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THE HYDROLYSIS AND SAFETY ASSESSMENT OF FOOD FLAVOURING ESTERS

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ABSTRACT

FACULTY OF MEDICINE HEALTH AND BIOLOGICAL SCIENCES
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by Neil Robert Buck

There are currently approximately 1800 food flavouring additives in common usage, many of which are ester-type compounds which may be hydrolysed to their parent alcohol and carboxylic acids following ingestion. The metabolism and toxicity of the majority of flavouring esters have not been investigated, and their presence in the food supply is accepted by regulatory bodies due partly to predictions concerning their hydrolytic metabolism. These predictions are that flavouring esters may be predicted to be rapidly and completely hydrolysed, and that data available upon the hydrolysis of a single member of a structurally related group of compounds may be used as indicative of the hydrolysis of the other compounds within the group. However, concern has been raised about the validity of making such predictions because few data are available upon the hydrolysis of xenobiotic esters, and the identity and activity of tissue esterases remain poorly characterised.

To facilitate the safety assessment process, a predictive model of flavouring ester hydrolysis has been constructed through an evaluation of the hydrolysis of 44 esters of monoterpene alcohols (citronellol, geraniol, nerol, linalool, and α-terpineol), cinnamyl alcohol, cinnamic acid and furfuryl alcohol. This was achieved by investigating the rates of hydrolysis in artificial gastrointestinal fluids, rat and human tissue homogenates and following single dose oral and intraperitoneal administration to rats. Substrate specificity was found to be similar in each system investigated and was due to defined structural criteria (which are numbered in order of decreasing importance):

1. Steric hindrance of nucleophilic attack by esterases on the ester bond, by hydrocarbon groups present on carbon atoms directly adjacent to the ester bond.
2. The presence of bulky and rigid groups.
3. Substrate specificity as a result of the linear length of the alcohol and carboxylic acid carbon chains.

The serine esterases of rat liver which hydrolysed selected flavouring esters were found to consist of 2 isoenzymes: one has low affinity but high stereoselectivity for (S)-linalyl esters; the other has high affinity but is not stereoselective for linalyl esters. The low affinity but not the high affinity isoenzyme was found to be highly sensitive to inhibition by phenylmethylsulfonyl fluoride.

The acid-catalysed reactions, absorption across rat intestine and metabolism of the economically important monoterpene alcohols were investigated as supporting studies. Linalool, geraniol and nerol undergo a series of structural isomerisations in acidic solution to form α-terpineol. In vitro studies with preparations of rat liver illustrated that linalool is readily oxidised by cytochrome P-450 and also conjugated with glucuronic acid. In vivo studies with rats demonstrated the rapid sequestration of lipophilic esters into adipose tissue and confirmed the glucuronic acid conjugation of citronellol, linalool and α-terpineol following the hydrolysis of their esters.

Overall these studies add increased confidence to the metabolic predictions necessary during the safety assessment of food flavouring esters.

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If anyone does ever read this looking for wisdom, note but these lines; long journeys take a long time so be reflective about where you find your quality of life (don't be fooled, this is the only thing that matters), take too many long journeys and you will have missed everything.

Abbreviations

ADI	Acceptable daily intake
AUC	Area under the curve
BEMA	British Essence Manufacturers' Association
BIBRA	British Industrial Biological Research Association
BNPP	Bis-(p-nitrophenol) phosphate
CHX/THN	Cyclohexane containing 75 μM 1,2,3,4-tetrahydronaphthalene
CNS	Central nervous system
DIPF	Diisopropylphosphofluoridate
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediamine tetraacetic acid
EFFA	European Flavour and Fragrance Association
EU	European Union
Expts.	Experiments
FAA	Food Additives Amendment
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration
FDIC	Food and Drinks Industries Council
FEMA	Flavor and Extract Manufacturers' Association
FEXPAN	Flavor and Extract Manufacturers' Association Expert Panel
FFDCA	Federal Food, Drug and Cosmetic Act 1958
GRAS	Generally Recognised as Safe
IFRA	International Fragrance Association
ILSI	International Life Science Institute
IOFI	International Organisation of the Flavour Industry
JECFA	Joint Expert Panel on Food Additives
Min	Minute
NAD	β-Nicotinamide adenine dinuncleotide
NADP	β-Nicotinamide adenine dinuncleotide phosphate
NDGA	Nordihydroguaiaretic acid
NOAEL	No observed adverse effect level
NTE	Neuropathy target esterase
OP	Organophosphate
PA	Picoamps
PADI	Possible average daily intake
PAPS	3'-Phosphoadenosine-5'-phosphosulphate
PLUM	Priority-low – usage minimum
PMSF	Phenylmethylsulphonyl fluoride
RIFM	Research Institute for Fragrance Materials
SCF	European Union Scientific Committee on Food
SLR	Scientific Literature Review
TIU	Toxicologically insignificant usage
Tris-HCl	(Tris[hydroxymethyl]-aminomethane hydrochloride)
Tris-Maleate	(Mono[tris(hydroxymethyl)aminomethane]maleate)
UDPGA	Uridine 5'-diphosphoglucuronic acid
WHO	World Health Organisation

Definitions

A-esterase

An esterase enzyme the catalytic mechanism of which does not operate via a mechanism involving the acylation of a serine residue present at the active site.

B-esterase

An esterase enzyme the catalytic mechanism of which operates via acylation and subsequent deacylation of a serine residue at the active site.

Consumption ratio

The ratio of the amount of a compound consumed as a result of it being a natural component of the diet, to the amount consumed as a result of its use as a food additive, when it is consumed by the same population over the same period of time.

Functional carbon atom

The carbon atom to which the functional group or ester bond is attached (for example, the functional alkyl carbon atom of an ester refers to the carbon atom of the alkyl moiety on which the ester bond is located).

Nature identical

A compound which is produced via an artificial synthetic process, but which is identical in structure to a compound which is produced via a metabolic process.

CHAPTER 1

HYDROLYTIC METABOLISM OF FOOD FLAVOURING ESTERS: REVIEW & STUDY RATIONALE

1.0 Hydrolytic Metabolism of Food Flavouring Esters: Review & Study Rationale

1.1 Introduction

Flavour is the main factor influencing the consumer choice of food, and one of the main issues confronting today's food industry is the development of new products for the consumer market[1]. A rapidly growing demand for pre-processed, low fat and other speciality products presents significant challenges for flavour technologists, however these trends also coexist with increasing public concern over the use of 'chemicals in the food supply' such as flavourings and other food additives.

A number of factors lend uniqueness to the safety evaluation and regulation of food flavouring compounds:

- The addition of flavouring additives is generally self-limiting. Flavours typically constitute between 10^{-8} to less than 10^{-14} % of food[2]. As such the consumption of flavouring compounds represent less than 0.01 % the consumption of all food additives[3; 4] (calculated on a *per capita* intake basis; see A.4.3.2 on exposure estimation).
- A large number of flavouring additives (approximately 1800) are currently in use, the annual quantities of production of most of which are very limited[5].
- Most of these compounds have been in use for a considerable period of time but have never been toxicologically tested for safety.
- The consumption of many flavouring compounds as a result of their presence as natural components in the diet, may be greater than their consumption as a result of their use as food additives[6; 7] (therefore, for many flavouring compounds the 'consumption ratio' is greater than 1; see A.4.3.2).
- The pace of flavour research and product development is accelerating creating an increasing number of new flavouring compounds.
- New types of use for established flavouring compounds are being increasingly applied.

Several organisations have undertaken safety evaluations of food flavouring additives, however the most prominent are the Expert Panel of the Flavor and Extract Manufacturers' Association (FEMA), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). FEMA is a trade association which represents the flavouring industry in the USA (the largest in the world), it has statutory regulatory powers and has traditionally assessed flavouring compounds under the general paradigm of '*evaluating for lack of evidence of hazard*'. JECFA is an international body of experts and serves an advisory role, it classically assesses food additives under the paradigm of '*evaluating for evidence of safety*'[8]. With the development by JECFA of a new food flavouring additive safety evaluation protocol[9], both of these programmes have recently become more harmonised. The European Union Scientific Committee on Food (SCF) is currently considering the safety evaluations of both organisations in it s production of a list of permitted food additives[10]. An examination of flavouring additive safety assessment and risk management is given in Appendix I.

Flavouring additives are present in foods at low levels, and due to the large number of flavouring compounds currently at market and their limited production, it would be an onerous burden upon producers and society if all compounds were required to undergo a full toxicological assessment for safety. As such, in-order to establish the safety of a flavouring compound in the absence of toxicological data, information may be used concerning history of usage, exposure and consumption pattern, structure, and predicted metabolism (the pathways and consequences of metabolism must be known or predictable). This has been the foundation of the procedures used by FEMA since the 1960s.

Many flavouring compounds are esters which may be hydrolysed to their component alcohols and acids by gastrointestinal fluids and microflora, the intestinal mucosa, the blood and/or the liver. In the absence of metabolic information, predictions are made upon the hydrolysis of the compounds and safety assessment is frequently based upon the known and presumed toxicology of the parent alkyl and acyl moieties. For groups of structurally related esters, such information upon one compound may be taken as being representative of the entire group. However, only a limited number of hydrolysis studies have been conducted concerning any compound undergoing

toxicological assessment for safety[11], and of the small number and generally poor quality hydrolysis studies concerning flavouring compounds, wide differences in hydrolytic fate between different esters are indicated (see 1.3).

Furthermore, as discussed below, it may be unwise to make general predictions concerning esterases because these enzymes, particularly the carboxylesterases, are very poorly characterised. Available information indicates that serine carboxylesterases and lipases may often be indistinguishable, and non-serine carboxylesterases may be associated with lipid metabolism and have an important role in the hydrolytic activity of the blood and liver. In addition, and somewhat speculatively, although a number of inducible isoenzymes have been described, the majority of constitutively expressed serine carboxylesterases in a number of tissues may be composed of just two independently expressed isoenzymes with distinct substrate specificity and enzymic characteristics.

In order to help remove uncertainty in the safety evaluation of flavouring esters and better protect the health of consumers, it is necessary to improve the characterisation of hydrolytic metabolism for these compounds, and as such build a more comprehensive model for use in future safety evaluations.

1.2 The Esterases

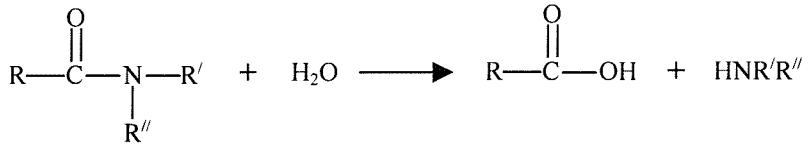
Many proteins capable of hydrolysing ester bonds are present in biological material of all kinds (micro-organisms, plants, invertebrates and vertebrates)[12], and in the higher vertebrates esterases have been identified in most tissues[13; 14]. These esterases catalyse the hydrolysis of a wide range of structurally diverse xenobiotics, such as medicines, pesticides, environmental chemicals, food additives, and endogenous substances[15]. Some isoenzymes serve a defined biological function, as indicated by their substrate specificity and distribution within organisms. However, most esterases have a wide substrate specificity indicating that they have broad biological functions. Enzymes which are known to hydrolyse ester linkages include:

- Proteases & peptidases
- Cholinesterases
- Lipases
- Carboxylesterases

Each of these enzymes are usually assayed by their ability to hydrolyse simple ester compounds[13]. By convention, only the latter two have commonly been referred to as esterases, although their physiological roles remain largely unknown. However, it is increasingly becoming apparent that the specificity of esterases depends more on the nature of R, R/, and R// than on the atom (O, S, or N) adjacent to the carboxyl group (see figure 1.1). Carboxylesterases will hydrolyse carboxythioester and carboxyamide linkages in addition to carboxylester groups (although carboxyamide groups are usually more stable than carboxylesters to enzymatic hydrolysis)[16], and thus amidases are also often referred to as esterases. This group of enzymes are major determinants of the pharmacokinetic behaviour of most xenobiotic agents containing ester, thioester or amide bonds[13; 15].

A. Carboxylester hydrolysis

B. Carboxyamide hydrolysis



C. Carboxythioester hydrolysis

Figure 1.1: Reactions of esterase-mediated hydrolysis. R, R' and R" = hydrocarbon chains.

The hydrolysis of esters may also occur *in vivo* via purely chemical processes: such as in acidic gastric fluids, or via interactions with other molecules. For example, human serum albumin exhibits esterase activity by binding the acyl leaving group, aspirin may be hydrolysed in this manner[14; 17]. Although the significance is uncertain, immunoglobulin molecules have also been shown capable of this effect[18]. The major determinant of the form and rate of such hydrolysis is the structure and inherent stability of the ester reactant. Whilst non-enzymatic hydrolysis may be relatively simple to predict, the actions of tissue esterases are not. It should be noted that in general the tissue carboxylesterases remain uncharacterised.

Due to the wide tissue distribution and lack of classification of esterases, in the following text a discussion is given of attempts to characterise the enzymes via substrate specificity and structure, prior to a discussion of their distribution and function. Available data on stereoselective substrate specificity has not been used to-date for classification purposes, but limited data does indicate the possibility of using stereoselective hydrolysis as an important probe to identify the tissue distributions of individual enzymes. As such a separate discussion of substrate specificity due to stereoselectivity precedes a discussion of esterase distribution.

1.2.1 Characterisation and Classification

1.2.1.1 Substrate Specificity

As a result of the wide substrate specificity of many esterases, their abundant distribution (see 1.2.4), and the apparent existence of multiple isoenzymes, debate continues regarding classification and nomenclature. In 1952 the late Professor Norman Aldridge identified, as a part of his PhD thesis, two types of esterases which he named A and B[19; 20]. Type A are not inhibited, and can hydrolyse, the organophosphate compound (OP) diethyl 4-nitrophenyl phosphate (a microsomal metabolite of parathion named paraoxon - up to concentrations of 10^{-3} M), whereas type B are extremely sensitive to paraoxon (being inhibited by concentrations as low as 10^{-8} M). However, classification has been complicated by the more recent finding that some B-esterases are not inhibited by OP compounds, and it has been suggested

that this may be as a result of the requirement for substrate negative charges for access to the catalytic centre. As such, the definition of A-esterases is now stated as those which hydrolyse uncharged esters and are not inhibited by OP compounds and other acylating inhibitors[12]. This is a large group of esterases which hydrolyse a diverse range of compounds[21].

The inhibition profiles of A- and B-esterases clearly suggests two distinct enzymatic mechanisms (see 1.2.2). Paraoxon binds so tightly to B-esterases that detoxication has very recently been demonstrated *in vitro* and *in vivo* in rats to be dependent upon both A- and B-esterases, in that following initial exposure and prior to their total inhibition, B-esterases may be the most important factor in protecting against paraoxon toxicity[22]. In 1957 a third class of esterase was identified, C-esterases which are neither inhibited nor can hydrolyse paraoxon[23], and the three classes have subsequently been shown to be selective for certain types of substrate (although none have been shown to be specific)[13]. Table 1.1 illustrates this simple classification scheme[24]. Although substrate specificity is now regarded as a poor indicator of enzyme identity, current literature may still classify arylesterase as a distinct form of A-esterase.

Nearly half a century following Aldridge's discoveries, little advance has been made in respect of the classification of carboxylesterases. Of the information which is available on the esterases / amidases, the data almost exclusively concerns the B-esterase class. However, over 80 % of B-esterases remain uncharacterised[25], very little is known of the A-esterases and the C-esterases remain entirely uncharacterised.

Esterase	Paraoxon	Preferred substrate
A-esterase	Substrate	Aromatic esters
B-esterase	Inhibitor	Aliphatic esters
C-esterase	No influence	Acetyl esters

Table 1.1: Simple esterase classification scheme[24]

Table 1.2 illustrates a current classification of the major groups of B-esterases based upon substrate specificity[13; 26]. Liver B-esterases are the most prominent group of all 'non-specific' ester-cleaving enzymes, however it is commonly believed that they can

be separated into isoenzymes that have different substrate specificity. Among the enzymes given in table 1.2, the carboxylesterases / amidases, cholinesterases, monoacylglycerol lipases and arylamidases are all closely related enzymes. Individual members of these groups are periodically found to be identical, thereby adding confusion to classification schemes. It is also often not clear what the main physiological substrates are, for example many lipases may in-fact be better classified as carboxylesterases (and *vice-versa*), while many cholinesterases may share less similarity with different cholinesterases than with other members of the B-esterase group. Mentlein *et al* have purified five serine esterases from rat liver microsomes; all of the enzymes had activity with simple aliphatic and aromatic esters in addition to hydrolysing endogenous compounds such as long-chain acyl-CoA, monoacyl glycerol, phospholipids, acyl carnitine, and retinyl palmitate[27]. Although individual Enzyme Commission numbers have been assigned to esterases (for example 'non-specific' serine-carboxylesterase is classified as EC 3.1.1.1), it is uncertain whether classifications represent individual enzymes.

Common name	Characteristic substrates	Remarks
Carboxylesterase / amidase	Simple aliphatic and aromatic esters, thioesters, aromatic amides	Heterogeneous group, many individual enzymes with differing specificity
Acetylcholinesterase	Acetylcholine	Specific, but will act on other esters and amides
Cholinesterase	Choline compounds	Assayed with butyrylcholine, otherwise same as carboxylesterase
Triacylglycerol lipase	Glycerol di- and tri-esters of long chain fatty acids	Not all lipases are B-esterases
Lysophospholipase	Lysophospholipids, simple aliphatic and aromatic esters	Microsomal enzymes, possibly identical to carboxylesterases
Monoacylglycerol lipase	Monoglycerides of long chain fatty acids, aromatic amides and simple aliphatic and aromatic esters	Possibly the physiological role of some of the 'unspecific' carboxylesterases
Dipeptidyl-aminopeptidase IV	Glycylprolyl-β-naphthylamide and other synthetic peptide derivatives	
Serine proteases	Proteins, also synthetic esters and amides	Individual well characterised enzymes
Carboxypeptidase	Peptides, also synthetic esters and amides	Only limited number are B-esterases
Formamidase	Formic acid esters and amides	
Arylacylamidase	Same as carboxylesterase	Not all members are serine hydrolases

Table 1.2: Classes of B-esterases based upon substrate specificity[13; 26]

The most extensively characterised B-esterases (besides proteases and peptidases) are the lipases and particularly the cholinesterases. Although some recent work has been undertaken, the major efforts to characterise esterases other than cholinesterases principally took place during the 1960s and 1970s, and were particularly directed toward liver and gastrointestinal enzymes. A discussion is given below of attempts to characterise the substrate specificity of hepatic esterases (in conjunction with limited relevant data concerning plasma and salivary esterases), followed by a discussion of pancreatic esterases.

1.2.1.1.1 Hepatic Esterases

Through differential inhibition studies at least seven carboxylesterase isoenzymes have now been found to occur in the liver[28; 29]. Early work upon the substrate specificity of liver esterases (both microsomal and cytosolic, usually from the horse) demonstrated that hydrophilic esters are poor substrates in comparison to hydrophobic compounds[16], and Hung *et al* have recently shown this effect with diflunisal and salicylic acid O-acyl esters using perfused *in situ* rat liver[30; 31]. Other work however, has indicated that the trend of increased substrate specificity with increasing lipophilicity may not hold true in all cases[32]. Interpreting such studies is complicated by the fact that substrate preference may possibly be more a factor of different structural motifs between substrates rather than lipophilicity *per se*.

Between 1973-1979 Junge *et al* attempted to characterise carboxylesterases[33-35], and showed by isoelectric focusing the presence of two groups of liver carboxylesterases which they termed H- and L-forms based upon their high and low molecular weights[26]. The H-forms were inactivated faster by paraoxon and phenylmethylsulfonyl fluoride and showed maximum reaction rates with simple methanol esters with acyl chain lengths C_3 to C_5 (in a series of C_1 to C_6), whereas the L-forms showed maximal rates with substrates of acyl chain length C_5 to C_6 (and were believed to be important in fatty acid metabolism). Under the experimental conditions employed, both H- and L-forms showed similar profiles in their affinities for the various substrates which ranged between K_M = 0.3 - 10 mM (whilst the V_{max} ranged between 150-800 μmol/min/mg protein in the case of the H-forms and 1-330 μmol/min/mg protein in the case of the L-forms). In a similar set of investigations with acetic acid esters with

alkyl chain lengths of C_1 to C_8 the K_M profiles were similar for both H- and L-forms and ranged between 0.03 - 1 mM (whilst the V_{max} ranged between 20-410 μmol/min/mg protein in the case of the H-forms and 10-90 μmol/min/mg protein in the case of the L-forms). It was additionally discovered in these investigations that the H-forms had up to a 5-fold faster turnover of *p*-nitrophenylacetate in comparison to the L-forms, and that the L-forms showed more amidase activity than the H-forms. A decade after this work Hosokawa *et al* identified three major carboxylesterase isoenzymes in rat liver microsomes[36] which showed distinct substrate specificities and regulation characteristics (see 1.2.6). Two were found to be low molecular weight (~61,000) and were termed RL1 and RL2, one was of higher molecular weight (~174,000) and was termed RH1. RL1 was shown to posses the highest activity toward *p*-nitrophenylacetate and long-chain acyl-CoA, RL2 was most active toward acetanilide and RH1 showed most activity toward butanilicaine.

Inhibition studies using soman have shown the existence of two major carboxylesterases in rat plasma[37], and more recently Morgan *et al* have identified, with the use of polyclonal antibodies, two liver microsomal B-esterases (from a variety of mammals) which together account for the liver's entire hydrolytic activity toward one of the now 'standard' carboxylesterase substrates *p*-nitrophenylacetate (see 1.2.4 on the distribution of these enzymes)[38; 39]. These enzymes have been designated hydrolases A and B (known henceforth as esterases Ba and Bb respectively), esterase Ba is inhibited more readily by phenylmethylsulfonyl fluoride and was shown to have high affinity (K_M ~25 μM) and esterase Bb low affinity (K_M ~400 μM) for *p*-nitrophenylacetate. Further differences in substrate specificity were observed in that esterase Bb, but not esterase Ba, has high amidase activity toward acetanilide, where as esterase Ba, but not esterase Bb, will catalyse the transesterification of cocaine (a methylester) to ethylcocaine[38; 39] (see 1.2.5 on transesterification).

Human salivary esterases have also been separated into two main groups of proteins by the isoelectric focusing technique[40], but it is important to note that the technique has since been shown to be extremely difficult to interpret in the case of carboxylesterases due to the formation of artefacts such as agglomerations of proteins

and carbohydrates[41]. As such it is possible that 'groups' of enzymes may in fact represent different physical forms of individual species. It is as such possible to suggest that the H form of rat liver esterase identified by Junge *et al*[26] is identical to the RL1 isoenzyme identified by Hosokawa *et al*[36] and the Ba isoenzyme identified by Morgan *et al*[39]. Similarly, the L form may be identical to the RL2 and Bb isoenzymes.

Irrespective of the tissue under study, with simple esters (up to acyl and alkyl carbon chain-lengths of 6), it has now been concluded that enzyme reactivity (V_{max}) is mainly governed by the structure of the acyl residue, whereas enzyme affinity (K_M) is not strongly influenced by the acyl group's structure, but rather, is dependent upon the structure of the alkyl group[41; 42]. In general, when comparisons are made of esterases in different species and tissues and with multiple types of ester substrate, V_{max} appears to be far more variable than K_M[43]. A review of the published literature reveals that the maximum values for V_{max} and K_M are found to be esterase-dependent[41], although it would appear that a chain length of 4 is the preferred size for both alkyl and acyl moieties[16]. Furthermore, esters of branched chain alcohols are found to be poorer substrates than compounds containing straight-chain alkyl residues[44]. In the case of cholinesterases, butyrylcholinesterases have been found to have a broad substrate specificity in respect of the size of the acyl group, whereas acetylcholinesterases show a marked reduction in catalysis between propionylcholine and butyrylcholine[42; 45].

