenic acids. This result paralleled that found for epidermal strips from infected plants except that it occurred earlier in dark incubated plants.

Thus the changes observed in polar lipid fatty acid structure in diseased plants are the same as those in plants undergoing artificially induced premature senescence. In other host-parasite combinations powdery mildew infection causes premature senescence (Finney, 1978) but the mechanism by which this is achieved is not known. Presumably the stress associated with supporting a parasitic symbiont induces premature ageing. It seems unlikely that the shading of the leaf by the mycelium could itself be responsible.

The changes in lipid composition recorded in this study most likely result from premature senescence and there is nothing to suggest that they

are connected with specific permeability changes or parasite nutrition. Indeed the pathogen becomes established and grows for a period of 14 days before any changes in the lipid fractions are detectable thus the parasite is able to obtain nutrients for growth and sporulation long before any changes to lipid composition are observed in the membrane and so the mechanism by which it is able to redirect the host metabolites must be sought elsewhere. There are a number of possibilities such as alterations in specific membrane proteins, enhancement of membrane permeability to certain metabolites, the establishment of a metabolic sink in which depletion of nutrients in the epidermal cells causes the passive diffusion of these metabolites from adjacent cells due to altered concentration gradients, and finally local changes in the lipid composition of the membrane resulting in increased metabolite diffusion without any corresponding observable change in membrane structure. A further point is the restricted surface area of host membrane directly in contact with the parasite, so that any parasitism-associated changes to membrane composition are likely to be heavily diluted and are probably undetectable using the methodology adopted here.

Lösel (1978) speculated in Poa pratensis infected by Puccinia poarum the oil droplets found in host cells around the infection sites constitute a concentrated sink of host photosynthate adjacent to the fungus which helps to maintain translocation gradients towards the site of infection. Similarly, fungal lipids represent a parasite sink for host assimilate in Poa poarum as has been demonstrated in Tussilago farfara (Lösel and Lewis, 1974).

The changes in the neutral lipid composition of dark incubated plants exactly duplicate those in infected plants except that they become statistically significant ($P \leq 0.5\%$) earlier. This contrasts with the work of Lösel and Lewis (1974) who found that the increases in triglycerides and free fatty acids were greater in infected plants. They also found that in senescent plants there was a decrease in chloroplast lipids but no increase in PC and PE which they found in infected tissue. Thus the two experiments seem to produce conflicting results. This is probably attributable to the different host-pathogen combinations under study.

So the conclusion from this part of the work is that the changes in fatty acid saturation in lipid bound fatty acids were a reflection of the premature senescence of the tissue caused by infection by E. pisi.

The progress of senescence was followed by measuring the chlorophyll, amino N and protein N content of stem epidermal strips. The results showed that there was a significant reduction in the protein-bound nitrogen in dark incubated plants, a significant increase in the amino acid nitrogen content in infected plants and a decrease in chlorophyll in both infected and dark incubated plants compared with the light grown healthy plants. The reduction in chlorophyll content was greater and occurred earlier in dark-incubated plants.

In the absence of infection there is only a small increase in the amino N content of the leaves which senesce while still attached to the plant (Finney, 1979). Finney (1979) reported a similar result in wheat infected by Erysiphe graminis. He suggested that it was caused by the retention of soluble nitrogen around the pustules. He thought that the increase in amino N was responsible for the maintenance of protein content in senescing diseased leaves since yellowing leaves retain the ability to synthesize protein. This agrees with the maintenance of protein content in infected but not darkened plants suggesting that the parasite is able to suppress these aspects of senescence which would be to its detriment.

Chlorophyll loss from epidermal cells of darkened plants was much more rapid than from infected plants. Therefore although infection by Erysiphe pisi does promote senescence, the process as a whole is less rapid than in darkened plants.

The results from these experiments suggests that infection causes a general acceleration of senescence and that the changes in the polar lipid fatty acid composition are a reflection of this rather than a change induced by the pathogen in order to obtain nutrients from the host. At present it is only possible to speculate as to how the pathogen alters the host's physiology in order to obtain nutrients as with the available methodology it is not possible to test for fine changes in membrane structure.

6. Conclusion

The most pertinent conclusion from these experiments is that infection by Erysiphe pisi has little effect on the lipid composition in pea plants. In fact up to 24 days after infection there is no change at all in the leaf lipids and the only changes that were observed in this study were a loss of chloroplast lipids, an increase in the unsaturation of the polar lipid-bound fatty acids and an alteration in the composition of the neutral lipid classes in pea stem epidermal cells. The changes that occurred were not restricted to any particular membrane.