1.2.1.1.2 Pancreatic Esterases

The best characterised 'non-specific' esterases in respect of substrate preference are porcine and human pancreatic lipases[44; 46]. The hydrolytic activity of the gastrointestinal tract is extremely important in determining the fate of many ingested compounds, and has a significant contribution to presystemic elimination[47-49]. Pancreatic fluid contains at least three lipolytic enzymes; cholesterol / carboxyl ester hydrolase, phopholipase A_2, and pancreatic lipase[35; 50-52]. Pancreatic lipase is a B-esterase that is unusual in that it appears to be at it s most active at the oil / water interfaces of micelles formed in the duodenum, where it is classically associated with the hydrolysis of triglycerides[53]. Adaptations for this function include an unusually large size with opposing hydrophobic and hydrophilic areas and free surface sulfhydryl moieties[54]. Enzymic structure is stabilised by bile salts, principally

taurocholates, which also aid in the formation of fat micelles. However, high concentrations of bile salts will inhibit the enzyme, and as such a small peptide co-factor is secreted by the pancreas which helps overcome this effect[55-58]. Two forms of pancreatic lipase have been isolated, L_A and L_B which have an almost complete specificity for the α-position of glycerides[44].

There have been extensive studies upon substrate specificity which have shown that pancreatic lipases have no stereoselectivity in respect to the hydrolysis of fatty acid esters[59] (see 1.2.3 on the stereoselectivity of esterases). Esters of primary alcohols are hydrolysed more readily than esters of secondary or tertiary alcohols, and the presence of electron withdrawing groups on the alkyl moiety have been shown to increase the rate of hydrolysis[60; 61]. In micellar experiments Mattson and Volpenhein found that the rate of hydrolyses of fatty acid esters increases as the alkyl chain length increases, and concluded that this effect was possibly due to substrate orientation at the oil / water interface[62]. It was also shown that chain branching at the first carbon atom of the alkyl moiety created severe steric hindrance to hydrolysis, much more so than branching at the second carbon position. This was subsequently also found to hold true in respect of studies upon the acyl fatty acid moiety[44; 63]. As predicted from studies upon other esterases, the structure of the acyl moiety however, has been found to have greater importance in determining catalytic rate than the alkyl moiety.

It is interesting to note that the product 'Olestra', developed by Procter and Gamble Inc. as a calorie-free cooking fat replacement consists of six hydrocarbon chains (hexa- hepta- and/or octa-) esterified to sucrose. The steric hindrance around individual ester bonds is such that the product is not hydrolysed in the intestine and is not absorbed[64-66].

Pancreatic lipase has no specific requirement for esters with fatty acid chains, and for a variety of substrates with different alkyl moieties there has been shown to be a clear trend in acyl chain preference[44; 63]. Acyl carbon chains containing double bonds are usually hydrolysed slower than corresponding chains without double bonds, an effect presumably due to chain rigidity and the ability to fit into the enzyme. Like many other esterases studied, butyric esters are hydrolysed substantially faster than the

lower or higher substrate in an homologous series of increasing acyl chain length, however increasingly longer chains show a gradual increase in reaction rate[67]. An exception however are the pentanoic acid esters which always appear to be particularly poor substrates, and similarly to the alkyl chain investigations, this has been explained as a function of the substrate's possible orientation at the oil / water interface[44]. Substrates with a acyl chain length of less than 5 may not present steric hindrance at the catalytic site, whereas substrates with a chain length of more than 5 may be sufficiently large to be bent away from the catalytic site back toward the oil phase of the interface.

1.2.1.2 Catalytic Site Composition

The large body of work upon insecticidal and chemical warfare agents has lead to the elucidation of the mechanism of OP and carbamate esterase inhibition. B-esterases are now commonly regarded as serine esterases, in which a serine moiety is acylated and subsequently hydrolysed in an acid catalysed hydrolytic mechanism (see 1.2.2). All cholinesterases belong to the serine B-esterase superfamily[42]. Microsomal liver and intestinal carboxylesterases have generally been found to be B-esterases[41]. Apart from glycosidases, all lipases (such as pancreatic lipases) hydrolyse ester or amide bonds and have been found almost entirely to be serine esterases[44] (it is important to note that besides the usual presence on lipases of a hydrophobic recognition site, no clear distinction between lipases and carboxylesterases currently exists[68]).

The enzymic mechanism of A-esterases is not currently known and no specific inhibitors are available. They are not thought to operate through an acylated serine intermediate. Table 1.3 summarises the most useful inhibitors of B-esterase activity currently available[12; 14; 18; 45; 69; 70].

Carboxylesterases from numerous sources have been found to be inhibited by nordihydroguaiaretic acid (NDGA) and related ligands (isolated from the creosote bush *Larrea divaricata*)[71]. This general inhibition may be due to the amphipathic character of NDGA, which allows the compound to accommodate to a variety of binding sites in numerous proteins. However, norisoguaiacin was found not to inhibit acetylcholinesterases (serine- or B-esterases) to the degree of a second unidentified

'highly-sensitive' esterase class (which included esterases from guinea pig kidney microsomes but not from the cytoplasmic fraction). Although very little information is available, there has been a recent suggestion that it may be possible to differentiate serine carboxylesterase isoenzymes by selective inhibition by known inhibitors of serine proteases such as PMSF[39]. It would be particularly interesting to investigate such possibilities.

Inhibitor	Inhibits
Boric acid Diisopropylphosphofluoridate (DIPF) Phenylmethylsulfonyl fluoride (PMSF)	Serine proteases and possibly many other serine esterases
Paraoxon	Serine esterases
Bis-(p-nitrophenol) phosphate (BNPP)	Serine carboxylesterases only
Physostigmine (Eserine)	Cholinesterase & slightly inhibitory to serine carboxylesterase
Mercuric chloride p-Chloromercurobenzoate	Enzymes with an –SH group (cysteine) at their active centre
Ethylenediamine tetraacetic acid (EDTA)	Enzymes requiring metal ions
Bis-(3-trimethyl-ammonium-5-hydroxyphenoxyl)-1,3 propane	Acetylcholinesterases only
10-(1-diethylamino propionyl) phenothiazine	Cholinesterases apart from acetylcholinesterases

Table 1.3: Commonly utilised esterase inhibitors[12; 14; 18; 45; 69; 70].

In general A-esterases often require metal ions such as calcium or magnesium, and interestingly the hydrolysis of long chain triacylglycerides by serine lipases is assisted by the presence of calcium ions which aid the removal of the long chain fatty acids as calcium salts[44]. A-esterases also appear to be sensitive to inhibition by metallic salts such as mercury, organomercury, nickel and copper which have an affinity for sulfhydryl groups[12; 22]. This suggests a similar mode of action to cysteine, calcium-activated cysteine, or metal proteases such as the well characterised papain, calpain or carboxypeptidase A respectively (for which inhibitors are well characterised)[72; 73]. It would be useful to assess the characteristics of A-esterases in relationship to proteases and peptidases, which themselves often possess hydrolytic activity toward simple ester and amide compounds (the majority of proteases / peptidases belong to one of four major classes: the serine proteases such as trypsin; zinc proteases which contain a divalent zinc ion at the active centre such as carboxypeptidase A; cysteine (thiol) proteases which contain a cysteine residue at the active centre such as papain; and the

acid proteases such as pepsin which contains two aspartate residues at the active centre, one of which is required to be ionised by a low pH for the enzyme to be active[18]).

Although evidence is lacking, there is some current speculation that the physiological role of A-esterases may generally be associated with lipid metabolism (for example, it could be suggested that perhaps the role of A-esterases is not hydrolysis but esterification to form fatty acid conjugates or mixed triglycerides). If esterases are to be further characterised, there exists a current pressing need to identify inhibitors of A-esterase activity. During the 1980s some effort was taken in identifying and investigating inhibitors of calcium-dependent and thiol proteinases[73-79], but studies have not examined the effects of similar compounds upon esterase activity.

1.2.1.3 Recent Progress

In the serine esterase family, two different codons are utilised in-order to code for serine[41; 42]. These two cannot be converted into each other by single nucleotide mutations, and thus it is likely that the evolutionary origins of the two are distinct. The active centre serine for most carboxylesterases is coded for by TCx, other serine esterases (for example thrombin, Factor IX, and plasminogen) posses a codon of the type AGx[41]. It is interesting to note that a single nucleotide change in either can lead to codons for threonine and cysteine respectively, and that a genetically related diverse family of proteins has now been discovered whose functional capacities extend beyond simple hydrolase activity. The amino acid sequence of cholinesterases do not show strong global homology with other serine hydrolases beyond the active centre, with the carboxy-terminal region being more closely related to thyroglobulin[42]. The relationship between A-, B- and C- esterases is currently unknown.

Recently, work concerning sequence homology and evolutionary relationships has been taken further to suggest a novel carboxylesterase classification scheme. Satoh and Hosokawa have created a phylogenetic tree for mammalian carboxylesterases in which it is suggested that 4 gene families exist (CES 1, CES 2, CES 3, and CES 4), with structural identities of more than 70 % within each subfamily[41]. This novel

classification scheme is hampered by the quite limited number of esterases which have been characterised.

The CES 1 family includes the isoenzymes which show more than 60 % homology with human carboxylesterase, and is divided into 3 subfamilies (CES 1A, 1B and 1C). CES 1A is further subdivided into CES 1A1, 1A2 and 1A3, CES 1A1 contains the major forms of human carboxylesterase. CES 1A2 contains the major forms of other mammalian carboxylesterases, and CES 1A3 contains egasyn (an accessory protein to β-glucuronidase). The CES 1B subfamily includes isoenzymes which are known to hydrolyse long-chain acyl-CoA esters, members of the 1C subfamily are similar but share a greater homology to human liver carboxylesterases and are secretory. The CES 2 family appear generally to be *N,O* acetyltransferases, the physiological function of members of family CES 3 is not known. The CES 4 family has the least homology with human liver carboxylesterase and contains isoenzymes which have a different general structure to other members of the superfamily.

1.2.2 Structure and Catalytic Mechanism

Mammalian liver, kidney and intestinal serine carboxylesterases appear to usually consist of trimeric units of molecular weight of approximately 60,000, and each unit has been found to have one active site[41]. Hosakawa *et al* has purified 10 carboxylesterases from the hepatic microsomes of a variety of mammals and shown that although their molecular weights vary little, their isoelectric points range from 4.7 to 6.5[28]. Furthermore, it was indicated that the isoenzymes were glycoproteins bearing a sugar moiety of the mannose type. Natsuka *et al* subsequently determined that one subunit of liver microsome A-type carboxylesterase isoenzyme contains one oligomannose-type sugar chain[80]. A number of mammalian hepatic carboxylesterases have since been shown to be glycosylated[39]. The glycosylation of cholinesterases has not been shown to effect catalytic parameters, but does increase thermal stability[42].

A number of carboxylesterase genes have been cloned and the amino acid sequence about the active site serine of several B-esterases has been studied[41]. Mammalian liver and kidney carboxylesterases have an active site sequence Gly-Glu-Ser-Ala-Gly, which is similar to that of mammalian serine proteases, and it would appear, the entire

serine hydrolase superfamily. Carboxylesterases have four cysteines which may be involved in specific disulphide bonds, these cysteines and a proposed catalytic triad (Ser, Glu, and His; see figure 1.2) are conserved in all mammals studied. The catalytic mechanism for B-esterases which has been proposed is based upon investigations involving site directed mutagenesis, and the body of work upon acetylcholinesterases. This body of work includes the solution to the three dimensional structure of the acetylcholinesterase from the electric organ of the fish *Torpedo californica*[81; 82].

Satoh and Hosokawa have isolated and studied the cDNA sequences of carboxylesterases from 10 animal species and found high homology between them all, particularly in respect of the active site region[83]. The structure of *Geotrichum* lipase has been shown to posses the same folding patterns and catalytic triad positional alignment as *Torpedo* acetylcholinesterase, and a common folding pattern is seen in the cholinesterase family[84]. Surprisingly, a serine carboxypeptidase from wheat, a dienelactone hydrolase from *Psuedomonas*, and a haloalkane dehalogenase from *Xanthobacter* also show the same folding pattern, despite the absence of sequence homology. The structures of these proteins have converged to contain not only similar folding patterns, but also the same catalytic triad[42]. It is believed that this arrangement increases the nucleophilicity of the catalytic serine and of the reactant water molecule[81] (see figure 1.2a and d).

Many esterases (particularly acetylcholinesterases) operate with their natural substrates at close to the maximum diffusion-limited rate[18] (they approach catalytic perfection) were k_{cat}/K_M is between 10^8 and 10^9 M^{-1} s^{-1}. It is interesting to note that in the case of cholinesterases access to the catalytic site appears to be via a predominantly hydrophobic and aromatic channel[82]. This would naturally facilitate the rapid movement of hydrophobic substrates and the more hydrophilic products between the external milieu and the catalytic centre. Perhaps the variation seen in K_M when the carbon chain-length of the substrate's alkyl residue is altered, is in-part due to interaction with this hydrophobic channel. There is continued speculation over the purpose of the peripheral anionic site in cholinesterases, it would appear that acetylcholinesterases may be inhibited by physical obstruction of the catalytic gorge by compounds which bind to the anionic site[42]. It is believed that the site may, for example, facilitate substrate transfer down the gorge, or may act as a sensor to

maintain constant catalytic rates over a range of ionic strengths. Pancreatic lipase is known to posses a free peripheral sulfhydryl group near the entrance to the catalytic site[44]; perhaps this moiety has a function additional to it's presumed role in aiding the orientation of the protein at the oil / water interface.

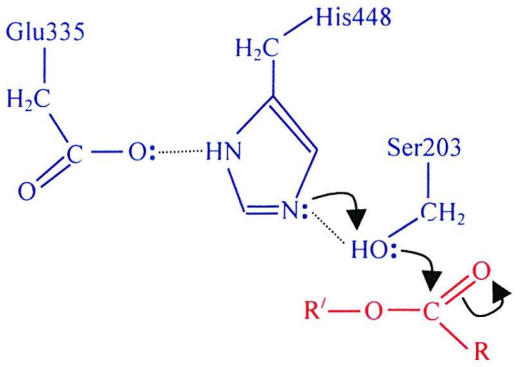
Figure 1.2 shows the proposed catalytic mechanism of serine esterases, based mainly upon information available for acetylcholinesterases[41; 42; 45], where low-barrier hydrogen bonds facilitate the acylation of Ser203. Although the catalytic mechanisms of other B-esterases have not been fully elucidated, in the case of pancreatic carboxyl ester hydrolase (the physiological function of which is probably as a lipase), it has been illustrated that an acylation / deacylation mechanism is employed[85-88].

In the proposed mechanism of figure 1.2, a proton is transferred to His448 from Ser203 (figure 1.2a) and then transferred to the acyl carbonyl group of the substrate (figure 1.2b). The mechanism is facilitated, during the first tetrahedral intermediate stage, by low-barrier hydrogen bonds between the tetrahedral oxyanion and peptide N-H bonds of Gly123 and Gly124 (figure 1.2b). This sufficiently stabilises the oxyanion intermediate to enable progression of the catalytic mechanism. During both tetrahedral intermediate stages, low-barrier hydrogen bonds between His448 and Glu335 stabilise the transition states (figures 1.2b and 1.2e). Formation of the acyl-enzyme complex requires removal of a proton from His448 (figure 1.2b), and the tetrahedral intermediate is disrupted (figure 1.2c). Once the unbound reaction product (the alcohol) has diffused away, a deacylation step occurs that is essentially the reverse of the acylation step, and a water molecule substitutes for the alcohol group of the original substrate (figures 1.2c to 1.2f).

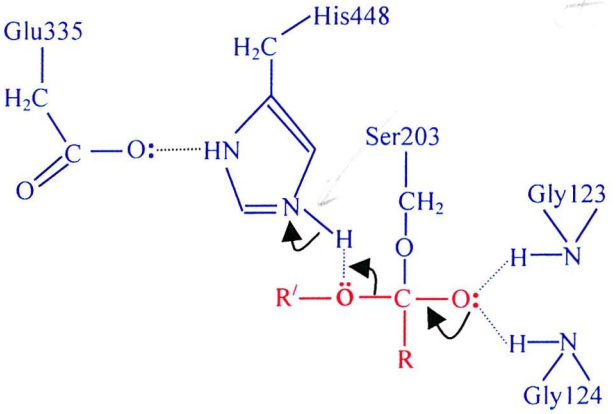
In the presence of acceptors other than water (for example, alcohols) carboxylesterases can act as trans-acylation or esterisation catalysts[13] (a feature currently being researched toward exploitation by the chemical and biotechnology industries[89]). As a high concentration of acceptor is usually required, such trans-acylation is not generally thought to serve a physiological role. However, recent findings indicate that transacylation by 'esterases' may have important implications in the processing of lipid and cholesterol esters[90] (see 1.2.5).

Figure 1.2: Proposed catalytic mechanism of B-esterases[41; 42; 45]. Curved arrows represent the movement of electrons. Enzyme = blue, ester = red, water = black.

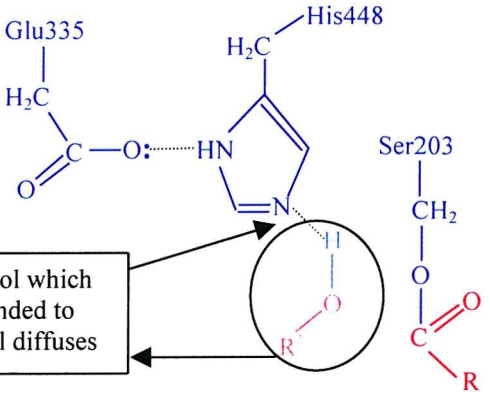
1.2a: Nucleophilic attack upon the substrate



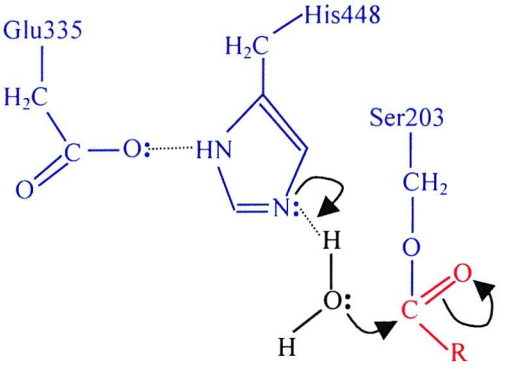
1.2b: First tetrahedral intermediate



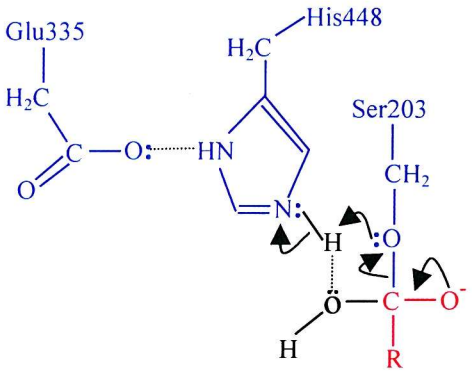
1.2c: Acyl-enzyme complex



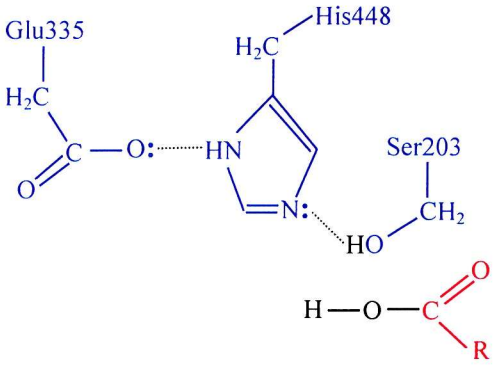
1.2d: Nucleophilic attack by H_2O



1.2e: Second tetrahedral intermediate



1.2f: Catalysis complete



Although the catalytic mechanism of A-esterases has not been identified, the involvement of an acylated cysteine and a displacement reaction involving an activated water molecule is possible. Of particular interest, is the finding that cytosolic aldehyde dehydrogenase, which requires NAD^+ for dehydrogenase activity and operates through an acylated cysteine intermediate, has NAD^+-independent hydrolytic activity with a range of ester substrates[91].

It is interesting to speculate that differences observed in V_{max} for B-esterases, as a result of varying the carbon chain-length of the acyl moiety (see 1.2.1.1), may be partly due to stabilising effects upon the tetrahedral intermediate stages of the catalytic mechanism. It has also been proposed that differences in the amino acid sequence directly around the catalytic triad may account for some degree of substrate specificity. In support of this, Satoh and Hosokawa have identified a cysteine residue, which is not involved in a di-sulphide bond, adjacent to Glu335 in carboxylesterases which show a particular affinity toward long-chain acyl-CoA esters[41]. Interestingly,

lipases also possess a free sulfhydryl group close to the catalytic centre[44], however, any association between these findings and substrate specificity remains contentious (and adding confusion, it should be remembered that the difference between lipases and carboxylesterases is not distinct).

The simplest enzyme kinetics are governed by three constants which are specific for substrate and reaction conditions, these are represented for B-esterases in figure 1.3.

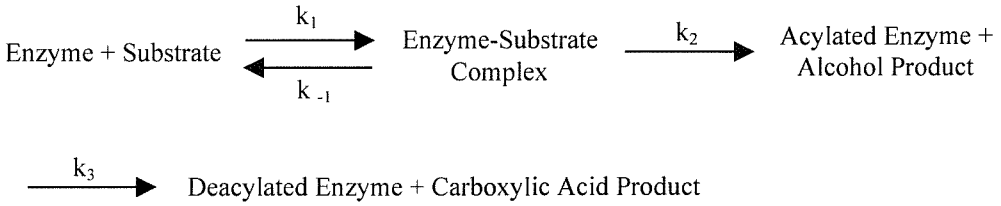


Figure 1.3: B-esterase kinetic constants

Evidence suggests that the acylation and deacylation steps in the catalytic mechanism of cholinesterases occur at comparable rates at V_{max}[42], which indicates that k_2 and k_3 are of comparable magnitude. Both together are more rapid than the essentially diffusion-determined rate limiting step of k_1. It should be noted that for B-esterases K_M, as determined from a Lineweaver-Burk plot, may not directly indicate the strength of enzyme-substrate binding as k_{-1} may not be greater than k_2+k_3 (note the hydrophobic channel discussed above), but represents the substrate concentration which results in $\frac{1}{2}k_{cat}$ (half the active sites are filled).

The inhibition of B-esterases by OP compounds is due to the reversible or permanent phosphorylation of the catalytic serine. Unlike the acylated enzyme intermediate illustrated in figure 1.2c, the more strongly bonded and structurally distinct phosphoryl group on serine is hydrolysed slowly, if at all, and so the catalytic site is blocked. Thus the k_3 step of figure 1.3 becomes rate limiting. Decarbamoylation is also the rate limiting step following inhibition with carbamate compounds, however k_3 is faster than with OP inhibition. The OP-inhibited phosphorylated enzyme may become more permanently inhibited if a group attached to the phosphorus atom by a phospho-ether bond is lost, in a process known as ageing. The ageing of sarin (nerve

gas) inhibited esterases for example, produces a charge which perturbates the active site. Ageing may occur by a purely chemical process, or may be facilitated by the hydrolytic action of the enzyme itself[92].

1.2.3 Stereoselectivity

Little is known of the stereoselective substrate specificity of esterases. Stereoselectivity may however prove to be valuable in examining the structure and catalytic mechanism of the enzymes. Furthermore, available data indicates that in the future stereoselectivity may enable a better knowledge of the tissue distribution of esterase isoenzymes.

Current interest in stereoselective processes is increasing mainly due to the development of new chiral chemistry techniques, which are enabling the investigation of stereoselective enzymic processes and eutomer / distomer drug mixtures. Apart from isomerases, biological stereoselectivity is determined predominantly by steric effects concerned with substrate / enzyme interactions, and may occur during active absorption, distribution, metabolism (phase I and II) and elimination of xenobiotics. For lipophilic esters stereoselective effects would not be anticipated during the absorption process. As discussed above, plasma binding proteins such as albumin, have been shown to possess esterolytic activity. A distinct catalytic site associated with this activity has been demonstrated in the case of human serum albumin which has further been shown to elicit stereoselectivity for the glucuronic acid conjugate of (R)-ketoprofen[93] and a number of other drug metabolites[94]. Little information is available concerning stereoselective active renal tubule secretion and reabsorption of esters or their metabolites[94]. In the case of enantiomeric ester compounds, the most important stereoselective processes are likely (if at all) to occur during phase I reactions (particularly enzymic hydrolysis) and conjugation (assuming that the chiral centre is maintained following phase I metabolism). Despite early investigations with ethyl esters of racemic mixtures of amino acids which did not indicate stereoselective hydrolysis[16], the limited data currently available generally does indicate the possibility of esterase stereoselectivity.

It is likely that stereoselective hydrolysis by esterases may be possible only when the centre of chirality is adjacent to the ester group interacting with the catalytic site of the enzyme. An exception to this would appear to occur in compounds with *cis / trans* isomerisation; for example in 1973 the hydrolysis of certain pyrethroid insecticides was found to be stereoselective for the *trans* isomer[95]. The double carbon bond producing the isomerisation is a feature present upon the unusual tetramethylcyclopropane acyl moiety some 3 carbon atoms distant from the ester bond.

Stereoselective hydrolysis of optically active compounds by carboxylesterases has since been indicated by studies of the hydrolysis of cocaine[96], atropine[94], 3-hydroxy-3-methyl alkanoic acid[97], esmolol[98], ester prodrugs of propranolol[99; 100], ibuprofen and flurbiprofen[101]. Stereoisomers of organophosphorus compounds have been shown to be hydrolysed at different rates by A-esterases[102; 103].

Yang *et al* have conducted some incisive work with oxazepam 3-acetate, and reported that tissue-specific enantiomeric differences are observed between esterases in rat (male Sprague-Dawley) intestinal mucosa cytosol, rat and human hepatic microsomes and rat brain S9 fraction[104; 105]. Interestingly, in the tissues studied it was demonstrated that A- and B- esterases often illustrated distinct enantiomer hydrolytic selectivity for the substrate[104], and that the separate stereoisomers of oxazepam 3-acetate could either inhibit or stimulate the hydrolysis of each other in a tissue-specific manner[105]. During the same period, Srinivas *et al* investigated the enantioselective hydrolysis of methylphenidate by the blood of seven mammalian species. Enantioselective differences were observed between the different species, and interestingly, opposite hydrolytic stereoselectivity was observed between human plasma and red blood cells[106]. These investigations are the only reports of such differences, and are clear indicators to the importance of such studies for the better characterisation of esterases toward understanding hydrolytic processes.

Some circumstantial evidence concerning chiral effects also exists; stereoselective hydrolysis of the monoterpene food flavouring compounds linalyl acetate and α-terpinyl acetate has been demonstrated by esterases from bacterial and yeast

sources[107; 108]. It is clear that a great deal of work remains to be undertaken to understand the processes involved, and the implications of hydrolytic stereoselectivity.

1.2.4 Distribution

1.2.4.1 Tissue Distribution and Cellular Localisation

Compounded by the scarcity of information regarding function, there continues to be debate concerning precise esterase localisation and the number of isoenzymes commonly found in, for example, the endoplasmic reticulum and cytosol of many tissues. Data upon age and sex differences are particularly lacking, and despite the considerable efforts of Hosokawa and Satoh in particular[15; 28; 36; 41; 83; 109], little distribution information remains known.

B-esterases have been demonstrated in almost all mammalian tissues with the highest concentration of esterase activity toward simple aliphatic and aromatic substrates localised in the liver[41]. By electron microscopic autoradiography of liver slices, the bulk of B-esterases have been found to be localised in hepatocytes[13], where 60-80 % of carboxylesterase activity is localised to the microsomal fraction[26] (it is estimated that approximately 3 % of total rat liver microsomal protein is comprised of esterases[13; 38]). B-esterases are also found in: serum, macrophages, monocytes, red blood cells, muscle, the brain and the central nervous system (particularly endothelial cells of the blood / brain barrier system), the kidneys, small intestinal epithelial cells and other areas of the gastro-intestinal tract (including the saliva and other digestive fluids), the lung tissue, the testis, and the adipose tissue[13; 15; 17; 40; 41; 110-117].

Morgan *et al* have identified two serine hydrolases with high and low affinity toward *p*-nitrophenylacetate (termed Ba and Bb respectively, as discussed in 1.2.1.1.1) which are independently expressed in a wide variety of extrahepatic tissues in rats, and were found in all mammals studied[38; 39]. Interestingly, esterases Ba and Bb were found to comprise approximately 1.5 and 0.5 % of total rat liver microsomal protein respectively. Additionally, esterase Ba was found in high concentration in rat testicular microsomes and esterase Bb was found in high concentration in kidney

microsomes. Esterases Ba and Bb have been sequenced and cloned by Morgan *et al* and shown to represent distinct enzymes which share 70 % amino acid sequence identity. It is clear from the distribution and quantity of these two microsomal enzymes that they are possibly extremely important in determining the esterolytic activity of several tissues. This is despite the facts that the investigations of Morgan *et al* utilised a limited range of substrates, and that additional esterases, with both high and low affinity for *p*-nitrophenylacetate have been identified in various tissues, which are not esterases Ba or Bb.

Non-serine A-esterases have similarly been found in numerous tissues, notably including the lipoprotein fraction of sheep blood and human blood; lending some suggestion of their possible function as lipases[12]. Approximately 5 % of liver homogenate esterase activity is due to A-esterases[26], the carboxylesterases of liver lysosomes contribute little to the general esterolytic activity of the liver, however, they have been found to be A-esterases[13; 41].

Following cell fractionation by differential centrifugation, the carboxylesterase / amidase activities of the liver, kidney, and brain were found predominantly in the microsomal and mitochondrial fraction, although some cytoplasmic activity was also detected[13; 26; 118]. Robbi and Beaufay have purified four carboxylesterases and identified their presence on the luminal side of the endoplasmic reticulum (ER)[119-121].

It is currently believed that a number of serum esterases originate in the liver. In support of this a number of reports have demonstrated the presence or absence of the tetrapeptide carboxy-terminal ER retention sequence in hepatocyte esterases, which are respectively either microsomal or secretory[120-124]. Blood esterases have been found to not posses the ER retention sequence[123; 124], and it seems clear that some esterases have this consensus sequence, whilst others do not. Kroetz *et al* in 1993 elucidated the cDNA sequence for two human liver carboxylesterases and demonstrated the predicted carboxy-terminal ER retention sequences[125].

1.2.4.2 Inter- and Intra-Species Variation

Studies have shown that despite wide substrate specificity, hydrolysis activity varies significantly between mammalian species, and hydrolysis rates do not parallel each other. For example, the methyl ester group of cocaine is hydrolysed in human serum but not rat serum[126], atropine (tropic acid ester) is toxic to human beings whilst rabbits are able to hydrolyse and inactivate it[127], phenacetin-hydrolysing activity is much higher in pigs than it is in rabbits, but the relationship is inverse for the hydrolysis of tributyrin[13]. The relationship of A- to B-esterase activity is also different between species, as demonstrated by Aldridge in 1953 who showed that for *p*-nitrophenyl esters in serum, A-esterase activity is predominant in rabbits, B-esterase activity predominates in rats and the activities of both are similar in horses[19].

Hosokawa *et al* have compared liver carboxylesterase activity from a number of species, and found that total activity, probably as a result of isoenzyme concentrations, in the rat most closely resembles that of human liver samples[28]. However, studies by McCracken *et al* upon insecticidal substrates have determined that overall esterase activity is higher in the rat than in the human[69; 128], and work upon an homologous series of 30 α- and β-naphthyl esters has indicated that rat liver esterase activity is approximately five times that of the mouse or human[32]. Luttrell *et al* have demonstrated wide differences in the rate of meperidine hydrolysis by B-esterases in hepatic microsomes, with activity increasing in the order; guinea pig<human<mouse<rat<dog[129]. However, Aungst *et al* have investigated the hydrolysis of esters of nalbuphine and found that rat whole liver homogenate is 1.8 times more active than dog whole liver homogenate in hydrolysing the anthranilate ester. Furthermore, plasma was found to have activity toward the acetylsalicylate ester in the order; human<dog<monkey<rat, with at least a 120 fold difference existing between rat and human plasma[130]. However, Morgan *et al* have demonstrated using serum at 25°C that the rate of hydrolysis of 2.4 mM acetylsalicylic acid increases in the order; dog<rabbit≤cat≤human<rat [and additionally that the rate of hydrolysis (k) in serum from the 18 human subjects varied by a standard deviation of 0.028 around a mean of 0.047 minutes^{-1}][131]. Also notable was the finding that in the case of the substrate employed by McCracken *et al* (fluazifop-butyl) plasma carboxylesterase

activity (V_{max}) in the rat was 66 times higher than in humans[69; 128]. These data have clear implications particularly in respect to 'first-pass' effects toward the extrapolation of animal metabolic data for human safety assessment purposes. Although inter-species variation is highly dependent on the ester substrate investigated, the rat would appear to be a poor model of human hydrolytic metabolism.

In a recent review of drugs which are metabolised predominantly (70-100 %) via hydrolysis (cocaine, heroin, aspirin, etomidate, esmolol, fosprinolol, remifentanol, and flumazenil), the differences between human and animal clearance values were calculated. In all cases animal clearance was more rapid than human clearance. The dog was found to be the best model with a value of between 1-3 fold difference to human clearance, the rabbit had a value of 2-4 fold difference, and the rat had a value of 10-12 fold difference[132].

Species differences identified in the hydrolysis of cinnamyl anthranilate are of interest from the perspective of the safety assessment of food flavouring compounds. In a chronic carcinogenicity bioassay cinnamyl anthranilate was hepatocarcinogenic in mice (and was withdrawn from market[133])[134], and was subsequently found to be a mouse-specific peroxisome proliferator[135-137]. Further *in vivo* and *in vitro* studies identified that the hydrolysis (and inactivation) of cinnamyl anthranilate decreases in the order; rat $\approx$ human > mouse[138].

Species differences in the susceptibility of xenobiotics to hydrolysis not only have potential implications with respect to the toxicity of esters, but are clearly also important in determining pathways of metabolism. The profile of up to 30 different hepatic microsomal metabolites of the calcium antagonist mibefradil has been shown to be dependent on hydrolytic susceptibility. Mibefradil has been shown to be readily hydrolysed by the hepatic microsomes of cynomolgus monkeys and rabbits, is less readily hydrolysed by human microsomes and is poorly hydrolysed by rat and marmoset microsomes[139]. Furthermore, the oral administration to rats, monkeys (*Macaca fascicularis*) and humans of the nonsteroidal anti-inflammatory drug aceclofenac resulted in the excretion of metabolites indicative of hydrolysis in the rat, simultaneous hydrolysis and oxidation in the monkey, and oxidation only in humans[140].

A number of differences between rat and human plasma have been identified: human plasma appears to contain more cholinesterase activity than rat plasma; and human plasma contains more A-esterase activity than rat plasma, and at a level approximately proportional to human liver microsomes[69; 128]. It must however be emphasised that these investigations only examined a limited range of substrates, and although esterase substrate specificity is very wide, effects due to isoenzyme specificity should not be discounted.

In general, it would appear that inter-species plasma esterase activity is more variable than hepatic activity. This could be correlated to the observations that carboxylesterases in human and rat plasma appear to be quite distinct proteins (with different molecular weights and sub-unit composition)[141; 142], the ratio of A- to B-esterases in serum vary widely between species[19], enantioselectivity has been shown to differ between mammals[106] (as discussed in 1.2.3 above), and a high level of sequence homology exists between hepatic carboxylesterases in mammalian species[26; 38]. Limited evidence concerning substrate specificity and electrophoretic behaviour, indicates that intestinal mucosa esterase differs significantly between humans and mammalian experimental animals[143].

Nutritional status has been demonstrated to have a significant impact upon esterase activity. Digestive enzymes are known to adapt to the diet[144] and rats undergoing severe protein malnutrition show decreased levels of all pancreatic enzyme activities except phospholipase A2 and carboxyl ester hydrolase[145]. Furthermore, the quantity of intestinal mucosa esterase has been shown to increase in rats fed a high-fat diet, and to decrease in rats fed a fat-free diet or fasted[143]. Food intake has been shown to have a significant impact upon the bioavailability and presystemic clearance of many oral drugs[146; 147], as have age differences, disease-states[148] and the composition of the gut microflora[149-151]. All of these are factors which may have an influence upon the fate of xenobiotic esters. Furthermore, sex differences are likely as several esterases appear to be under the influence of hormonal regulation (see 1.2.6.2). A study by Lund-Pero *et al* has recently shown significant variation (18.4 fold) between 16 women in mammary non-specific steroidal esterase activity, in which there was a positive correlation between the esterase activity and age[116].

Human pancreatic lipase has been shown to appear in the 21st week of pregnancy and to increase substantially at birth[152]. The level of carboxylesterases in guinea pig foetuses is very low, but begins to increase at 8 days prior to birth[153; 154]. Male and female rats have low levels until weaning when levels increase dramatically until puberty[38; 155].

Young rats have been found to be more sensitive to OP actylcholinesterase inhibitors than older animals, and this has been correlated to the lower liver and plasma esterase activity found in young rats when compared to mature animals[155]. In adult but not young rats, gender related differences are seen in that female animals appear as susceptible to OP toxicity as adolescent animals of either sex[15] (female animals generally show lower liver and plasma esterase activity than males, but a number of substrates have been reported to be more readily hydrolysed by female rat plasma and liver[156]). It is known that cytochrome P-450 enzymes are important in both the activation and the deactivation of many OP compounds. As young and adult female rats generally demonstrate less active liver metabolism than adult males, it has been suggested that the cause of the observed increase in OP susceptibility is due to low deactivation[155]. However, it has also been shown that activation is lower in young and adult female rats than adult male animals[157], thus microsomal cytochrome P-450 mediated activation / deactivation probably does not play a role in the documented age and gender related differences[69]. Such differences are probably more readily explained when viewed with regard to the very recent findings that the amount of peripheral esterase activity is critical in determining the level of paraoxon-induced toxicity in rats[158], and that both B-esterases and A-esterases are important in detoxifying paraoxon (via binding to B-esterases and hydrolysis by A-esterases)[22].

In case reports of human anticholinesterase poisonings (mainly comprised of carbamate poisoning incidents) children display more central nervous system depression than do adults[155]. Assuming that most of the age-related sensitivity in rats is due to the developmental profile of esterases, and humans possess less esterase activity than rats, it is possible that humans, particularly children, may be more sensitive to the toxicity of esterase inhibitors than rats, and that age-related toxicity differences may not be as exaggerated as in the rat.

Very little information is available concerning the genetic polymorphisms of carboxylesterases, however differences in the enzymic kinetics of purified esterases from several human livers have been reported[159], and polymorphism of plasma esterases has been reported in dogs[160]. Interestingly, the gene for carboxyl ester hydrolase of pancreatic fluid is located at a hypervariable region at the most distal part of the long arm of human chromosome 9[161]. Important recent work has identified allozymes of the human non-serine esterase known as paraoxonase, and identified that polymorphisms at amino acid position 192 (where arginine or glutamine may be found) affects the enzyme's catalytic properties[162]. Although much work remains, a number of attempts have been made to correlate the neurological symptoms of Gulf War veterans and farmers (who may have been exposed to organophosphate acetylcholinesterase inhibitors) with paraoxonase genotypes[163; 164].

1.2.5 Function

Although their physiological functions are almost entirely unknown, esterases will catalyse the hydrolysis of endogenous compounds such as short- and long-chain acyl-glycerols, long-chain acylcarnitine, and long-chain acyl-CoA esters[41]. The hydrolysis of fatty acid esters and steroids has been demonstrated in both rat pancreas[165] and kidney[166]. The A-esterase known as 'paraoxonase' appears often to be associated with the fractions of plasma which contain lipoprotein[12], and has been shown to be lowered in patients undergoing myocardial infarction[167; 168]. Enzymes capable of the hydrolytic metabolism of the fat soluble micronutrient vitamin A have been found in several tissues[169-171]. Interestingly, an enzyme from porcine liver which when expressed in Chinese hamster ovary cells illustrated high affinity for acyl coenzyme A:cholesterol acyltransferase activity, has been found to be identical to porcine and human liver carboxylesterase[90]. This may be an important suggestion that 'esterases' are important in activities other than ester hydrolysis.

The recent finding that the level of peripheral esterase activity is crucial in determining susceptibility to paraoxon-induced toxicity[158], indicates the possible importance of widely distributed non-specific esterases in protecting against toxic esters. Supporting this is that a serine esterase identical to a liver microsomal

carboxylesterase has been found to be expressed in human lung macrophages[172]. It is possible to predict that further esterase roles exist in respect of the processing of intra- and inter-cellular compounds. For example, histological staining for 'non-specific esterase' has long been recognised as a marker of phagocytic cells, and esterases are vital to the functioning of the innate immune system. Maki *et al* have determined changes in the profiles of three major microsomal rat liver carboxylesterases (which they had previously named RH1, RL1 and RL2[36], see 1.2.1.1.1) following partial hepatectomy and hepatocarcinogenesis, and suggested that RL2 is involved in the regulation of protein kinase C activity by metabolising its activators[173].

Esterases have important functions in the central nervous system, for example rat brain has been shown to contain amidases possibly related to acetylcholinesterases which are inhibited by serotonin and related compounds[174]. Interestingly, certain OP compounds such as tri-orthocresyl phosphate cause a delayed degenerative neuropathy through inhibition of 'neuropathy target esterase' (NTE)[92; 175; 176]. It is currently believed that many phosphate, phosphorothioate and to a lesser degree carbamate compounds would cause the condition if sufficient *in vivo* concentrations could be reached before the onset of severe acute toxicity. The precise function of NTE remains to be elucidated[12], however it is clear that it holds a crucial role in the maintenance of large diameter neuronal cell axons, and it has been suggested that it is an ion channel in the endoplasmic reticulum[25].

1.2.5.1 Pharmacological and Toxicological Aspects

Apart from acetylcholinesterases and proteases, the esterases remain poorly characterised, and investigations into the effects of inhibitors *in vivo* have yielded no clear evidence for physiological function. However, studies using animal models suggest that the bioactivation of xenobiotics is catalysed primarily by esterases and other phase I metabolising enzymes. As it would appear that carboxylesterase isoenzymes have broadly overlapping substrate specificities, competitive esterase inhibition may be of clinical significance in combined drug therapies. Furthermore, hydrolysis may also compete with other detoxication reactions[14]. In most cases the hydrolysis of an ester or amide bond in a toxic compound leads to detoxification because of increased hydrophilicity and accelerated excretion[13]. Instances where

hydrolysis results in an increase in toxicity include the action of liver carboxylesterases upon phenacetin[177], N-acyl anilides such as the herbicide propanil[178], and the food flavouring allyl esters[179].

The action of many carboxylester and carboxyamide drugs and xenobiotics is terminated partly by enzymic hydrolysis[14; 26; 111; 180; 181]. The most studied examples include procaine[182], aspirin[183], caramiphen[184], propanidid[185], steroid hormone esters[186], atropine (liver atropinesterase is present in some, but not all, rabbit strains)[187], pyrethroid insecticides (esterases contribute significantly to the development of insect resistance to OP, carbamate and pyrethroid insecticides)[95], lactones[188], acetanilide[189], lidocaine[190], butanilcaine[191], N-acetylated sulfonamide[192], N-2-fluorenylacetamide[193], chloramphenicol and isocarboxazid[26], candoxatrilat[194], and acetylcarbromal[16]. Hydrolytic metabolism may account for significant first-pass elimination for a number of drugs, for example pethidine[195; 196], and drugs which are metabolised predominantly (70-100 %) via de-esterification include cocaine, aspirin, etomidate, esmolol, fosprinolol, remifentanol, and flumazenil[197]. The amoebicidal drug diloxanide furoate is largely hydrolysed (and inactivated) in the intestinal lumen and mucosa[198]. The pharmacological effects of suxamethonium (a depolarising muscle relaxant) are limited by rapid hydrolysis to choline by plasma esterases[14]. In 1960 J.C. Stoddart demonstrated the important relationship between plasma cholinesterase activity and the dose of suxamethonium required for muscle relaxation[199].

Although the enzyme systems involved have not been characterised, some drugs may only become active following hydrolysis. Examples include heroin[200], lovastatin[201], oxazepam[202], carbimazole[14], capecitabine[203], irinotecan[204] and clofibrate[182]. Prodrug esters have been specifically designed to provide improved pharmacological properties. For example, esterification of bitolterol with two 4-methylbenzoic acid residues not only prolongs bronchodilatory action, but also reduces cardiovascular side effects[205], and the slowly hydrolysed dipivalyl esters of adrenaline have been successfully used to treat glaucoma[206].

Prodrug esters may be hydrolysed in the intestinal mucosa, liver, serum, and locally at their site of action to either limit or prolong pharmacological effects[207]. Normally,

esterification with small aliphatic or aromatic carboxylic acids, such as with ampicillin and sulfonamides, reduces polarity and therefore promotes absorption through the intestinal wall[17; 208], and in the case of aspirin may additionally reduce local irritancy effects[209]. Esterification may also be employed to protect against the acidic pH of the stomach, as is the case with erythromycin-like compounds[17]. Drugs which exhibit high first-pass conjugative metabolism and low bioavailability such as nalbuphine and other opioids, may be esterified with charged phenoic groups such as anthranilate which protect from conjugation in the intestines and liver. The fraction of prodrug which enters the systemic circulation unhydrolysed and unconjugated may subsequently undergo hydrolysis and activation[130]. The absorption of topicocorticosteroids into the skin is enhanced when applied as steroid esters, which are subsequently hydrolysed, and activated, following absorption[210]. Lipophilic prodrug esters may also be administered by intramuscular injection in oil, which serves as a slow-release reservoir the contents of which are activated upon release by local esterases. Hormones and antipsychotics have been administered in this manner[211].

Although very little information is available, a few attempts have been made to determine and systematise the hydrolysis rates of ester prodrugs by varying the size of acyl residues (it is generally held that an increase in size results in a decrease in hydrolysis rate)[17; 212]. Furthermore, certain ester drugs show a high degree of stability in human plasma, possibly due to steric effects. For example atropine is stable in human plasma, however it is not stable in rabbit plasma[14; 127] (see 1.2.4.2 on inter-species variation).

A field of current ester prodrug research is that of targeting drugs to esterases present at particular sites. With the use of prodrugs which are hydrolysed by known isoenzymes, it may become possible to target chemotherapeutic compounds to specific sites and produce, for example, localised cytotoxicity[213].

1.2.6 Regulation

1.2.6.1 Transcriptional Regulation

The regulatory mechanisms of carboxylesterase expression remains unclear, however the 5′ flanking region of a human liver carboxylesterase has been cloned[214], and the sequence contains potential binding sites for GATA-1, CTF site, sterol-dependent transcription factor NF-1, sterol regulatory element, and macrophage and B-cell specific factors PU1, and Sp1. However, no upstream TATA-box was identified which suggests that the gene is regulated by a TATA-less promoter or some other novel mechanism. Recently, Langmann *et al* have reported that transcription factor Sp1 will mediate differentiation-induced expression of a carboxylesterase gene in the monocyte leukaemia cell line THP-1[215].

1.2.6.2 Hormonal Regulation

The response of esterase activity / synthesis to hormones appears to be dependent upon the hormone, tissue, and individual esterase, and there may be a complex hormonal influence on specific esterases[37]. For example, insulin and glucagon have an opposite effect on the B-esterases of rat liver, insulin increases and glucagon decreases the amount of microsomal liver esterases[216]. In addition quantitative differences between sexes in rats in the esterolytic activity of the liver, along with distinct isoenzyme patterns, have been reported (see 1.2.4.2 on intra-species differences).