These results demonstrate the high level of compatibility between the parasite and its host. It is in the nature of biotrophy that a successful parasite must coexist with its host, such that it is able to grow, sporulate and reinfect other host plants before the primary host dies. It is to the parasite's ecological and evolutionary advantage to cause as little cellular damage as possible to its host whilst still making use of the host's metabolites for its own purposes. The fact that Erysiphe pisi causes no change to the lipid composition of whole leaves and that the changes it causes to the lipid composition of the stem epidermal cells are small shows how it is a successful biotrophic parasite.

The original aim of the project was to study the lipid changes in diseased plants in order to obtain an insight into the mechanism of the infection process. The results from these experiments show that, while some changes in lipid structure do occur, they are a general response to infection rather than the means by which the fungus is able to obtain nutrients from the host. The changes in lipid composition in infected epidermis do not occur until the parasite is present on the host for 10 days, during which time it has grown and sporulated and has thus already been able to redirect the host metabolites. The changes in polar lipid-bound fatty acid saturation merely reflect the fact that the plants are undergoing premature senescence rather than changes more fundamental to the infection process. The fact that these changes in fatty acid saturation occur in every membrane fraction also suggests that this is a general response to infection. It is likely that this premature senescence leads to a general increase in the permeability of the membranes as is

found in bean leaves infected by Uromyces phaseoli (Hoppe and Heitefuss 1974a).

The other change that occurs to the lipid composition as a result of infection is an alteration in the composition of the neutral lipid classes, namely an increase in the amount of triglycerides and free fatty acids and a decrease in the amount of sterol esters, monoglycerides and diglycerides. It is possible that this is because of a redirection of the neutral lipids which are found in infected cells in mildewed peas. This can not be proved until either a method for isolating these structures from the cell can be devised or these lipids can be marked in some way for observation through an electron microscope. At present, however, these techniques are not available.

Having ascertained that the lipid changes in these experiments were the result of premature senescence of the tissue the question still remains as to how the parasite is able to redirect the host's metabolites for its own purposes. There are three possible explanations. Firstly that there is an alteration of specific membrane protein resulting in increased permeability to certain metabolites. It is the composition of the membrane proteins that is responsible for all active transport of metabolites across membranes and so alteration in these proteins would change the permeability characteristics of the membrane. This could be extremely difficult to measure as it might only need small changes in the membrane proteins to give this effect. The second explanation is that a metabolic sink in the shape of the parasite could provide a sufficient gradient to drive the movement of metabolites by diffusion alone. Finally smaller local changes could occur which would lead to increased metabolite^t diffusion either passively or actively by altering the environment in which the protein is acting. At present it is not possible to state whether any, all or none of these occur.

To investigate the mechanism by which membrane structure, in particular that of the extrahaustorial membrane is altered following infection, methods for separating the individual membranes will be needed. This in turn requires that each major membrane system be characterized and recognisable by some easily measured marker, ideally a

high catalytic activity enzyme. If this was possible it would enable a study of the different membranes, including fungal membranes and the extrahaustorial membrane, to be attempted. It would then be possible to see how each of the membranes was adapted to fulfill its function.

If this study was conducted in conjunction with a pulse chase radiolabelling experiment at an early stage in the infection process, it should be possible to see at what stage in the procedure the fungus was first able to obtain nutrients from the host and how this related to the presence and structure of particular membranes. In this way it might be possible to achieve some understanding of how the parasite obtains its nutrients from the host.

It would also be of interest to make a study of enzyme activity during the early stages of powdery mildew infection to attempt to detect which enzymes are responsible for any change in membrane structure which may occur. Obviously methods of extreme sensitivity would be essential for such an approach: immunocytochemistry probably offers the most fruitful approach. Various workers (Lumsden and Bateman, 1968, and Hoppe and Heitefuss, 1974b) have demonstrated increased phospholipase activity upon infection but this has not been related either to the breakdown of phospholipids or to nutrient transfer mechanisms. More work is necessary to ascertain whether localised increases in phospholipases have any affect on membrane permeability and nutrient transfer.

The other way in which membrane permeability can be altered in plant diseases is by toxins and it would be interesting to see whether E. pisi produces any such molecules. Toxins have an uncertain role in plant disease and on theoretical grounds it seems unlikely that any biotrophic pathogen would secrete general cellular toxins in anything other than very carefully controlled minimal quantities.

From the results of the present experiments it can be concluded that infection of pea plants by Erysiphe pisi causes premature senescence of the epidermal cells which is amongst other things revealed as a decrease in chloroplast lipids and an increase in unsaturation of polar lipid-bound fatty acids. This study does not demonstrate which changes in membrane

structure are necessary to permit nutrient transfer from host to pathogen. Further work based upon the lines suggested above should resolve this problem in the future.

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