The kidneys of mice contain several testosterone-dependent esterases[217], and an oestrogen-dependent posthparin esterase has been demonstrated in human serum[218]. In males the latter esterase is expressed at a relatively high level and is independent of age, whereas a lower expression level in women increases with age. Oestrogen has been shown to have an inhibitory effect upon a number of hepatic carboxylesterases[36], and castrated male rats have been shown to possess lower levels of carboxylesterases in comparison to control animals[219]. The three isoenzymes (RL1, RL2 and RH1) purified by Hosokawa *et al* from rat liver microsomes (see 1.2.1.1.1) were found to

exhibit sex differences, RL1, which is highest in male rats, was found to be particularly testosterone-dependent[109].

Certain hepatic carboxylesterase isoenzymes appear to be regulated, in part, by pituitary hormones. β-Endorphin has been shown to decrease activity in male rats[220]. Hypophysectomy and thiouracil feeding in both male and female rats has been found to effect carboxylesterases in a substrate specific manner (for example, in male rats malathion hydrolase activity decreased whilst isocarboxazid hydrolase activity increased)[37; 221]. Hypophysectomised rats have also been shown to have reduced plasma lipoprotein lipase activity, which is however up-regulated along with hepatic lipases by growth hormone[222]. Furthermore, the activity of a lipase identified in rat skeletal muscle has been found to be increased in response to adrenaline[223], and adipose tissue lipoprotein lipase activity in human males has been shown to be suppressed by testosterone[224].

1.2.6.3 Enzyme Induction / Suppression

Hepatic microsomal and cytosolic esterases have been shown to be inducible by treatment with chemicals known to induce other xenobiotic-metabolising enzymes. Compounds which have been studied extensively as inducers of cytochrome P-450 isoenzymes also induce carboxylesterases[109; 225-229]. The administration of phenobarbital has been shown to induce hepatic microsomal carboxylesterases (and protect from the toxicity of procaine in rats)[230], but does not appear to induce intestinal mucosa esterase[143]. The induction of hepatocyte cytosolic acyl-CoA hydrolase activity has been demonstrated with clofibrate[231]. Although there is a scarcity of studies in the field, peroxisome proliferators, which exhibit a wide diversity of chemical structures, also appear to increase the quantities of carboxylesterase isoenzymes[109; 232].

Confusing the general findings of esterase induction by well documented cytochrome P-450 inducing compounds, Morgan *et al* have examined the induction and inhibition of esterases Ba and Bb (see 1.2.1.1.1 on these isoenzymes), which may be major contributors to general rat hepatic microsomal hydrolytic activity (assuming that the

enzymes possess wide substrate specificity). Treatment of mature male animals with 11 known microsomal enzyme inducers caused little or no induction, with only clofibrate causing a moderate increase in liver esterase Ba and Bb activity (30-35 %). Treatment with β-naphthoflavone, pregnenolone-16α-carbonitrile or dexamethasone suppressed the levels of both enzymes[38]. Although the lack of induction could possibly be partly attributed to the minimal period of pre-treatment (4 days in all cases), phenobarbitone and dexamethasone treatment did cause the induction of at least one esterase which was not previously constitutively expressed. As such it could be postulated that a limited number of hepatic microsomal esterases are constitutively expressed which are considerably less inducible than a number of further esterases which are not constitutively expressed. The available evidence further suggests that the ability to express additional esterases is more limited in the intestinal mucosa than it is in the liver.

Limited evidence exists for induction by esterase substrates such as the combined chronic administration of enalapril / diltiazem (daily administration of up to 6.25 / 75 mg/kg/day for 27 weeks)[156]. As discussed in 1.2.4.2, nutrition has an effect upon digestive enzymes, and in 1974 Machovich demonstrated that pancreatic esterases in 4-8 day old rats are inducible by repeated daily intragastric administration of sesame oil[233]. This may have implications with regard to the interpretation of *in vivo* studies of ester compounds which are administered in this manner.

Carboxylesterases have been shown to be inhibited by a variety of compounds. Suppression of serum aspirin esterase activity has been observed in women with habitual aspirin intake[234], and disulfiram treatment of rats has been shown to inhibit hepatic microsomal and plasma activity[235]. As is often the case with inhibitors of cytochrome P-450 isoenzymes, esterase inhibitors may in-fact also subsequently lead to induction, the OP compounds tetrachlorvinphos and soman have been shown to result in both outcomes[236; 237].

1.3 Hydrolytic Metabolism of Food Flavouring Esters

There exists little data on the hydrolytic metabolism of flavouring esters, furthermore of the small number of studies presented below, most have not been published or subject to peer review.

1.3.1 Hydrolysis by Artificial Gastrointestinal Fluids

In the 1960s - 1970s the flavour industry sought to investigate the presystemic metabolism of flavouring esters using artificial gastrointestinal fluid models prepared either according to Pharmacopoeia Helvetica VI, United States Pharmacopoeia XVI or similar formulations (with incubations at 37°C). The only protein contained in artificial gastric fluid was pepsin, artificial pancreatic fluid contained porcine pancreatin (dried acetone precipitate of whole pancreas aqueous homogenate).

Table 1.4 summarises the data available in industrial and published reports for the purpose of comparing hydrolysis rates within individual studies (methodologies varied between studies). Further contemporary work included a number of reports upon more limited ranges (1-2) of flavouring esters[238-242]. Epoxy flavouring esters have been shown to be poorly hydrolysed in artificial gastric and pancreatic fluids (based upon the investigation of 4 compounds)[243], and the degree of ring-opening of flavouring lactones in artificial pancreatic fluid has been shown to be substrate specific and highly variable (4 lactones showed between 20-90 % ring opening following 60 minutes incubation, γ-undecalactone and dibutyl butyrolactone were the least and most hydrolysed substrates respectively)[244].

It is possible only to suggest limited associations between hydrolysis rates and the structures of the substrates investigated (see below) due to the range of unrelated compounds studied. However, large variations were seen in the rates of hydrolysis of different substrates. From the limited range of flavouring esters investigated, hydrolysis rates within individual studies varied by at least 30 times in the case of artificial gastric fluid experiments, and by at least 700 times in the case of artificial pancreatic fluid experiments. It should be noted that no account was made in these

investigations of hydrolytic effects due to the acidity of artificial gastric fluid in comparison to possible enzymic hydrolysis by that medium.

Report (Number of Esters Studied)	Hydrolysis in Artificial Gastric Fluid	Hydrolysis in Artificial Pancreatic Fluid
Gangolli and Shilling 1968 (9 esters)	$t_{½}$ = 2.9 to 99 minutes rapid = ethyl nonanoate slow = methyl anthranilate	$t_{½}$ = 0.09 to 69 minutes rapid = ethyl butyrate slow = methyl anthranilate
Leegwater and van Straten 1974, and published by Grundschober 1977 (26 esters)	-	In 2 hours 0 – 100 % hydrolysis rapid = isoamyl furylpropionate slow = dimethylbenzylcarbinyl acetate
Longland *et al* 1977 (16 esters)	$t_{½}$ = 150 to 6000 minutes rapid = isoamyl caproate slow = methyl anthranilate	$t_{½}$ = 2 to 4200 minutes rapid = allyl caproate slow = methyl anthranilate

Table 1.4: Summarised data on ester hydrolysis in artificial gastrointestinal fluids. Rapid = most rapidly hydrolysed substrate in the series investigated, slow = least rapidly hydrolysed substrate in the series investigated[245-248].

An increasing carbon number of the carboxylic acid component did not appear to influence the rate of hydrolysis of ethyl esters in artificial gastric fluid, at least up to ethyl laurate (ethyl dodecanoate). Esters of acetic acid appeared to be hydrolysed by artificial pancreatic fluid more slowly than esters of higher straight chain aliphatic acids. Branched chain aliphatic esters differed considerably in their rates of hydrolysis in artificial pancreatic fluid, some were hydrolysed as fast as straight chained esters, others more slowly.

1.3.2 Hydrolysis by Tissue Preparations

Two studies have examined the hydrolytic metabolism of flavouring esters in liver and intestinal homogenates. In both cases this was combined with investigations into hydrolysis in artificial gastrointestinal fluids, in order to define the most appropriate hydrolytic model for human safety assessment purposes. Rat liver and intestinal homogenates were found to have greater hydrolytic activity towards the substrates investigated than artificial pancreatic fluid. The liver and intestinal homogenate concentrations and incubation methodologies were not consistent between studies,

however table 1.5 illustrates that within individual studies there was substantial variation in the hydrolysis rates of different esters.

Report (Number of Esters Studied)	Hydrolysis in Rat Liver Homogenate	Hydrolysis in Rat Intestinal Homogenate
Longland *et al* 1977 (8 esters)	10 % whole homogenate. $t_{½}$ = 0.04 to 1600 minutes rapid = benzyl isobutyrate slow = methyl anthranilate	10 % mucosal homogenate. $t_{½}$ = 0.07 to 148 minutes rapid = benzyl isobutyrate slow = methyl anthranilate
Leegwater and van Straten 1974, and published by Grundschober 1977 (3 esters in liver homogenate expts. 8 esters in intestinal homogenate expts.)	Enzyme preparation from 10 % whole homogenate. In 2 hours, 20 to 100 % hydrolysis rapid = methyl-*N*-methylanthranilate slow = dimethylbenzylcarbinyl acetate	Enzyme preparation from 0.4 % jejunum homogenate. In 2 hours, 0 to 100 % hydrolysis rapid = iso-amyl acetate slow = dimethylbenzylcarbinyl acetate

Table 1.5: Summarised data on ester hydrolysis in rat liver and intestinal preparations. Rapid = most rapidly hydrolysed substrate in the series investigated, slow = least rapidly hydrolysed substrate in the series investigated[247; 248].

The degree of ring-opening of 3 flavouring lactones during incubations in 4 % rat liver homogenates has been studied; between 32 and 93 % ring opening occurred following a 1 hour incubation period (γ-undecalactone and γ-valerolactone were the least and most hydrolysed substrates respectively)[249].

1.3.3 In Vivo Hydrolysis Studies

During the safety evaluation of food flavouring esters, if predictions of extensive hydrolytic metabolism are accurate it would be anticipated that available data would indicate similar toxicity profiles for the esters as for their parent alcohol / carboxylic acid moieties. However, due to observations of the lack of distinct toxicologies, available data does not directly support hydrolytic predictions. For example in the case of simple esters of monoterpenoid alcohols; in summary linalyl and terpinyl esters have very low acute toxicities[250] as does linalool and α-terpineol[251-254]. Sub-chronic or chronic toxicity tests have been undertaken with linalyl, geranyl, terpinyl

and citronellyl acetates[255-257], and a sub-chronic bioassay of linalool and citronellol has been reported[255], in all cases no evident toxicity was identified.

If predictions of extensive hydrolytic metabolism of esters are accurate, following the administration of ester substrates hydrolytic products should be detected or metabolites should be produced which are the same as those identified following the administration of the parent alcohol / carboxylic acid. For example, in an early study (1948) by Paul *et al*, the glycine conjugate of furoic acid was detected in the urine of rats collected for 6 hours after a single oral dose (20 mg) of furfuryl alcohol or furfuryl propionate[258]. However, there is a general lack of metabolic data following the administration of esters substrates, except principally in the case of esters of cinnamyl alcohol / cinnamic acid.

The absorption and metabolism of 3 flavouring esters (methyl cinnamate[259], cinnamyl anthranilate[136], and propyl anthranilate[260]) have been investigated *in vivo*, and 4 compounds [iso-amyl-3-(2'-furyl) propionate, allyl phenylacetate, methyl-*N*-methyl anthranilate and dimethyl benzyl carbinyl acetate] have been assessed for intestinal absorption in the guinea pig[261].

The absorption, distribution and metabolism of methyl cinnamate was investigated in rats (female Birmingham Wistar, 250-400 g) and rabbits (female White New Zealand, 3-4 kg) following administration by stomach tube (0.62 mmoles in rats, 3.1 mmoles in rabbits)[259]. In rats the ester was rapidly absorbed (50 % disappearance from the gut within 10-15 minutes), at no time not more than 9 % of the ester was detected as it's hydrolysis products in the stomach, and not more than 5 % of the dose was detected in the lower part of the gut. No ester was detected in the peripheral blood of dosed rabbits or rats, and only traces were detected in portal and heart blood samples in dosed rats. Importantly, over a period of 24 hours following the oral administration to rats, hippuric acid (66 % of the dose) and benzoylglucuronide (5 % of the dose) were excreted in the urine. This distribution of metabolites is nearly identical to that obtained following dosing with cinnamic acid, and indicates that hydrolysis of the ester precedes metabolism of the acid[259].

Data are available concerning the hydrolytic metabolism of cinnamyl anthranilate which, as described in 1.2.4.2 has been investigated due to the high incidence of hepatocellular carcinoma found in mice (but not rats) treated with high doses (up to 30,000 ppm in the diet)[262], and it's reported proliferation effects on mouse hepatic peroxisomes (the compound has been subsequently withdrawn from use as a food flavouring additive). At low doses in rodents, cinnamyl anthranilate was hydrolysed to cinnamyl alcohol and anthranilic acid, however, urinary excretion of the intact ester was reported after intraperitoneal injection into male Fischer 344 mice of doses at and above 20 mg/kg body weight and following the dietary administration to male and female $B6C3F_1$ mice of doses at and above 100 mg/kg body weight. A human volunteer study (males 22-35 years old, 65-95 kg, non-smokers) using a single oral dose of 250 mg failed to detect any intact ester in the urine[136].

Analysis of the gut contents of rats (female Birmingham Wistar, 250-400 g) at intervals after administration of propyl anthranilate (up to 3.2 mmol/kg body weight) via stomach tube suggested that the ester is rapidly absorbed from the stomach, with less than 7 % detected in the small intestine at any time point (up to a maximum of 24 hours). Unchanged propyl anthranilate was readily detected in the blood (up to 0.5 μmol/ml 240 minutes following a dose of 2.9 mmol/kg body weight). Furthermore, following a dose of 2.9 mmol/kg body weight in the rat and rabbit (female White New Zealand, 3-4 kg) 9 % and 5.3 % of the dose respectively was found to be excreted unchanged in the urine[260].

An operative procedure upon live guinea pigs has been employed to assess the absorption and subsequent portal blood levels of four flavouring esters following direct intestinal administration[261]. Concentrations of substrates were prepared in saline solution, and 5 ml/kg body weight was administered. 70 ppm iso-amyl-3-(2'-furyl) propionate, 270 ppm allyl phenylacetate and 25 ppm methyl-*N*-methyl anthranilate in saline solution were completely absorbed from the intestinal lumen in less than 30 minutes and free ester was not detected in the blood. Following the introduction into the intestinal lumen of as little as 13 ppm dimethyl benzyl carbinyl acetate in saline solution, intact ester was detected in blood samples.

1.3.4 Analysis and Summary of Investigations into the Hydrolysis and Absorption of Flavouring Esters

In summary, flavouring esters have generally been shown to be hydrolysed relatively slowly by artificial gastric fluids, therefore absorption of unhydrolysed esters across the stomach would be expected. If straight chain aliphatic esters reach the intestine intact they are likely to be hydrolysed rapidly in pancreatic fluid or by the intestinal mucosa (all the members of this group of esters which have been tested have shown half-lives of less than 10 minutes in artificial pancreatic fluid, and were rapidly hydrolysed by homogenates of intestinal tissue). Straight chain aliphatic esters also appear to be hydrolysed rapidly by liver homogenates, and as such it may be appropriate to base safety assessments upon the alcohol and acid hydrolysis products. Acetates may be an exception however, as they tended to be hydrolysed more slowly than other straight chained aliphatic esters. Branched chain and aromatic esters generally appear to be more poorly hydrolysed than straight chained esters. In these cases, it may not be as appropriate to base safety evaluations upon the parent alcohols and acids.

It would appear from the experimental data that the absorption of flavouring esters across gastrointestinal surfaces is rapid when the substrates are introduced in a liquid media, and that rates of hydrolysis during or subsequent to absorption vary substantially between substrates. Although data are lacking, it would be anticipated that the order of lipophilicity of the compounds employed in the *in vivo* oral administration studies[136; 259; 263] would be methyl cinnamate>propyl anthranilate>cinnamyl anthranilate. The results indicate that methyl cinnamate is rapidly absorbed, and subsequently rapidly hydrolysed by the gut mucosa, blood or the liver. Propyl anthranilate would appear, similarly to methyl cinnamate, to be rapidly absorbed from the stomach, however, it was not as rapidly hydrolysed during or subsequent to absorption. The rate of stomach absorption of cinnamyl anthranilate is not ascertainable from the published data. The dose levels at which intact ester was found in the urine of mice are substantially higher than the levels expected from normal estimated weekly human exposure (prior to withdrawal from market)[3]. However, studies with cinnamyl anthranilate raise questions concerning the possible saturability of esterase activity, in addition to potential esterase species differences

(see 1.2.4.2), and consequently the relevance of extrapolating high dose animal studies to assess the safety of human exposure to esters. It is interesting to hypothesize that cinnamyl anthranilate may be a poorer substrate for B-esterases than it is for A-esterases which have historically been associated with arylesterase activity.

It is important to note that flavouring esters may be highly lipophilic, and in all of the above studies absorption across intestinal surfaces was shown to be rapid. As such, excluding the possibility of retention in the gut due to sequestration in lipid food material, it is possible that flavouring esters are often absorbed across the stomach and avoid hydrolysis by pancreatic fluid or the intestinal mucosa. This gives increased importance to the hydrolytic activity of the blood and liver. There is a paucity of information concerning the hydrolytic fate of ingested esters, and a lack of characterisation of esterase enzymes (see 1.2). A considerable quantity of work is required to provide greater confidence in the safety evaluation of ester food flavouring additives.

1.4 Study Rationale and Selection of Compounds

Currently, there are approximately 1800 food flavouring additives in common usage and esters of carboxylic acids and alcohols are frequently used as artificial or nature-identical flavourings. However there exists little toxicological data concerning the food flavouring compounds currently at market. In-order to help establish the safety of food flavouring esters in the absence of toxicological data, information may be used concerning the predicted pathways and consequences of metabolism. If a presumption is made that rapid and complete presystemic hydrolysis occurs (or indeed if some relevant hydrolytic data is available), safety evaluations may be based upon the available metabolic and toxicological data for the carboxylic acid and alcohol hydrolysis products (for an account of the safety assessment and risk management of flavouring additives see Appendix I). The safety evaluation of food flavouring ester compounds is frequently based upon the assumptions that:

- Hydrolytic metabolism may be predicted with reasonable certainty.
- Knowledge or assumptions concerning the hydrolytic metabolism of one of a number of structurally related esters, may be treated as representative of the entire group.

These assumptions may not be accurate as esterases, particularly the carboxylesterases are poorly characterised (see 1.2), and from the limited available data (see 1.3) the diverse group of esters which are utilised as food flavouring additives may undergo substantially different rates of enzymic hydrolysis.

The recently implemented JECFA decision tree protocol for food flavouring additives (see Appendix I A.5.1) requires an answer to the question 'can the substance be predicted to be metabolised to innocuous products ?'. This clearly requires information concerning hydrolysis in the case of flavouring esters, and an affirmative response could only be based upon assumptions for which little supporting data is available.

To investigate this uncertainty in current safety evaluation procedures, an objective was to construct a predictive model of ester hydrolysis. For this purpose a number of flavouring esters were selected for study based upon investigating the effects upon hydrolysis of four structural criteria:

- The effect of steric hindrance directly around the ester bond.
- The effects of acyl and alkyl moiety branched and aromatic carbon chains.
- The effects of acyl and alkyl moiety carbon chain lengths.
- The effects of acyl and alkyl moiety unsaturation.

Attempts would also be made to characterise the esterase enzymes hydrolysing selected compounds with the use of acylating inhibitors and by examining stereoselectivity.

It was considered that investigations with commonly used flavouring additives (which could be obtained without significant difficulty) would be preferred to investigations

with substances of limited use. Furthermore, if possible, it was decided that a series of flavouring compounds should be selected for which forthcoming safety evaluations were to rely in-part upon predictions concerning hydrolytic metabolism.

The selection of compounds for study was based upon investigating the structural criteria outlined above. The compounds selected were esters of monoterpenoid alcohols (esters of citronellol, geraniol, nerol, linalool and α-terpineol), cinnamyl alcohol and cinnamic acid, and furfuryl alcohol. A compendium of the Kekulé structures of the alcohols is given in 1.4.1 below (in addition to a list of the carboxylic acid moieties). Cinnamyl and furfuryl alcohols possess cyclic structures with varying degrees of unsaturation. All the selected esters of monoterpenoid alcohols possess unsaturated branched chain structures. Linalool and α-terpineol are tertiary alcohols which have additional chain branching on the carbon atom to which the functional group is bonded [α-terpineol (which is cyclic) has two methyl groups, and linalool has an allyl and a methyl group at this position (which therefore is a chiral centre)]. Citronellol, geraniol and nerol have no carbon chain branching at this position, geraniol and nerol are *trans* / *cis* isomers. Esters of cinnamic acid were selected for study so that comparisons in hydrolytic fate could be made with esters of cinnamyl alcohol, and to enable an assessment of the effects of alkyl carbon chain length.

It is possible that during and/or following the hydrolysis of monoterpenoid esters the alcohol products may undergo structural isomerisations in solution[264-268]. It was determined to investigate the structural isomerisations of monoterpenoid alcohols (particularly linalool), in addition to the intestinal absorption and metabolic fate of this important group of food flavouring additives. Available data on the toxicology and metabolism of monoterpenoid alcohols is given in 4.1.2 and 4.1.3 respectively.

The objective of the investigations presented below was to identify trends in the hydrolytic metabolism of flavouring esters which are related to structural features of the compounds. This was towards constructing a predictive model of hydrolytic metabolism, and as such better validate the assumptions currently made concerning such metabolism. This was approached by:

1. Undertaking an analysis of the behaviour of 44 selected esters of monoterpenoid alcohols, cinnamyl alcohol, cinnamic acid and furfuryl alcohol in artificial gastrointestinal fluids.
2. Selecting from 1, esters for the analysis of hydrolytic behaviour in rat gastrointestinal contents homogenates and preparations of rat and human tissue.
3. Selecting from 1 and 2, esters for the analysis of hydrolytic metabolism *in vivo* following single oral and intraperitoneal administration to rats.

Further supporting studies included:

- Investigation of the structural isomerisations and metabolism of monoterpenoid alcohols (particularly linalool).
- Assessment of the characteristics of intestinal absorption of selected monoterpenoid compounds using isolated rat intestinal preparations.
- Analysis of the esterase characteristics of artificial pancreatic fluid and preparations of rat liver using selected substrates and esterase-inhibiting compounds.

1.4.1 Compendium of Chemical Synonyms and Structures

Table 1.6 gives the common and structural names of the alcohol and acid moieties of the esters employed in this study, figure 1.4 gives the Kekulé structures of the alcohols employed, in addition the structures of *trans*-sobrerol and *l*-menthol are shown, these were not the subject of investigation but are discussed in the text below.

Common Name	Structural Name
Alcohols	
Linalool	3,7-Dimethylocta-1,6-dien-3-ol
α-Terpineol	α, α, 4-Trimethyl-3-cyclohexene-1-menthanol
Geraniol	(E)-3,7-Dimethyl-2,6-octadien-1-ol
Nerol	(Z)-3,7-Dimethyl-2,6-octadien-1-ol
Limonene (not an alcohol)	1-Methyl-4-(1-methylethenyl)cyclohexene
Terpinolene (not an alcohol)	1-Methyl-4-(1-methylethylidene)cyclohexene
Citronellol	3,7-Dimethyl-6-octen-1-ol
Cinnamyl alcohol	3-Phenyl-2-propen-1-ol
3-Phenylpropyl alcohol	3-Phenyl propan-1-ol
Furfuryl alcohol	2-Hydroxymethyl furan
Acids	
Formate	Methanoic acid
Acetate	Ethanoic acid
Propionate	Propionic acid
Butyrate	Butyric acid
Isobutyrate	2-Methyl propanoic acid
Valerate	Pentanoic acid
Isovalerate	3-Methyl butanoic acid
3-Methyl butanoate	3-Methyl butanoic acid
Hexanoate	Hexanoic acid
Octanoate	Octanoic acid
Caprylate	Octanoic acid
Benzoate	Carboxybenzene
Cinnamate	3-Phenyl propenoic acid
Anthranilate	1-Amino-2-carboxybenzene

Table 1.6: Common and structural names of the alcohol and acid moieties of the ester compounds investigated.

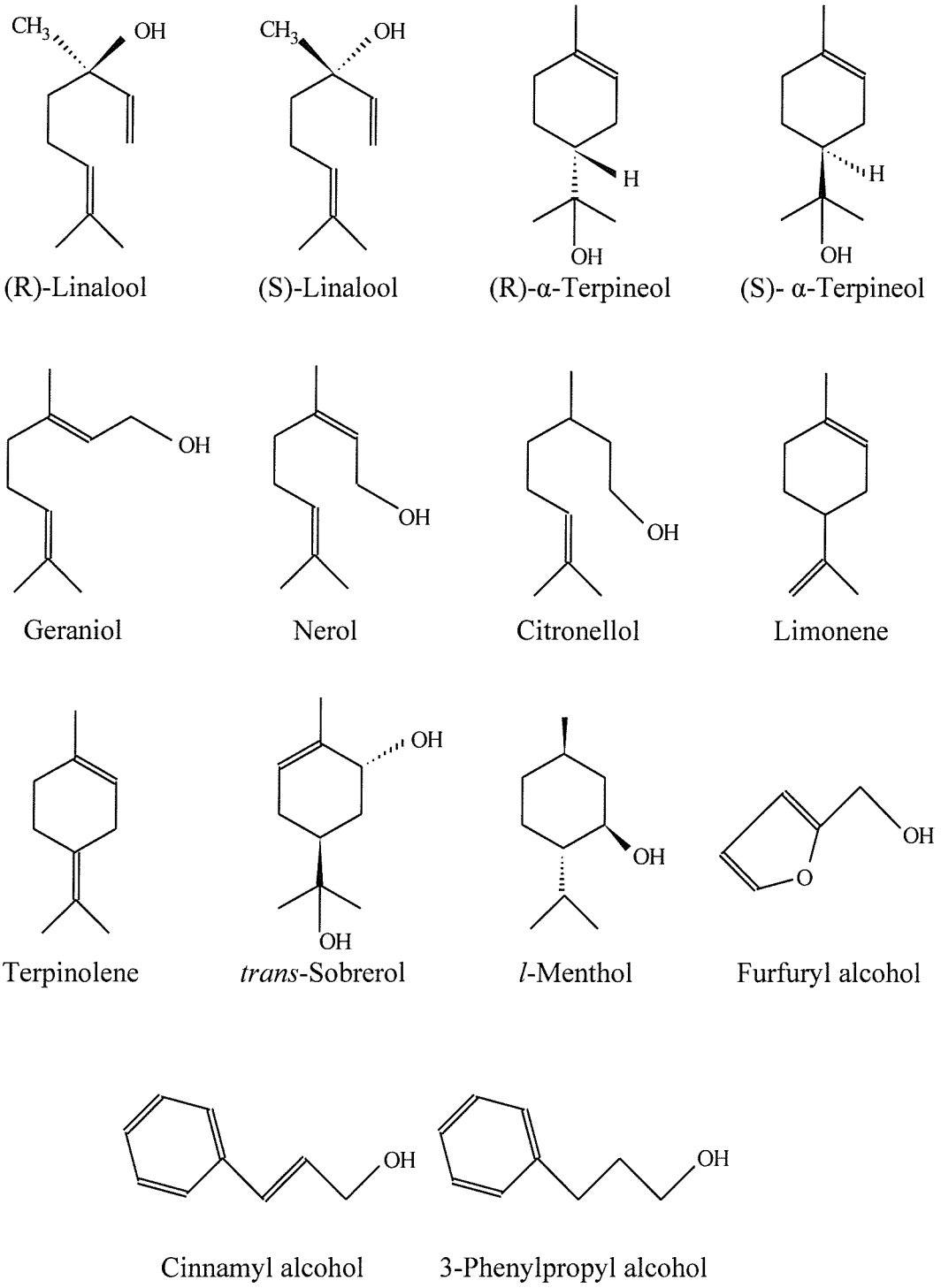


Figure 1.4: Structures of the alkyl moieties of the esters investigated. Orientation of the chiral centres of linalool and α-terpineol are indicated.

CHAPTER 2

GENERAL MATERIALS & METHODS

2.0 General Materials & Methods

2.1 Materials

BDH-Merck Ltd. (Merck House, Poole, Dorset, BH15 1TD, UK) supplied the AnalaR grade chemicals; calcium chloride (dihydrate), chloroform, dimethyl sulphoxide (DMSO), disodium hydrogen phosphate, glucose, hydrochloric acid (32 % w/w), magnesium sulphate, magnesium sulphate (7-hydrate), sodium chloride, sodium dihydrogen phosphate (dihydrate), sodium hydrogen carbonate, and sodium hydroxide.

Fisher Scientific UK Ltd. (Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG, UK) supplied Distol grade cyclohexane.

Sigma-Aldrich Company Ltd. (Fancy Road, Poole, Dorset, BH12 4QH, UK) supplied calcium chloride, 7-ethoxycoumarin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, β-glucuronidase, glycine, 7-hydroxycoumarin, β-nicotinamide adenine dinucleotide phosphate (NADP), oleic acid, pancreatin (specified to United States Pharmacopoeia), paraoxon (*O*, *O*-diethyl-*O*-[4-nitrophenyl]phosphate), pepsin, phenylmethylsulfonyl fluoride (PMSF), 3'-phosphoadenosine-5'-phosphosulphate (PAPS), potassium chloride, potassium dihydrogen phosphate, D-saccharic acid 1,4-lactone, sodium acetate, sodium taurocholate, sulphatase, 1,2,3,4-tetrahydronapthalene (THN), trichloroacetic acid, tris-HCl (tris[hydroxymethyl]-aminomethane hydrochloride), tris-maleate (mono[tris(hydroxymethyl)aminomethane]maleate), and uridine 5'-diphosphoglucuronic acid (UDPGA).

2.1.1 Flavouring Compounds

The availability of flavouring compounds was limited due to low volumes of production. This caused difficulty in obtaining compounds selected for study and necessitated compromise in the selection process. All the flavouring compounds

obtained were certified at least 98.5 % pure and were obtained from the following sources:

Bedoukian Research Inc. (Finance Drive, Danbury. C.T. 06810-4192, USA) provided as a gift (R)- and (S)- linalyl butyrate, methyl anthranilate, geranyl propionate, geranyl isobutyrate, geranyl caprylate, geranyl benzoate, neryl isobutyrate, citronellyl formate, citronellyl acetate, citronellyl propionate, citronellyl valerate, citronellyl anthranilate, and furfuryl propionate.

CTC Organics (792 Windsor Street, SW Atlanta G.A. 30315, USA) supplied linalyl hexanoate and linalyl octanoate.

The Flavor and Extract manufacturers' Association of the USA (Suite 925, 1620 I Street, NW, Washington, D.C. 20006, USA) provided furfuryl 3-methyl butanoate and 3-phenylpropyl cinnamate.

Fluka Chemicals (Messerschmittstr, 17, D-89231 Neu-Ulm, Germany) supplied citronellol, terpinyl acetate, cinnamic acid, (R)- and (S)- linalool, and (R)- and (S)- α-terpineol.

Greyhound Chromatography Ltd. (88 Grange Road West, Birkenhead, Merseyside, UK) supplied cinnamyl propionate.

International Flavor and Fragrances Inc. (150 Docks Corner Road, P.O. Box 439, Dayton, N.J. 08810-0439, USA) provided as a gift linalool, α-terpineol, geraniol, limonene, terpinolene, cinnamyl alcohol, furfuryl alcohol, linalyl formate, linalyl acetate, linalyl butyrate, linalyl cinnamate, geranyl acetate, neryl acetate, cinnamyl formate, cinnamyl acetate, methyl cinnamate, cinnamyl cinnamate, and furfuryl acetate.

Lancaster Synthesis (Eastgate, White Lund, Morecambe, UK) supplied propyl cinnamate, butyl cinnamate and cinnamyl anthranilate.

Pfaltz and Bauer Inc. (172 East Aurora Street, Waterbury. C.T. 06708, USA) supplied linalyl propionate, linalyl isovalerate, linalyl anthranilate, terpinyl formate, terpinyl propionate, terpinyl butyrate, terpinyl isobutyrate, geranyl formate, geranyl butyrate, citronellyl butyrate, citronellyl isobutyrate, and furfuryl butyrate.

Sigma Chemicals Co. (P.O Box 14508, St Louis M.O. 63178, USA) supplied nerol.

2.2 Methods

2.2.1 Gas Chromatographic Analysis

Gas chromatography was employed for analysis of flavouring compounds, with retention times in comparison to known standards used to identify individual compounds.

All injections were performed in duplicate, and in all cases cyclohexane containing, as an internal gas chromatography standard, 75 μM 1,2,3,4-tetrahydronaphthalene (CHX/THN) was employed for extraction and internal standardisation. No THN could be detected in the aqueous phase on analysis of a suspension of CHX/THN in distilled water incubated for 24 hours at 37°C, and recovery from the solvent fraction was 100 % when compared with freshly prepared CHX/THN. This result showed that no loss of internal standard occurs when in aqueous suspension. All the flavouring compounds under study, and their parent alkyl moieties, were shown to be stable in CHX/THN at 21°C for a period of at least 4 weeks, and were stable as stock solutions in dimethyl sulphoxide for at least 3 weeks.

Analysis was performed on a Chrompack CP-9003 (1999 dual-channel model) gas chromatograph (GC) using flame ionisation detection (901A detector model), the GC was fitted with a CP-9050 autosampler. Data acquisition was by direct digital output to the Maitre Chromatography System (Chrompack International nv) installed on an IBM-compatible computer, with a sampling frequency set at 16,667 Hz. In all instances the capillary column employed was WCOT fused silica, 50 m long, 0.32

mm internal diameter, 0.25 μm film thickness of 100 % methylsilicone (Chrompack CP-Sil 5CB). The carrier gas was hydrogen. Both the injection port and detector were maintained at 250°C. In all cases 0.5 μl injections were performed. The split / split-less injector was used in split-less mode with a 1.0 ml injection liner and a purge-time of 0.5 minutes following which a split flow of 30 ml/min was maintained (split ratio of 14.5:1). Automatic pressure regulators were set such that the hydrogen carrier gas flow rate was constant at 2.24 ml/min regardless of oven temperature (with an initial column head-pressure of 60 KPa).

The solvent-trapping effect was employed in order to focus solutes. In all cases the initial oven temperature was maintained at 60°C for two minutes, this was followed by a rise of 5°C per minute to the final oven temperature. Table 2.1 indicates the final oven temperature and duration of this temperature along with the retention times of the compounds under study. Figure 2.1 illustrates an example chromatogram of neryl isobutyrate following 0.1 minutes incubation in artificial pancreatic fluid.

Standard curves of substrate and product(s) were prepared in triplicate for each experiment (at concentrations of 2 times, 0.5 times and 0.05 times the maximum substrate concentration used in each experiment) utilising the appropriate incubation solutions (which in the case of preparations containing protein were deactivated by heating at 60°C for 4 minutes). All standard curves had a regression coefficient of at least 0.97. At all times investigations were conducted within the linear response range of the flame ionisation detector. Figure 2.2 illustrates an example standard curve of neryl isobutyrate in artificial pancreatic fluid.

Extraction efficiencies from aqueous solutions to CHX/THN were greater than 72 % in the case of all the experimental protocols employed. Chromatographic detection limits for all substrates and products were between 17-28 ng/ml cyclohexane for all biological samples analysed.

Table 2.1: Gas chromatographic conditions (following two minutes at 60°C and a 5°C / minute temperature increase); final oven temperatures and duration of final oven temperatures and retention times of flavouring compounds (in red parenthesis). Capillary column employed was Chrompack CP-Sil 5CB with a constant flow rate of hydrogen carrier gas of 2.24 ml/minute (initial column head-pressure = 60 KPa).

Duration of Final Oven Temperature (minutes)
(retention time [minutes])

Compound	Final Oven Temperature 140°C	160°C	190°C	195°C
Linalool	1 (10.55)			
α-Terpineol	1 (12.69)			
Geraniol	1 (14.35)			
Nerol	1 (13.71)			
Limonene	1 (8.75)			
Terpinolene	1 (10.25)			
Citronellol	1 (13.81)			
Cinnamyl alcohol	1 (15.25)			
3-Phenylpropyl alcohol	1 (13.44)			
Linalyl formate	1 (13.46)			
Linalyl acetate	1 (14.55)			
Linalyl propionate	1 (16.65)			
Linalyl butyrate		1 (18.75)		
Linalyl hexanoate			1 (23.09)	
Linalyl octanoate			1 (27.18)	
Linalyl isovalerate		1 (19.97)		
Linalyl cinnamate				7 (34.13)
Linalyl anthranilate			1 (23.86)	
Terpinyl formate	1 (15.69)			
Terpinyl acetate	1 (16.91)			
Terpinyl propionate		1 (19.10)		
Terpinyl butyrate		1 (21.15)		
Terpinyl isobutyrate		1 (20.02)		
Geranyl formate	1 (15.68)			
Geranyl acetate	1 (17.67)			
Geranyl propionate		1 (19.84)		
Geranyl butyrate		1 (21.89)		
Geranyl isobutyrate		1 (20.82)		
Geranyl caprylate				3 (30.01)
Geranyl benzoate				3 (30.05)
Neryl acetate	1 (17.22)			
Neryl isobutyrate		1 (20.30)		
Citronellyl formate	1 (14.99)			
Citronellyl acetate	1 (16.97)			
Citronellyl propionate		1 (19.20)		
Citronellyl butyrate		1 (21.27)		
Citronellyl isobutyrate		1 (20.28)		
Citronellyl valerate			1 (23.38)	
Citronellyl anthranilate				11 (36.14)
Cinnamyl formate	1 (16.47)			
Cinnamyl acetate	1 (18.78)			
Cinnamyl propionate		1 (21.09)		
Cinnamyl cinnamate				7 (35.16)
Cinnamyl anthranilate				16 (43.20)
3-Phenylpropyl cinnamate				16 (41.12)

Methyl cinnamate	1 (17.29)			
Propyl cinnamate		1 (21.74)		
Butyl cinnamate			1 (24.12)	
Methyl anthranilate	1 (18.02)			
Furfuryl acetate	1 (7.34)			
Furfuryl propionate	1 (9.47)			
Furfuryl butyrate	1 (11.97)			
Furfuryl 3-methyl butanoate	1 (13.30)			
Internal standard: THN	1 (11.88)			

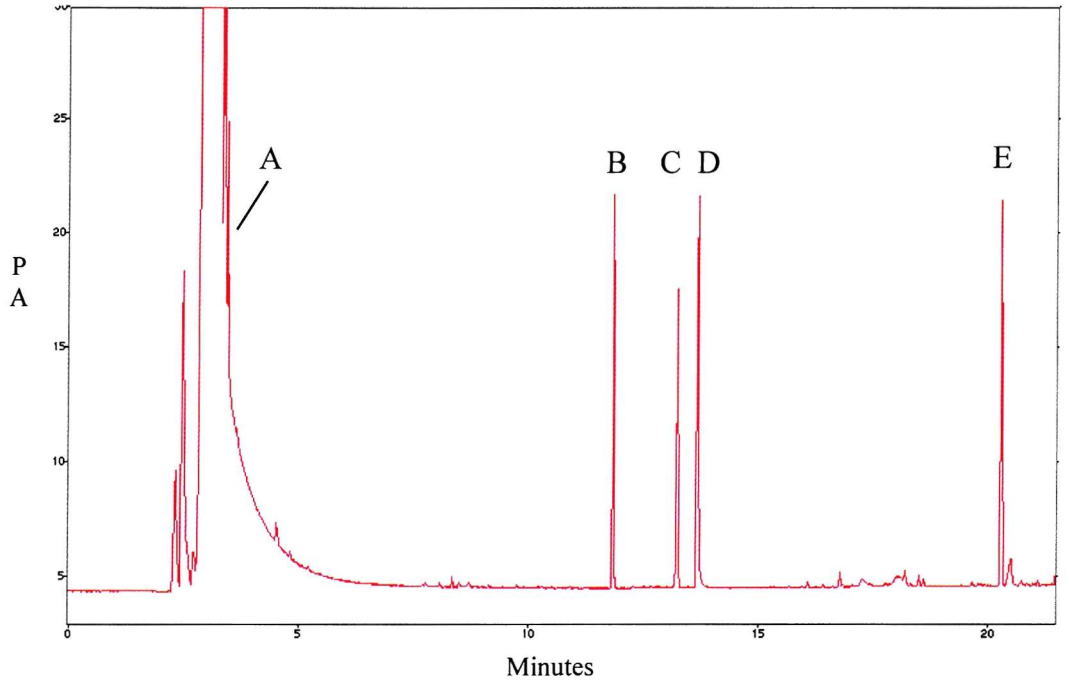


Figure 2.1: Example chromatogram of 50 μM neryl isobutyrate incubated at 37°C in artificial pancreatic fluid for 0.1 minutes. A = solvent front, B = THN internal standard, C = taurocholic acid, D = nerol, E = neryl isobutyrate.

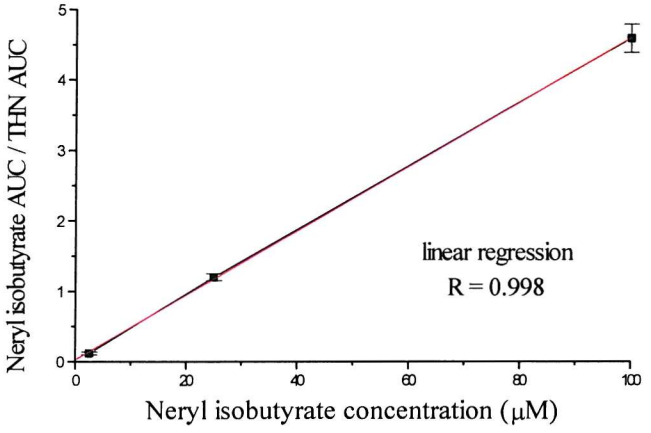


Figure 2.2: Standard curve of neryl isobutyrate in artificial pancreatic fluid.

2.2.1.1 Gas Chromatographic Analysis of Stereoenantiomers

The capillary column employed was WCOT fused silica, 25 m long, 0.25 mm internal diameter, 0.25 μm film thickness of β-cyclodextrin bonded directly to dimethylsiloxane (Chrompack CP-Chirasil-Dex CB). The carrier gas was hydrogen and detection was via flame ionisation. Both the injection port and detector were maintained at 250°C. In all cases 2 μl injections were performed. The split / split-less injector was used in split-less mode with a 1.0 ml injection liner and a purge-time of 0.5 minutes following which a split flow of 30 ml/min was maintained (split ratio of 11.2:1). Automatic pressure regulators were set such that the hydrogen carrier gas flow rate was constant at 2.78 ml/min regardless of oven temperature (with an initial column-head pressure of 90 KPa). The oven temperature was maintained at 60°C for 2 minutes following injection, this was followed by a rise in temperature of 5°C per minute up to 90°C which was the final temperature. The final temperature was maintained for 17 minutes for the analysis of (R)- and (S)- linalool, the retention times of which were 16.88 and 17.32 minutes respectively. The final temperature was maintained for 42 minutes for the analysis of (R)- and (S)- α-terpineol and (R)- and (S)- linalyl butyrate, the retention times of which were 40.73, 39.45, 43.38 and 42.80 minutes respectively. In all cases the retention time of the internal standard THN was 15.88 minutes.

2.2.2 Data Analysis

All data (digital and manual text) relating to individual experimental information was assigned a unique data number which was used for rapid identification and filing purposes. A duplicate archive of digital data was maintained on both optical and magnetic media, the individual duplicates were stored at different sites. All archives were appended with new data once per month from a running interim data backup, which itself was appended every day. Only one operator (N.R. Buck) had access to the primary hard disk data store which was isolated on a remote computer.

All computerised calculations were prepared using the software Excel 97 (Microsoft Inc.) with all graphs prepared using Microcal Origin version 3.5 (Microcal Inc.). Chemical structural rendering was performed using CS ChemOffice 3.2 software (CambridgeSoft Corp.).

Reaction rates were calculated from the initial linear portions of $\log^{10}$ concentration versus time graphs, unless otherwise stated. All errors are expressed as standard deviations around the mean. All statistical comparisons between data sets have been calculated using Student's T-Test (two-tailed, homoscedastic). All results are expressed to 2 significant figures [half lives are expressed to 2 significant figures but not more than 2 decimal places, reaction constants ($k_{formation}$ and moles / minute / mg protein) are expressed to 2 significant figures but not more than 4 decimal places].

2.2.3 Solubility Determination

The maximum solubility of the substrates at 37°C was determined by GC analysis. Solubilised substrate was extracted from a saturated solution in distilled water with CHX/THN, following 15 minutes incubation. All investigations were conducted in triplicate. Assessment was made against standards prepared, and extracted from distilled water. Comparison was also made to standards prepared in CHX/THN to identify any possible instability in an aqueous environment.

2.2.4 Investigations into the Effects of Artificial Gastric Fluid upon Substrates

The principle exocrine secretion of the human gastric glands is hydrochloric acid, common electrolyte salts of sodium and potassium, and up to ten pepsinogen isoenzymes[269]. Investigations into the behaviour and metabolism of flavouring compounds were conducted using an artificial model of human gastric fluid.

25 mM stock solutions of substrates in dimethyl sulphoxide were freshly prepared and added to incubation flasks (which were shaken) such that the final concentration of substrate was 50 μM, with dimethyl sulphoxide representing 0.2 % (v/v) of the final incubation mix.

Incubation flasks contained 50 ml of the following, pre-warmed for 10 minutes at 37 °C:

1. Neutral Control; neutralised artificial gastric fluid with no peptic enzyme
2. Acidic Control; artificial gastric fluid with no peptic enzyme
3. Test; artificial gastric fluid with peptic enzyme

All investigations were conducted in triplicate.

Artificial gastric fluid was prepared according to Pharmacopoeia Helvetica VI, and consisted of: sodium chloride 2.0g, in 1M HCl 80 ml and pepsin (porcine stomach mucosa, 3900 Units/mg) 3.2g made up to 1 litre with distilled water and adjusted with HCl to pH 1.2. Neutralised artificial gastric fluid contained no HCl and was adjusted with NaOH solution (pH 14) to pH 7.0. Artificial gastric fluid contained 3.2 mg protein / ml.

Flasks were incubated at 37°C in a shaking water bath, and 5 ml samples withdrawn at intervals of 0.1, 5, 10, 15, 30, 60, 120 and 180 minutes post substrate addition and placed in 10 ml glass tubes, which contained 1 ml CHX/THN, and vortexed. Subsequently, samples were immediately frozen on dry-ice.

For storage, all samples were maintained at -20°C for no more than 2 weeks, following which they were defrosted at room temperature followed by centrifugation at a relative centrifugal force of 6030 g prior to gas chromatographic analysis of the CHX/THN layer.

2.2.5 Investigations into the Effects of Artificial Pancreatic Fluid upon Substrates

Enzymes and precursors secreted by the human exocrine pancreas include trypsinogen, chymotrypsinogen, proelastase, procarboxypeptidases A and B, prophospholipase A, α-amylase, lipase (including carboxyl ester hydrolase), RNAse and DNAse[270]. Investigations into the behaviour and metabolism of flavouring

compounds were conducted using an artificial model of human pancreatic fluid. This contained porcine pancreatin which is the acetone precipitate of whole pancreas aqueous homogenate (prepared by the manufacturer according to the patented method of Hoek)[271].

25 mM stock solutions of substrates in dimethyl sulphoxide were freshly prepared and added to incubation flasks (which were shaken) such that the final concentration of substrate was 50 μM, with dimethyl sulphoxide representing 0.2 % (v/v) of the final incubation mix. In the case of investigations with a higher substrate concentration, more concentrated stock solutions were employed so that the concentration of dimethyl sulphoxide did not exceed 0.2 % (v/v).

Incubation flasks contained 50 ml of the following, pre-warmed at 37°C for 10 minutes:

1. Control; artificial pancreatic fluid solution with no pancreatin
2. Test; artificial pancreatic fluid solution with pancreatin

All incubations were conducted in triplicate.

Artificial pancreatic fluid was prepared according to Pharmacopoeia Helvetica VI, and consisted of: pancreatin (from porcine pancreas, activity certified to United States Pharmacopoeia specifications) 10 g, sodium taurocholate 0.5 g, disodium hydrogen phosphate 50.5 g, and sodium dihydrogen phosphate dihydrate 15.6 g, in 1 litre distilled water and adjusted with NaOH to pH 7.5. Artificial pancreatic fluid contained 2.3 mg protein / ml (23 % of pancreatin was protein material).

Flasks were incubated at 37 °C in a shaking water bath, and 5 ml samples withdrawn at time intervals of 0.1, 5, 10, 15, 30, 60, 120 and 180 minutes post substrate addition, and placed in 10 ml glass tubes which contained 1 ml CHX/THN and vortexed. Subsequently, samples were immediately frozen on dry-ice. In the case of 120 minute incubations (which were used in relation to enzyme inhibitor investigations, see 2.2.7) samples were withdrawn at intervals of 0.1, 5, 10, 15, 30, 60, 90 and 120 minutes.

For storage, all samples were maintained at -20°C for no more than 2 weeks, following which they were defrosted at room temperature followed by centrifugation at a relative centrifugal force of 6030 g prior to gas chromatographic analysis of the CHX/THN layer.

2.2.5.1 Confirmation of Porcine Pancreatin Activity

In the case of all artificial pancreatic fluid investigations, the porcine pancreatin was sourced from a single production batch and was from the single container in which it was supplied.

The activity of porcine pancreatin was verified by re-assessment of the hydrolysis rate of linalyl butyrate following the completion of all artificial pancreatic fluid investigations. Linalyl butyrate was amongst the first substrates to be investigated in artificial pancreatic fluid, during which a half-life of 4.8 ± 0.4 minutes was recorded. Following the completion of artificial pancreatic fluid investigations a half life for linalyl butyrate of 4.3 ± 0.5 minutes was recorded.

Substrate stocks were prepared in dimethyl sulphoxide prior to addition to incubations. To assess the possible effect of 0.2 % (v/v) dimethyl sulphoxide in artificial pancreatic fluid incubations, linalyl butyrate (from a solubilised stock in distilled water) was incubated in artificial pancreatic fluid without the presence of dimethyl sulphoxide. The resulting hydrolysis half life (4.7 ± 0.4 minutes) demonstrated that 0.2 % (v/v) dimethyl sulphoxide had no measurable effect upon hydrolysis rate.

2.2.6 Investigations into the Effects of Rat Tissue Homogenates

Investigations were conducted in triplicate in order to investigate the metabolism of substrates in homogenates of rat: stomach contents, intestinal contents, intestinal mucosal surface, blood and liver. The triplicate investigations were undertaken using tissues from 3 different animals with incubations conducted concurrently. To ensure the use of fresh tissue, all investigations were undertaken immediately following the

isolation of tissue. The concentration of homogenates used were dependent upon the individual series of investigations undertaken (this information is given with the results).

2.2.6.1 Homogenate Preparation

Male Wistar albino rats (250-300 g) were killing by cervical dislocation. All tissue samples were placed in pre-weighed vessels kept on ice.

The abdominal and thoracic cavities were opened and blood removed by cardiac puncture. Sections from all liver lobes were removed, and cut into strips. The stomach was cut open and the contents removed. Following this, 60 cm of small intestine (proximal to the stomach) was removed and cut into 10 cm sections.

Intestinal contents were extruded using forceps, and the intestinal sections were washed through with ice cold 'tissue buffer' [tris-HCl 50mM, KCl 0.154 M, $CaCl_2$ 0.1 mM, pH 7.4] using a 5 ml syringe. Longitudinal slices were made along the length of the intestinal sections, on a chilled glass slide, such that the intestinal mucosa was exposed. The gut mucosa was removed with lateral scraping force.

From the pre-weighed vessels, the weights of the samples (blood, liver, stomach contents, intestinal contents, and intestinal mucosal surface) were calculated, and the concentration of homogenates (w/v) was determined. Homogenates were made up in ice-cold tissue buffer. Homogenisation was carried out using a Janke & Kunkel Ultra-Turrax T25 using two 5 second bursts at 17000 revolutions / minute. A sample of each homogenate prepared was immediately stored frozen (-20°C) for use in the preparation of standard curves and for total protein determinations.

2.2.6.2 Incubations

An aliquot of each homogenate was diluted in tissue buffer (at 21°C) to the desired concentration to a total volume of 100 ml in the case of investigations of linalool and linalyl acetate, and to a total of 50 ml for all other tissue homogenate investigations

(unless otherwise stated). Incubation vessels were placed in a 37°C shaking water bath for 7 minutes to pre-warm.

Stock solutions of 25 mM substrate were freshly prepared in dimethyl sulphoxide and added to incubation mixtures such that the final substrate concentration was 50 μM, and such that the concentration of dimethyl sulphoxide was always below 0.2 % (v/v). To ensure this maximum concentration of dimethyl sulphoxide in the case of incubations with higher substrate concentrations, the concentration of substrates in the stock solutions were increased. Sealed incubation flasks were shaken, and 5 ml samples were withdrawn to 10 ml tubes containing 1 ml CHX/THN at 0.1, 5, 10, 15, 30, 60, 120 and 180 minutes, samples were vortexed and immediately frozen on dry ice. In the case of 120 minute investigations, samples were withdrawn at 0.1, 5, 10, 15, 30, 60, 90 and 120 minutes.

For storage, all samples were maintained at -20°C for a maximum of 2 weeks, following which they were centrifuged at a relative centrifugal force of 6030 g prior to gas chromatographic analysis of the CHX/THN layer.

2.2.7 Investigations into the Effects of Serine-Esterase / Protease Inhibitors

The experimental protocols for investigations of the hydrolysis of flavouring ester substrates by artificial pancreatic fluid and rat liver homogenate are given in 2.2.5 and 2.2.6 respectively. To study effects due to compounds with known serine esterase inhibiting activity, additional triplicate incubation sets were concurrently undertaken. These additional incubations contained the serine esterase inhibitor paraoxon (*O*, *O*-diethyl-*O*-[4-nitrophenyl]phosphate) or the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF).

2.2.7.1 Paraoxon

Following warming of incubation mixtures for 6 minutes in a shaking water bath at 37°C, paraoxon was added to incubations (from stock solutions in dimethyl sulphoxide) 4 minutes before the addition of substrate, such that the total

concentration of dimethyl sulphoxide was always below 0.2 % (v/v). The concentration of paraoxon in incubation mixtures of artificial pancreatic fluid and rat liver homogenate was 100 μM.

This concentration was chosen as B-esterases are almost completely inhibited by paraoxon from a concentration of 1 μM, whereas at 1 mM, some inhibition of A-esterases has been demonstrated[19; 20]. In the studies of McCracken *et al*, microsomal and cytosolic protein equivalent to up to 6 % (w/v) rat liver showed complete inhibition of B-esterases in the presence of 100 μM paraoxon, with remaining esterase activity corresponding to paraoxonase (A-esterase) activity[69; 128]. Pancreatin was present in artificial pancreatic fluid incubations at 1 % (w/v) concentration (2.3 mg/ml protein), whilst rat whole liver homogenate investigations contained a maximum of 5 % (w/v) tissue (approximately 10-15 mg/ml protein).

As paraoxon is a substrate for A-esterase(s) (the identity and number of paraoxon-metabolising enzymes remains unknown), in most investigations it was decided to use a higher concentration of flavouring ester substrate than paraoxon to help off-set any possible effects due to competitive inhibition of A-esterases. The majority of investigations utilised linalyl butyrate as substrate, this being the most preferred linalyl ester substrate as identified in investigations with artificial pancreatic fluid (see Chapter 3). Furthermore, due to the high rate of metabolism of linalyl butyrate, a concentration was required which would enable accurate quantification of the reaction rates which was within the solubility limit of the substrate. As such, the majority of investigations utilised a substrate concentration of 200 μM.

2.2.7.2 Phenylmethylsulphonyl Fluoride

Following the warming of incubation mixtures for 6 minutes in a shaking water bath at 37°C, PMSF was added to incubations (from stock solutions in dimethyl sulphoxide) 4 minutes before the addition of substrate, such that the total concentration of dimethyl sulphoxide was always below 0.2 % (v/v). The concentration of PMSF in incubation mixtures of artificial pancreatic fluid and rat liver homogenate was 100 μM.

This concentration was chosen as Morgan *et al* have shown differential inhibition of isoenzymes hydrolysing *p*-nitrophenylacetate[38; 39]. Using 25 μg/ml rat liver microsomal protein, a concentration of PMSF between 0.01 to 1 μM progressively inhibited up to approximately 50 % of total esterase activity (the isoenzyme with high affinity for *p*-nitrophenylacetate was inhibited). It was not until the concentration of PMSF exceeded 10 μM that the lower affinity isoenzyme became inhibited. Assuming that 20 % of total hepatic cellular protein is microsomal[272], the 1 % (w/v) rat liver homogenate utilised in this study contained approximately 0.6 mg/ml microsomal protein. As such, the concentration range at which only the high affinity esterase is inhibited would be anticipated to be between approximately 25 and 250 μM PMSF.

The concentration of PMSF utilised in investigations with artificial pancreatic fluid was also 100 μM. Investigations with rat liver utilised the low homogenate concentration of 1 % (w/v) in order to better elucidate the metabolic characteristics of the most rapidly hydrolysed linalyl ester; linalyl butyrate.

2.2.8 Protein Determination

The protein concentration of incubation mixtures was determined using the Sigma-Aldrich Lowry Micro-Protein Determination Kit number 690, and a Pharmacia Biotech Novaspec II spectrophotometer.

CHAPTER 3

HYDROLYSIS OF MONOTERPENE, CINNAMYL, CINNAMATE AND FURFURYL ESTERS BY ARTIFICIAL GASTROINTESTINAL FLUIDS

3.0 Hydrolysis of Monoterpene, Cinnamyl, Cinnamate and Furfuryl Esters by Artificial Gastrointestinal Fluids

3.1 Introduction

3.1.1 Background

As discussed in 1.4, for the purposes of their safety evaluation it has been assumed that ester food flavouring additives, such as the monoterpenoid, cinnamyl / cinnamic acid and furfuryl esters, would be readily hydrolysed to their component alcohols and carboxylic acids[250]. Therefore safety assessment has been based upon data available for these component moieties (an analysis of the toxicological and metabolic data available concerning monoterpenoid alcohols is given in 4.1.2 and 4.1.3 respectively). However, as described in 1.2, mammalian esterases are poorly characterised. Furthermore, as described in 1.3, the small amounts of information which are available relating to the hydrolysis of flavouring esters indicate large differences in the rates of metabolism between different compounds.

Available limited data on the metabolites produced following the administration to animals of propyl anthanilate[260], cinnamyl anthranilate[136], methyl cinnamate[259] and furfuryl propionate[258] (which is described in 1.3.3) indicates that hydrolysis is the principal route of metabolism of these esters, although esters of anthranilic acid may not be completely hydrolysed.

As described in 1.3, between 1968 and 1979 a number of studies were undertaken into the hydrolysis of a wide range of flavouring esters in artificial gastrointestinal fluids and tissue homogenates. Grundschober has reported the hydrolytic fate of a range of esters in artificial pancreatic fluid, and determined that the percentage of substrate hydrolysed following 2 hours incubation at 37°C decreased in the order citronellyl acetate (100 %) > benzyl cinnamate (80 %) > citronellyl phenylacetate (60 %)[247]. The hydrolysis of methyl anthranilate has been studied by Gangolli *et al*[245], Longland *et al*[248] and Leegwater *et al*[246] in artificial gastric and/or pancreatic fluids, and by

Leegwater *et al*[246] and Longland *et al*[248] in preparations of rat intestine and liver. In all the reports methyl anthranilate was found to be particularly poorly hydrolysed in comparison to the other esters investigated. As such, further to the available *in vivo* metabolic data discussed above, methyl anthranilate and a number of other appropriate esters of anthranilic acid have been included in the studies presented below.

3.1.2 Study Objectives

The fates of selected compounds in artificial gastrointestinal fluids were evaluated in order to undertake a screen of the characteristics of hydrolytic metabolism of food flavouring esters in order to select compounds for further *ex-vivo* and *in-vivo* studies, towards the construction of a metabolic model. In total 44 esters were selected for study, these included a wide range of esters of monoterpenoid alcohols (citronellol, geraniol, nerol, linalool and α-terpineol) in addition to a number of esters of cinnamyl alcohol, cinnamic acid and furfuryl alcohol. The structures of these compounds are given in 1.4.1, and as discussed in 1.4, the food flavouring esters were selected from those which were available, based upon investigating the effects of a number of structural criteria on the rate and characteristics of metabolism. Attempts were also made to distinguish esterase enzymes present in porcine pancreatin through the use of known inhibitors of serine-esterase and serine-protease activity.

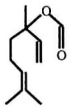
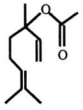
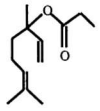
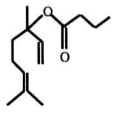
3.2 Results

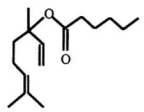
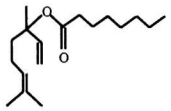
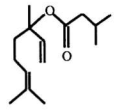
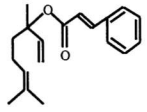
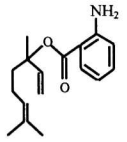
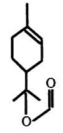
3.2.1 Hydrolysis of Monoterpene Esters by Artificial Gastrointestinal Fluids

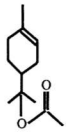
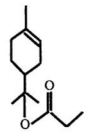
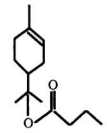
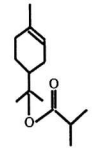
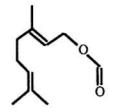
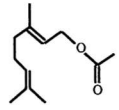
The effects of artificial gastric and pancreatic fluids were examined using a series of 30 food flavouring monoterpenoid esters. Table 3.1 summarises the results of these investigations (with reaction rates expressed as ester half lives ± standard deviation), following which an analysis of the results is presented. Table 3.1 illustrates that in the case of poorly hydrolysed substrates, half-lives were frequently longer in incubations containing enzyme(s) than in control incubations not containing enzyme(s). This was due to the sequestration of lipophilic esters in organic material and the inhibition of

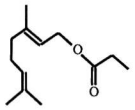
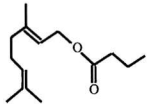
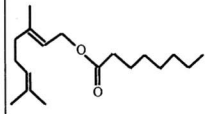
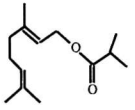
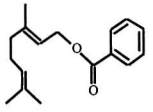
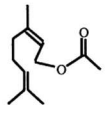
evaporation into the headspace of reaction vessels. In all cases the major alkyl hydrolysis products were identified and quantified (as determined from the molar quantities formed), and recorded with each hydrolysis rate given in table 3.1 is the detected alkyl hydrolysis product(s) (when no metabolites were detected this is indicated). When more than one hydrolysis products were detected, the major and minor products which were initially formed are indicated. The relationships between the quantity of products formed, as given in table 3.1, apply only in respect of the initial products formed, as monoterpene alcohols frequently underwent further reactions in acidic solutions to form secondary product(s).

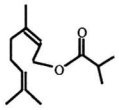
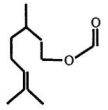
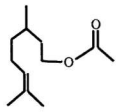
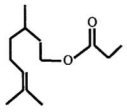
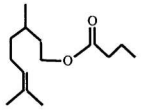
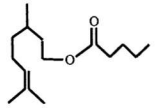
Table 3.1: Monoterpene flavouring esters incubated in artificial gastric and pancreatic fluids for 180 minutes, 37°C. Reaction rates calculated from the loss of parent ester. c = citronellol, g = geraniol, lim = limonene, l = linalool, n = nerol, t = α-terpineol, ter = terpinolene, - = no data.

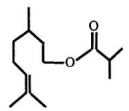
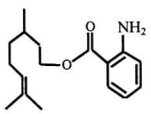
			$t_{½}$ (minutes) ± SD with initial metabolites identified in order of quantity produced				
			Artificial Gastric Fluid			**Artificial Pancreatic Fluid**	
Compound	**Structure**	**Solubility** H_2O, 37°C (mM)	Neutral, No Enzyme	Acidic, No Enzyme	Acidic, With Enzyme	No Pancreatin	With Pancreatin
Linalyl formate		3.50 ± 0.11	16 ± 0 l>t>g>n	0.28 ± 0.11 l>t>g>n	0.23 ± 0.04 l>t>g>n	19 ± 5 l>t>g>n	17 ± 1 l>t>n>g
Linalyl acetate		0.45 ± 0.22	110 ± 3 l=t>g	0.44 ± 0.11 l>t>g see figure 3.2A	0.31 ± 0.13 l>t>g see figure 3.2B	97 ± 7 l=t>g>n	83 ± 3 l>t>g=n
Linalyl propionate		0.70 ± 0.02	120 ± 10 No metabolites see figure 3.3A	1.5 ± 0.1 l>t>g>n see figure 3.3B	1.7 ± 0.2 l>t>g>n see figure 3.3C	72 ± 5 t>l>g>n see figure 3.11A	34 ± 0 l>t>g>n see figure 3.11B
Linalyl butyrate		0.68 ± 0.04	24 ± 3 l=t>g	4.7 ± 0.8 l>t>g>n	4.4 ± 1.1 l>t>g>n	50 ± 1 l=t	4.8 ± 0.4 l

Linalyl hexanoate		0.63 ± 0.03	32 ± 1 No metabolites	25 ± 4 l>t>g>n	27 ± 1 l>t>g>n	97 ± 21 t>l	11 ± 1 l
Linalyl octanoate		0.60 ± 0.04	190 ± 86 No metabolites	130 ± 64 l=t>g	230 ± 48 l=t>g	∞ No metabolites	9.1 ± 0.6 l
Linalyl isovalerate		0.38 ± 0.07	170 ± 27 t≥l>g	3.4 ± 0.1 l>t>g>n	3.9 ± 0.4 l>t>g>n	50 ± 4 l=t see figure 3.10A	440 ± 150 l>t>g>n see figure 3.10B
Linalyl cinnamate		3.65 ± 0.06	190 ± 70 l>t	80 ± 15 l>t>g>n	76 ± 2 l>t>g>n	2400 ± 3200 l	500 ± 40 l
Linalyl anthranilate		24.61 ± 3.01	∞ No metabolites	∞ No metabolites	∞ No metabolites	∞ No metabolites	∞ No metabolites
Terpinyl formate		1.81 ± 0.08	1000 ± 600 t>ter>lim	3.2 ± 0.1 t	3.7 ± 0.2 t	400 ± 100 t	470 ± 110 t

Terpinyl acetate		1.18 ± 0.09	870 ± 75 No metabolites	16 ± 0 t	16 ± 0 t	360 ± 60 No metabolites	380 ± 40 t
Terpinyl propionate		0.81 ± 0.03	340 ± 110 No metabolites	38 ± 1 t	39 ± 2 t	140 ± 7 No metabolites see figure 3.8A	170 ± 2 t see figure 3.8B
Terpinyl butyrate		0.53 ± 0.03	590 ± 330 No metabolites	57 ± 2 t	71 ± 3 t	130 ± 20 No metabolites	62 ± 4 t
Terpinyl isobutyrate		0.50 ± 0.03	240 ± 110 No metabolites see figure 3.4A	94 ± 6 t see figure 3.4B	68 ± 1 t see figure 3.4C	52 ± 1 No metabolites see figure 3.9A	270 ± 40 t see figure 3.9B
Geranyl formate		5.89 ± 0.12	200 ± 50 t>g>l>n	3.8 ± 0.1 l>g>t>n	4.3 ± 0.4 l>g>t>n	92 ± 7 g>t>n>l	0.12 ± 0.10 g>n>t≥l
Geranyl acetate		0.84 ± 0.09	650 ± 310 No metabolites see figure 3.5A	110 ± 20 l>g>t see figure 3.5B	72 ± 0 l>g>t see figure 3.5C	340 ± 30 g>t see figure 3.12A	0.23 ± 0.02 g>n see figure 3.12B

Geranyl propionate		0.94 ± 0.10	-	-	-	79 ± 6 g	0.03 ± 0.00 g>n
Geranyl butyrate		0.53 ± 0.03	270 ± 40 g	56 ± 12 l≥t≥g≥n	350 ± 140 l≥t≥g≥n	52 ± 7 g>n	0.02 ± 0.01 g>n>l≥t
Geranyl caprylate		0.24 ± 0.05	-	-	-	310 ± 70 No metabolites	0.02 ± 0.00 g>n
Geranyl isobutyrate		0.66 ± 0.07	-	-	-	53 ± 2 g	0.69 ± 0.06 g>n
Geranyl benzoate		0.94 ± 0.21	-	-	-	380 ± 390 No metabolites	1.0 ± 0.0 g>n
Neryl acetate		1.23 ± 0.10	520 ± 60 No metabolites see figure 3.6A	79 ± 7 t>l=n see figure 3.6B	60 ± 13 t>l>n see figure 3.6C	340 ± 40 t>n see figure 3.13A	0.23 ± 0.04 n see figure 3.13B

Neryl isobutyrate		0.47 ± 0.10	-	-	-	64 ± 20 t>n	0.77 ± 0.08 n
Citronellyl formate		0.82 ± 0.06	-	-	-	99 ± 5 c	0.03 ± 0.00 c
Citronellyl acetate		0.77 ± 0.11	-	-	-	140 ± 10 c	0.04 ± 0.00 c
Citronellyl propionate		0.69 ± 0.07	-	-	-	69 ± 9 c	0.02 ± 0.00 c
Citronellyl butyrate		0.58 ± 0.04	120 ± 70 No metabolites	54 ± 0 c	270 ± 60 c	62 ± 5 c>g see figure 3.14A	0.01 ± 0.00 c>g see figure 3.14B
Citronellyl valerate		0.16 ± 0.06	-	-	-	330 ± 70 c	0.14 ± 0.02 c

Citronellyl isobutyrate		0.51 ± 0.02	41 ± 9 c	52 ± 2 c	210 ± 30 c	57 ± 3 c	0.82 ± 0.03 c
Citronellyl anthranilate		11.82 ± 2.73	-	-	-	710 ± 40 No metabolites	340 ± 40 c

3.2.1.1 Hydrolysis of Monoterpenoid Esters by Artificial Gastric Fluid

The activity of artificial gastric fluid was due to the pH of the preparations, and peptic enzyme was found not to hydrolyse monoterpenoid esters (comparisons between the rates of hydrolysis of esters in acidic solutions containing and not containing pepsin are discussed below). Figure 3.1 illustrates the effect of artificial acidic gastric fluid not containing pepsin, upon the monoterpene esters investigated. The reaction rates shown represent the difference between acidic and neutral incubations in order to account for the evaporative loss of substrate (which occurred into the headspace of the sealed incubation vessels). In general, the stability of the compounds in acidic solution in comparison to neutral controls decreased in the order; citronellol esters > nerol esters ≥ α-terpineol esters ≥ geraniol esters > linalool esters. An increasing number of carbon atoms in the acyl group lent increasing stability in an acidic environment, with branched acyl chains seemingly being less stable in comparison to their linear equivalents in the case of linalyl esters, and more stable in the case of terpinyl and citronellyl esters.

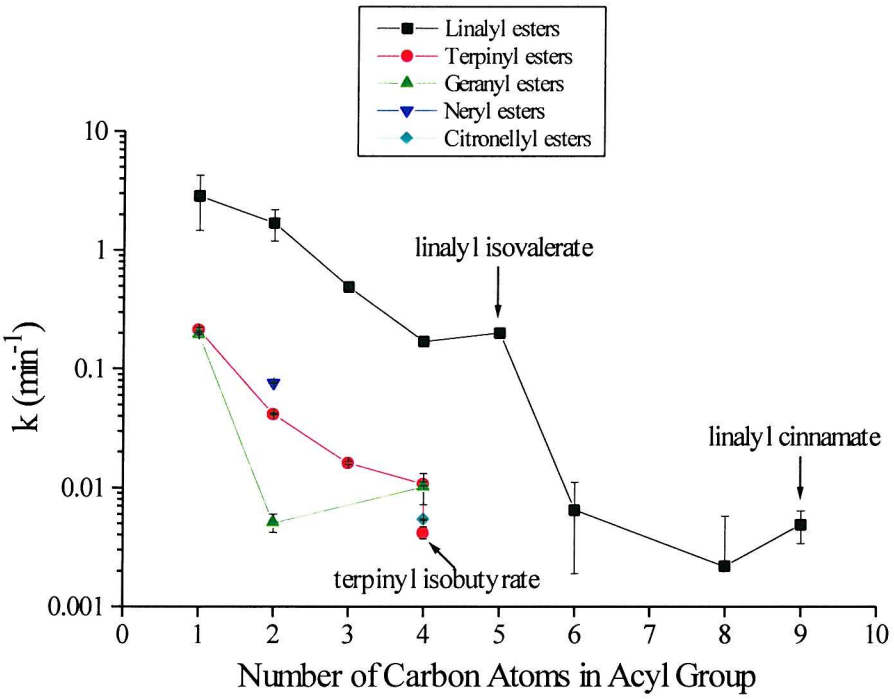


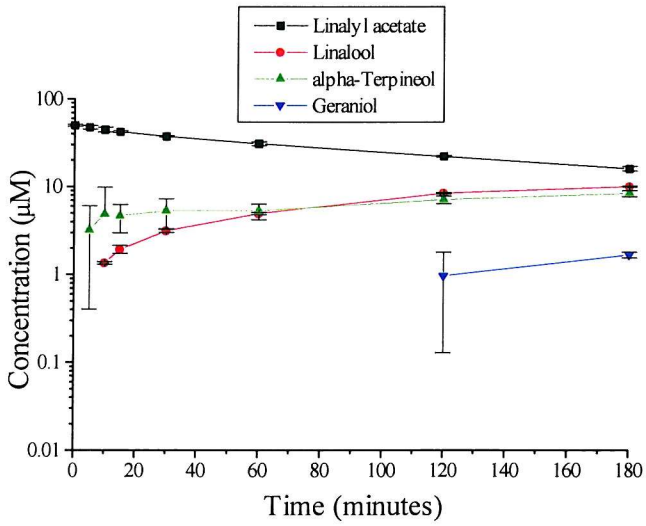
Figure 3.1: Rates of disappearance (k min^{-1}) of selected monoterpene esters in acidic artificial gastric fluid not containing pepsin – rates of disappearance (k min^{-1}) in neutralised artificial gastric fluid not containing pepsin (individual points = mean, error bars = SD). Compounds not containing a linear acyl chain are indicated. Compounds not included are linalyl anthranilate and citronellyl isobutyrate which were stable in acidic artificial gastric fluid.

3.2.1.1.1 Statistical Comparisons of the Rates of Hydrolysis of Monoterpenoid Esters in Artificial Gastric Fluids and Analysis of Reaction Products

In comparing neutralised artificial gastric fluid and acidic artificial gastric fluid not containing pepsin, all linalyl, α-terpinyl, geranyl, and neryl esters investigated were less stable in acidic solution (pH 1.2) in comparison to neutral solution [$P<0.05$, except terpinyl isobutyrate ($P<0.1$) and citronellyl butyrate ($P<0.2$)]. Citronellyl isobutyrate, linalyl octanoate and linalyl anthranilate were stable in acidic solution illustrating no statistically significant difference to substrates incubated in neutral artificial gastric fluid solution.

In an acidic environment, the hydrolysis of linalyl, terpinyl, geranyl and neryl esters frequently resulted in the formation of multiple alcohol products. However, citronellyl esters underwent acid-catalysed hydrolysis to form citronellol only. Hydrolysis of monoterpene esters in an acidic environment resulted in the formation of the same products as resulted from hydrolysis in neutral solution. However, in neutral solution all the products were formed concomitantly, whereas in acidic solution secondary alcohol products appeared to be formed from rearrangement of the primary product (parent alcohol), this is illustrated in the case of linalyl acetate in figure 3.2. As demonstrated in 4.3.1 and discussed in 4.4.1, further research to investigate the structural rearrangement of monoterpenoid alcohols illustrated that the ester hydrolysis products linalool, geraniol and nerol undergo rearrangement in an acidic environment to predominantly form α-terpineol.

A.



B.

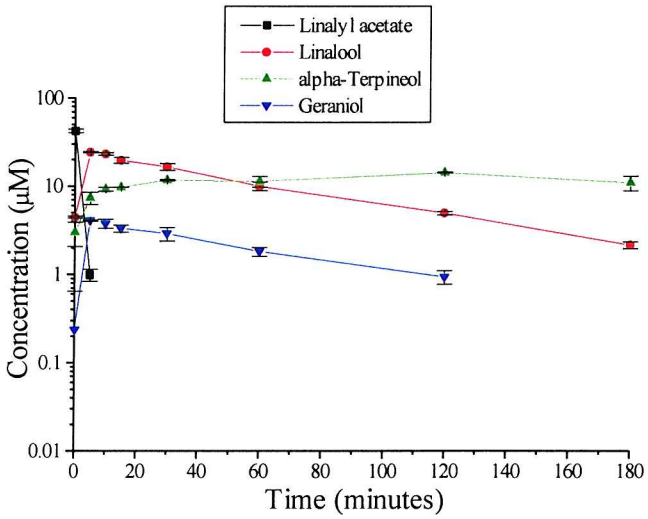
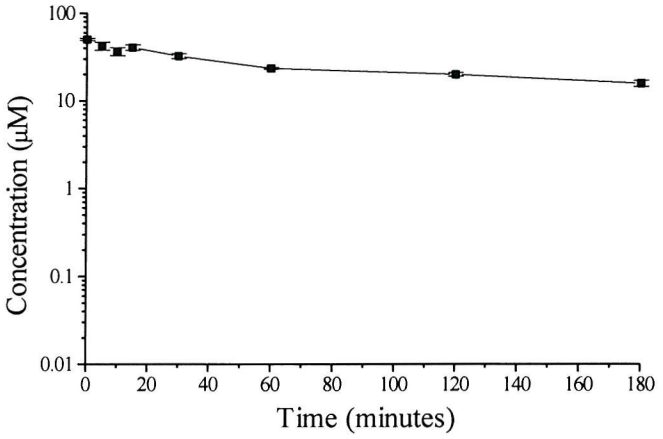


Figure 3.2: 50 µM linalyl acetate incubated in artificial gastric fluid 37°C for 180 minutes (mean ± SD) **A**. Neutralised artificial gastric fluid not containing pepsin. **B**. Acidic artificial gastric fluid not containing pepsin.

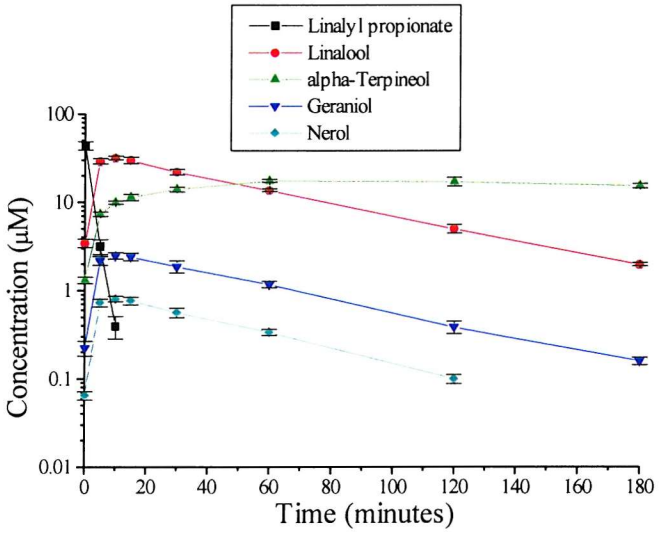
Linalyl esters in an acidic environment produced primarily the parent alcohol linalool with α-terpineol and smaller quantities of geraniol and nerol. Figure 3.3 illustrates the acid-catalysed hydrolysis of linalyl propionate. The acid-catalysed hydrolyses of terpinyl esters were found to produce only the alkyl group α-terpineol, except in the case of terpinyl formate which formed some additional limonene and terpinolene (which at no point accounted for more than 4 % of the initial substrate concentration). Figure 3.4 illustrates the acid-catalysed hydrolysis of terpinyl isobutyrate. The acid-catalysed hydrolysis of geranyl esters was found to produce primarily linalool, with geraniol, α-terpineol and some nerol. Figure 3.5 illustrates the acid-catalysed rearrangement of geranyl acetate. Neryl acetate was found to hydrolyse in acid to

primarily produce α-terpineol, with linalool and some nerol, as illustrated in figure 3.6.

A.



B.



C.

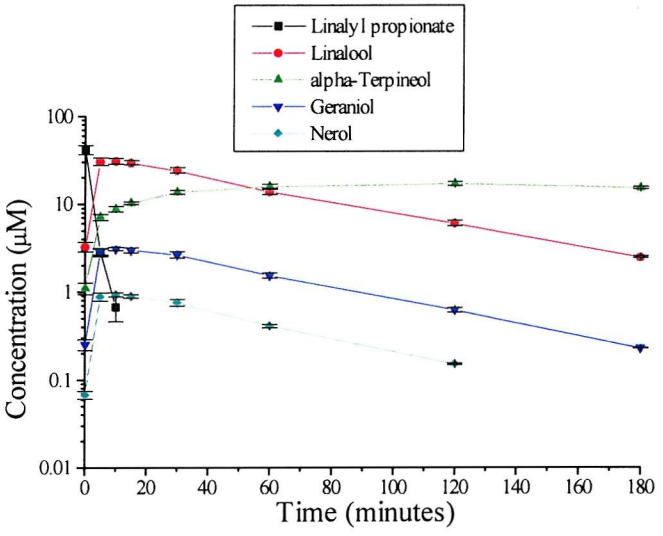
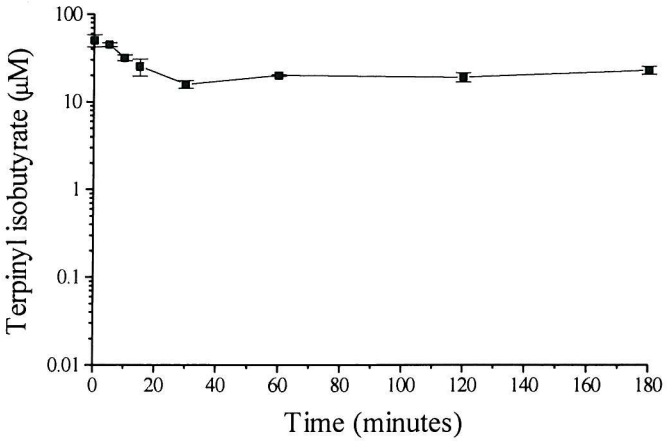
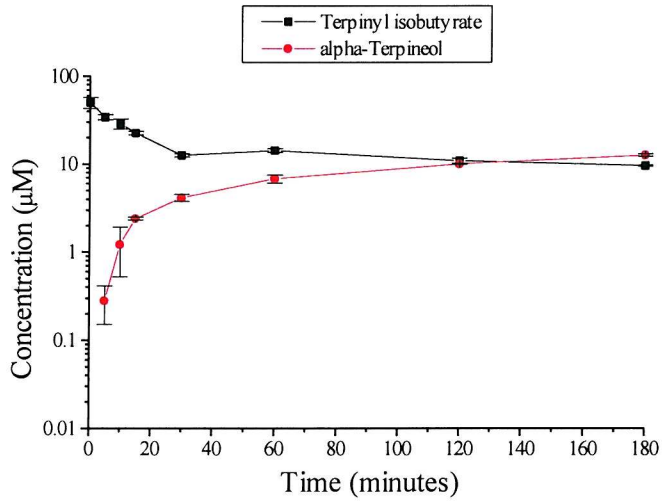


Figure 3.3: 50 μM linalyl propionate incubated in artificial gastric fluid 37°C for 180 minutes (mean ± SD) **A**. Neutralised artificial gastric fluid not containing pepsin. **B**. Acidic artificial gastric fluid not containing pepsin. **C**. Acidic artificial gastric fluid containing pepsin.

A.



B.



C.

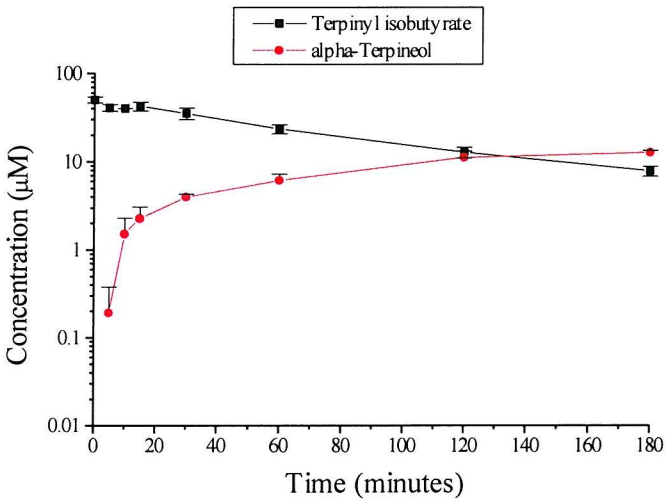
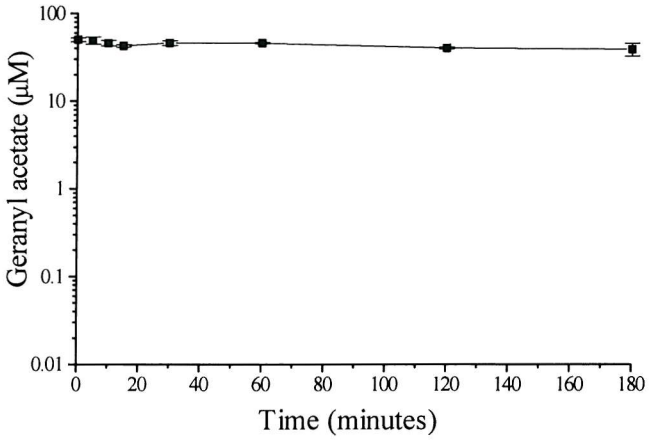
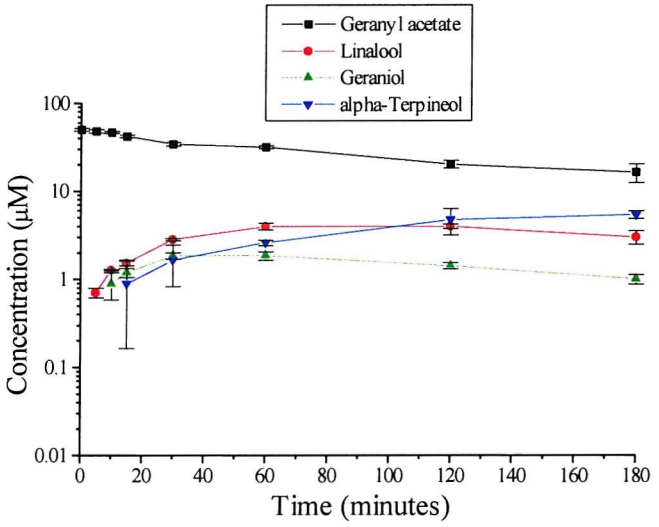


Figure 3.4: 50 µM terpinyl isobutyrate incubated in artificial gastric fluid 37°C for 180 minutes (mean ± SD) **A**. Neutralised artificial gastric fluid not containing pepsin. **B**. Acidic artificial gastric fluid not containing pepsin. **C**. Acidic artificial gastric fluid containing pepsin.

A.



B.



C.

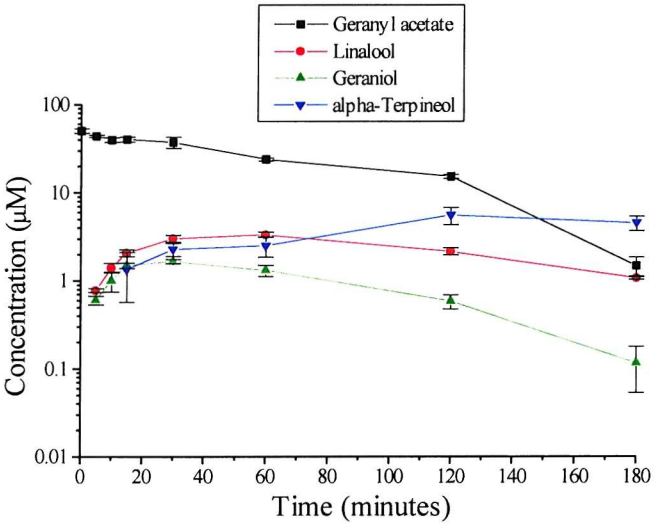
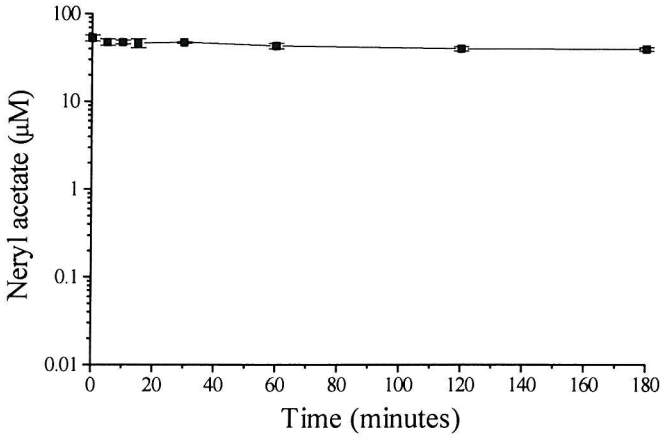
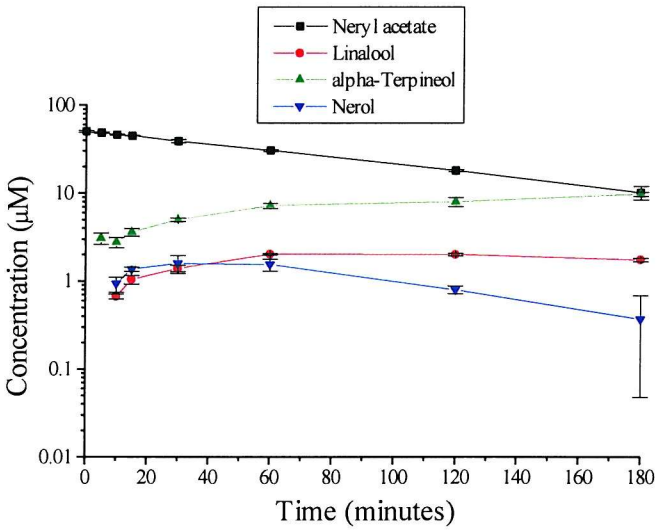


Figure 3.5: 50 µM geranyl acetate incubated in artificial gastric fluid 37°C for 180 minutes (mean ± SD) **A**. Neutralised artificial gastric fluid not containing pepsin. **B**. Acidic artificial gastric fluid not containing pepsin. **C**. Acidic artificial gastric fluid containing pepsin.

A.



B.



C.

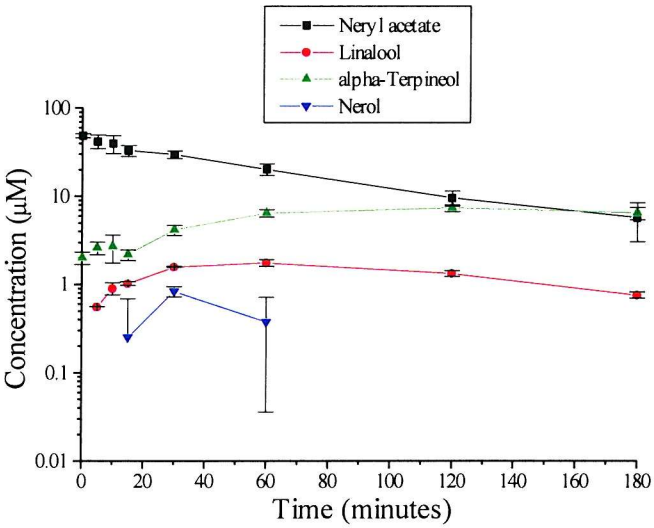


Figure 3.6: 50 μM neryl acetate incubated in artificial gastric fluid 37°C for 180 minutes (mean ± SD) **A**. Neutralised artificial gastric fluid not containing pepsin. **B**. Acidic artificial gastric fluid not containing pepsin. **C**. Acidic artificial gastric fluid containing pepsin.

As discussed above, in comparing acidic artificial gastric fluid containing and not containing pepsin, all monoterpene esters investigated showed no statistically significant differences, indicating the lack of enzymic hydrolysis. However, geranyl butyrate, terpinyl formate, terpinyl butyrate, citronellyl butyrate and citronellyl isobutyrate were more stable in acidic solution containing pepsin than they were in acidic solution not containing pepsin ($P<0.05$). Linalyl propionate (see figure 3.3) and geranyl formate were also more stable in acidic solutions containing enzyme than those not containing enzyme ($P<0.1$), as was linalyl isovalerate ($P<0.2$). For all the linalyl and geranyl esters investigated, which were hydrolysed to multiple alcohols, no significant differences were observed in the rates of alcohol products formed in acidic incubations containing and not containing pepsin (in example see figure 3.3; linalyl propionate), indicating that hydrolysis occurred via the same process in all cases. Longer half-lives in media containing enzyme were presumably due to the stability of these compounds in acid in conjunction with physical stabilisation of the lipophilic compounds against evaporation due to the presence of organic material. In contrast, terpinyl isobutyrate and geranyl acetate were less stable in artificial acidic gastric fluid containing pepsin than they were in artificial acidic gastric fluid not containing pepsin ($P<0.05$), see figures 3.4 and 3.5, and neryl acetate was also less stable ($P<0.1$), as illustrated in figure 3.6. However, as shown by figures 3.4 and 3.5, the $\log^{10}$ concentration versus time graphs for terpinyl isobutyrate and geranyl acetate illustrated late time-points which are not consistent with the initial first-order kinetics. If these portions of the graphs are not taken into account in the calculation of rate constants, both compounds did not show an increase in reaction rate when incubations containing pepsin were compared to acidic controls.

3.2.1.2 Hydrolysis of Monoterpenoid Esters by Artificial Pancreatic Fluid

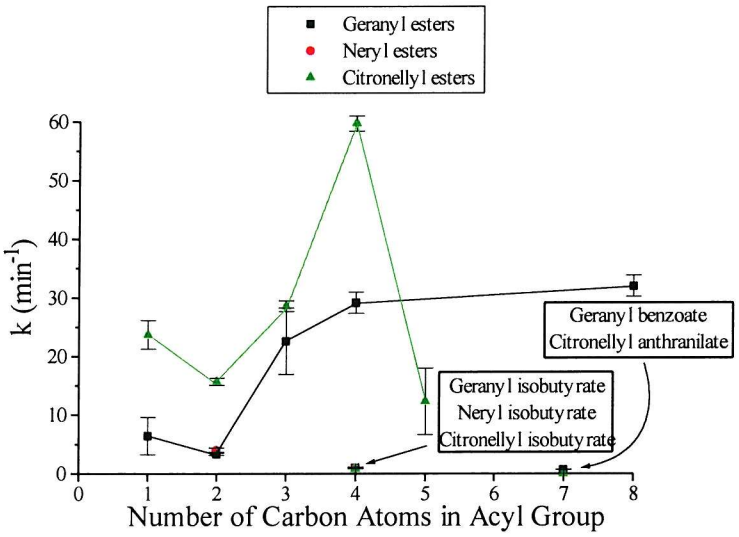
Porcine pancreatin hydrolysed most of the monoterpenoid esters investigated, the rate of hydrolysis was found to be substrate-specific. Figure 3.7A-C illustrates not only the distinct pancreatic esterase specificity lent by the substrates' acyl groups, but also illustrates the effects of steric hindrance around the ester bond due to groups attached to the functional alkyl carbon atoms. It should be noted that due to the anti-evaporative effect of pancreatic fluid (discussed below), figure 3.7 depicts the reaction rates of linalyl isovalerate, terpinyl formate, terpinyl acetate, terpinyl propionate and

terpinyl isobutyrate as derived from an analysis of the difference in rates of formation of the alcohol products (as opposed to the rates of hydrolysis of the parent compound) in artificial pancreatic fluid containing and not containing pancreatin.

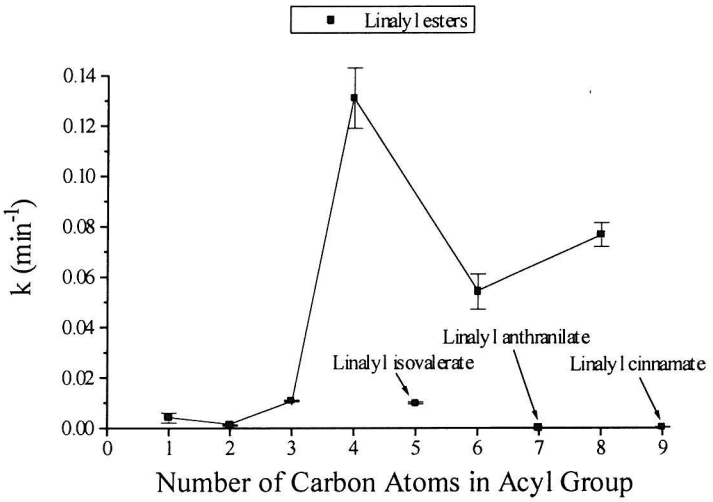
As illustrated in table 3.1 and in figure 3.7, wide differences were observed in the rates of reaction between groups of monoterpene esters, with reaction rates within groups dependent upon the size of the acyl moiety. Esters of butyric acid were metabolised most rapidly in the case of all monoterpene groups investigated. Based upon these rates, esters of butyric acid with no additional carbon chains attached to the functional alkyl carbon atom (geranyl, neryl and citronellyl) were metabolised on average approximately 330 times more rapidly than butyric acid esters with one methyl and one allyl group attached to the functional alkyl carbon atom (linalyl). Linalyl butyrate was metabolised approximately 25 times faster than butyric acid esters of compounds with two methyl groups attached to the functional carbon atom (terpinyl), and as such, terpinyl butyrate was metabolised approximately 8300 times more slowly than monoterpene butyric acid esters which had no additional carbon chains attached to the functional alkyl carbon atom.

The butyric acid ester of citronellol was hydrolysed approximately 2 times more rapidly than the comparable esters of geraniol and nerol. Geraniol and nerol are *trans* / *cis* isomers with the double bond two carbon atoms distant from the ester bond. Citronellol does not have a comparable double bond, and the greater resultant rotational flexibility is possibly the reason why esters of citronellol were hydrolysed more rapidly than esters of geraniol and nerol (the *cis* / *trans* isomerisation of nerol / geraniol had no measurable effect on hydrolysis rates).

A.



B.



C.

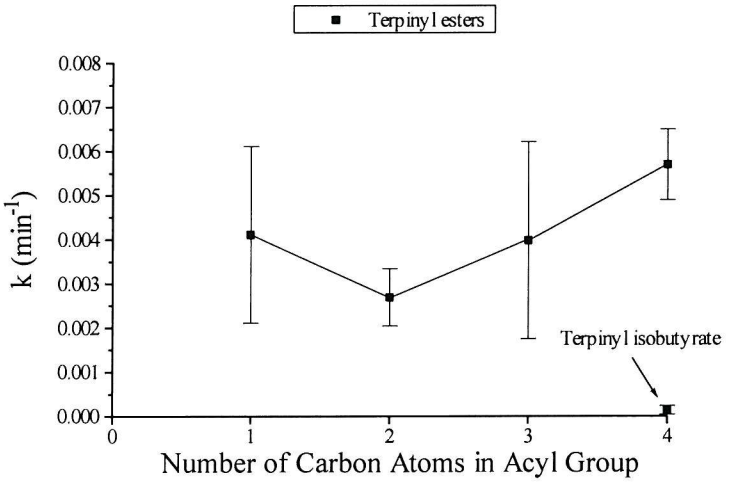


Figure 3.7: Reaction rates (k min^{-1}) of monoterpenoid esters incubated in artificial pancreatic fluid at 37°C for 180 minutes – reaction rates (k min^{-1}) in control buffer (individual points = mean, error bars = SD). Substrates with non-linear acyl groups are indicated. **A.** Substrates with no additional carbon chains attached to the functional alkyl carbon atom. **B.** Substrates with one methyl group and an allyl group attached to the functional alkyl carbon atom. **C.** Substrates with two methyl groups attached to the functional alkyl carbon atom.

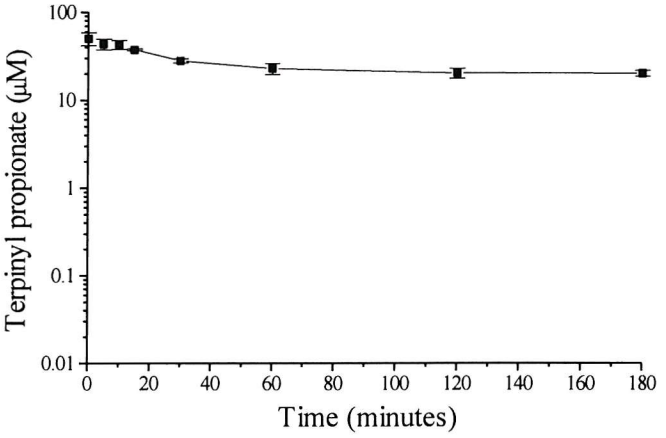
Distinct trends in catalytic specificity were observed in respect of the linear chain-length of the acyl moiety, which were consistent throughout all the monoterpene esters investigated (see figure 3.7). Esters of acetic acid were particularly poor substrates in comparison to esters of formic and propionic acid (although monoterpene formic acid esters were less stable compounds in aqueous solution than the other esters investigated). Butyric acid esters were considerably better substrates than shorter or longer chained substrates, although investigations of esters with linear acyl chains longer than six carbon atoms showed increasing reaction rate. Monoterpene esters with branched-chain acyl moieties showed substantially lower reaction rates in comparison to straight-chained isomers by at-least approximately 30 fold (in the case of geranyl butyrate and isobutyrate), although generally the difference was considerably larger.

3.2.1.2.1 Statistical Comparisons of the Rates of Hydrolysis of Monoterpenoid Esters in Artificial Pancreatic Fluids and Analysis of Reaction Products

In incubations of artificial pancreatic fluid, all the monoterpene esters investigated showed a significant increase ($P<0.05$) in their rates of disappearance from incubations containing pancreatin in comparison to those not containing pancreatin, except terpinyl formate, terpinyl acetate, linalyl formate, linalyl cinnamate and linalyl anthranilate where no statistical differences were observed between incubations. Terpinyl propionate, terpinyl isobutyrate and linalyl isovalerate showed a significant increase in half-lives ($P<0.05$) in artificial pancreatic fluid containing pancreatin in comparison to that not containing pancreatin. In respect of these substrates, table 3.1 and figures 3.8, 3.9 and 3.10 illustrates the formation of alkyl products in the presence of pancreatin in comparison to incubations not containing pancreatin. Linalyl isovalerate was the only substrate which was hydrolysed to form detectable quantities of the major alkyl product in both control and pancreatin-containing incubation systems. Figure 3.10C demonstrates that incubations containing pancreatin displayed a substantially larger product:substrate ratio than incubations not containing pancreatin (the difference being at its least a factor of 3.6 following 0.1 minutes incubation, and at its most 16.6 following 60 minutes incubation). These data therefore indicate that in the cases of terpinyl propionate, terpinyl isobutyrate and

linalyl isovalerate, the amount of physical stabilisation against evaporation lent by the presence of 1 % w/v pancreatin was substantially greater than the rate of hydrolysis as a result of the enzymic action of pancreatin (it should be noted that this effect will have additionally increased the linalool:linalyl isovalerate ratio for control incubations given in figure 3.10C, due to the evaporative loss of substrate).

A.



B.

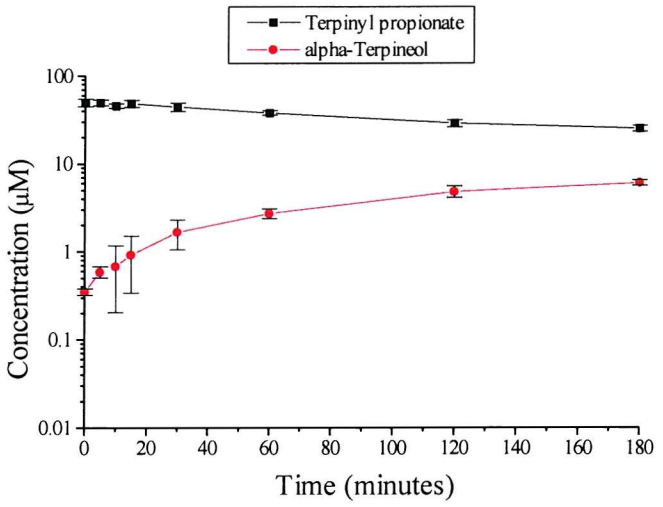
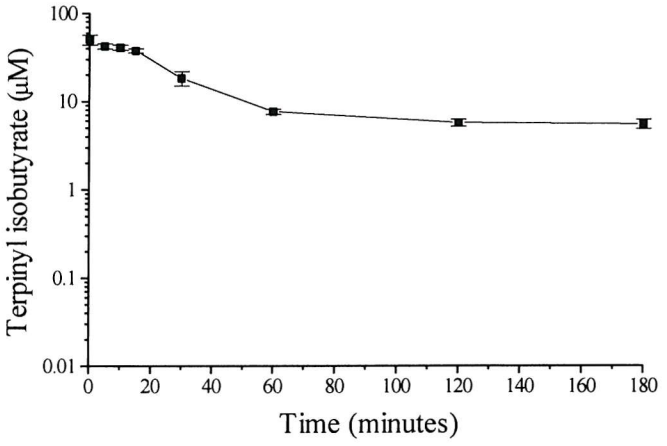


Figure 3.8: 50 µM terpinyl propionate incubated in artificial pancreatic fluid 37°C for 180 minutes (mean ± SD) **A.** Artificial pancreatic fluid not containing pancreatin. **B.** Artificial pancreatic fluid containing pancreatin.

A.



B.

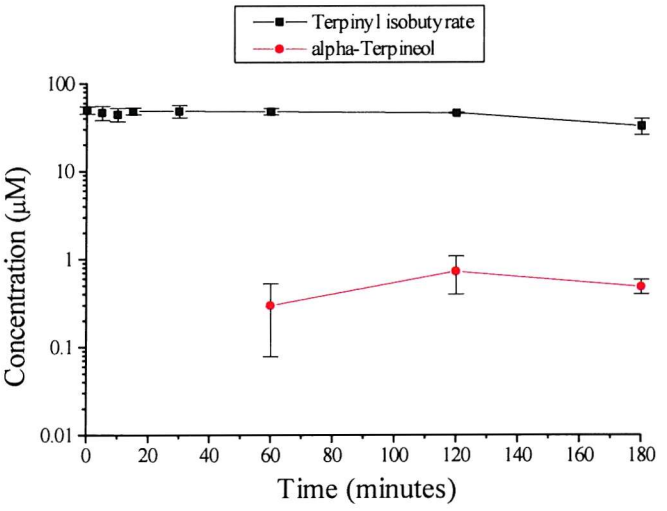
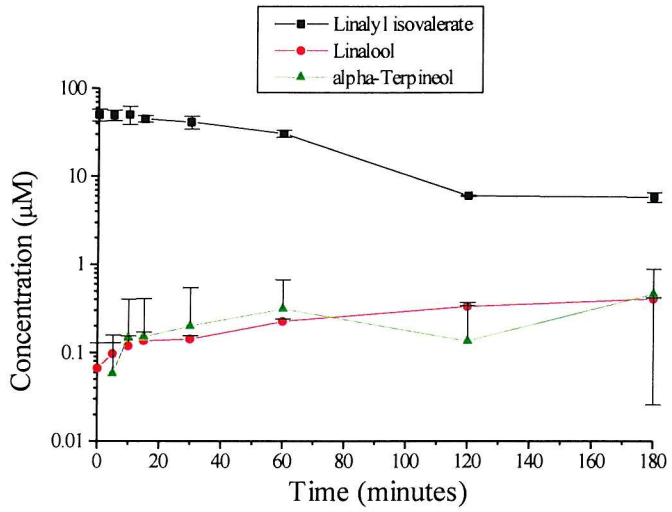
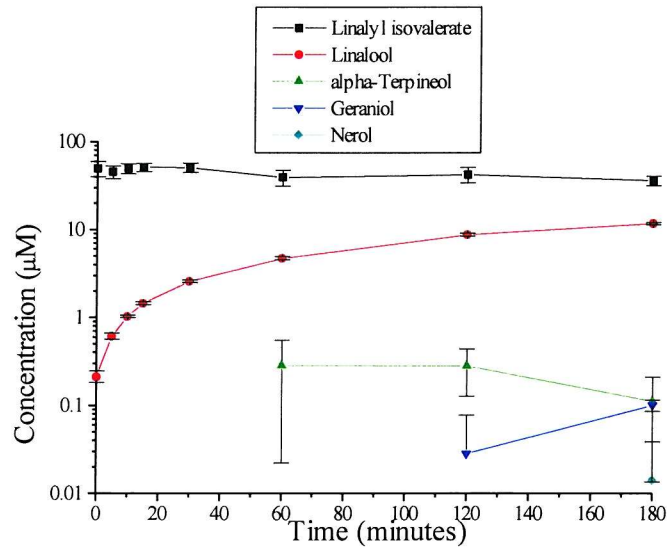


Figure 3.9: 50 μM terpinyl isobutyrate incubated in artificial pancreatic fluid 37°C for 180 minutes (mean ± SD) **A**. Artificial pancreatic fluid not containing pancreatin. **B**. Artificial pancreatic fluid containing pancreatin.

A.



B.



C.

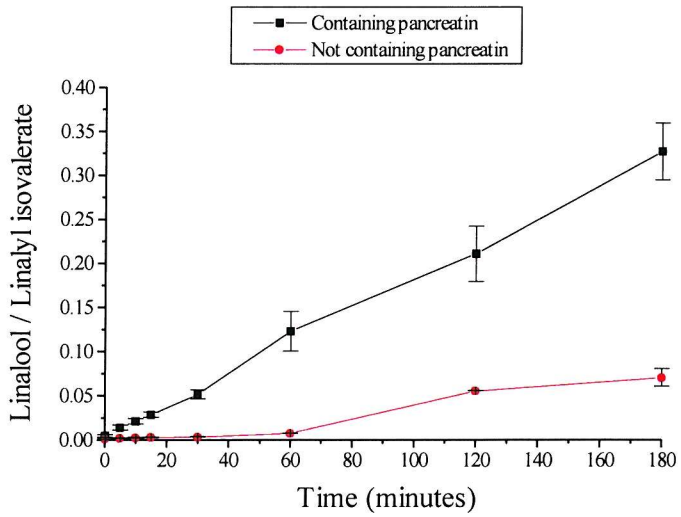


Figure 3.10: 50 μM linalyl isovalerate incubated in artificial pancreatic fluid 37°C for 180 minutes (mean ± SD) **A.** Artificial pancreatic fluid not containing pancreatin. **B.** Artificial pancreatic fluid containing pancreatin. **C.** Concentration of reaction product linalool divided by concentration of substrate linalyl isovalerate in respect of data from **A.** and **B.**

An analysis of reaction products is clearly of benefit when slow rates of hydrolysis are difficult to quantify. In the cases given above the degree of stabilisation of substrates against evaporation due to sequestration in organic material, in comparison to controls not containing organic material, is greater than the rate of hydrolysis of the substrate due to enzymic action. Due to the unusual chemistry of the monoterpenes under study (which has been further investigated in Chapter 4) it is a common feature throughout the investigations that the type and quantities of reaction products frequently differentiates enzymic from non-enzymic hydrolysis.

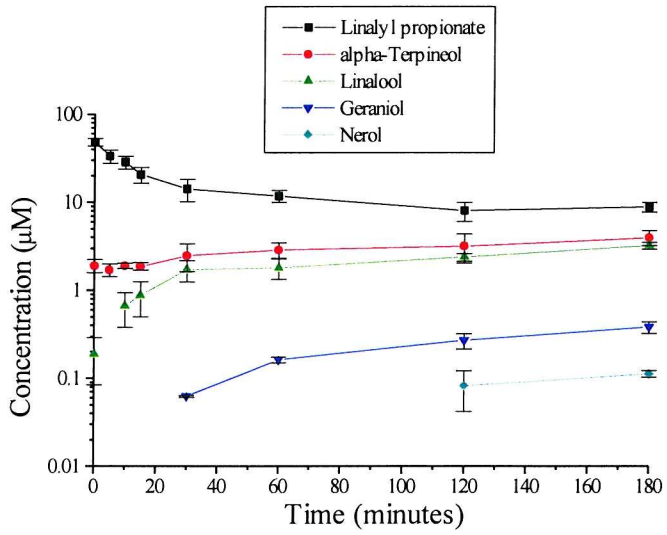
The hydrolysis of linalyl esters with an acyl-group carbon chain-length of less than 4 produced the alcohols linalool and α-terpineol with smaller quantities of geraniol and nerol. Except in the case of linalyl formate, the quantity of α-terpineol formed was always greater or equal to the quantity of linalool formed during incubations not containing pancreatin. In the case of incubations containing pancreatin, the quantity of linalool formed was always greater than α-terpineol, as illustrated with linalyl propionate in figure 3.11. This relationship was also true for linalyl esters with an acyl-group carbon chain-length of 4 or more, except that geraniol and nerol were generally not detected as reaction products. Linalyl isovalerate was an exception in that geraniol and nerol were identified as reaction products, as illustrated in figure 3.10.

The alkyl moiety of geranyl esters appeared to be more resistant than linalool to rearrangement during ester hydrolysis. Geraniol was always the major product of geranyl ester hydrolysis in incubations containing and not containing pancreatin, although smaller quantities of α-terpineol, linalool and nerol were also detected following the hydrolysis of some geranyl esters. However, in incubations containing pancreatin the quantity of nerol produced (the *cis*-isomer of geraniol) was always greater than the quantity of any other alcohol product except for geraniol, this was in contrast to incubations not containing pancreatin where more α-terpineol may be produced than nerol, as illustrated with geranyl acetate in figure 3.12.

During the non-enzymic hydrolysis of neryl acetate and neryl isobutyrate the quantity of α-terpineol produced was greater than the quantity of nerol produced, this was not the case during enzymic hydrolysis, as illustrated in figure 3.13.

The non-enzymic and enzymic hydrolysis of both terpinyl and citronellyl esters produced the parent alcohol only, except for citronellyl butyrate which formed some geraniol, as illustrated in figure 3.14. Citronellyl esters showed greater stability in aqueous solution than the other monoterpene esters investigated.

A.



B.

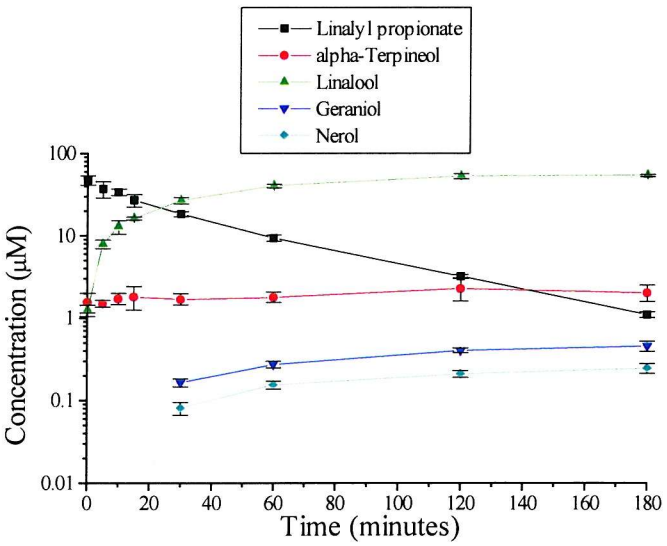
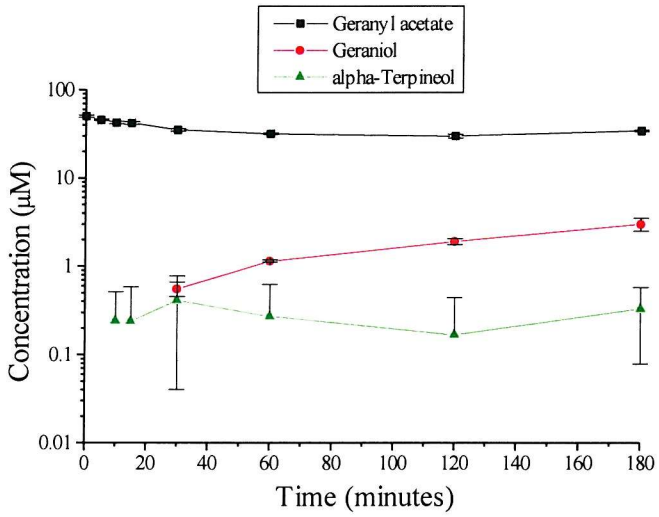


Figure 3.11: 50 μM linalyl propionate incubated in artificial pancreatic fluid 37°C for 180 minutes (mean ± SD) **A**. Artificial pancreatic fluid not containing pancreatin. **B**. Artificial pancreatic fluid containing pancreatin.

A.



B.

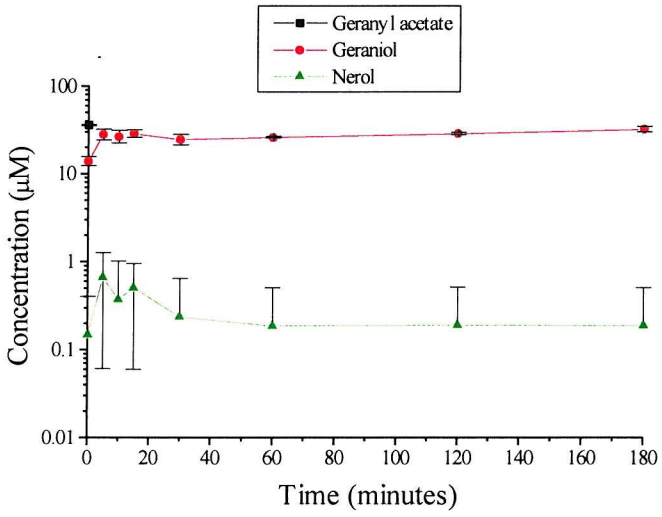
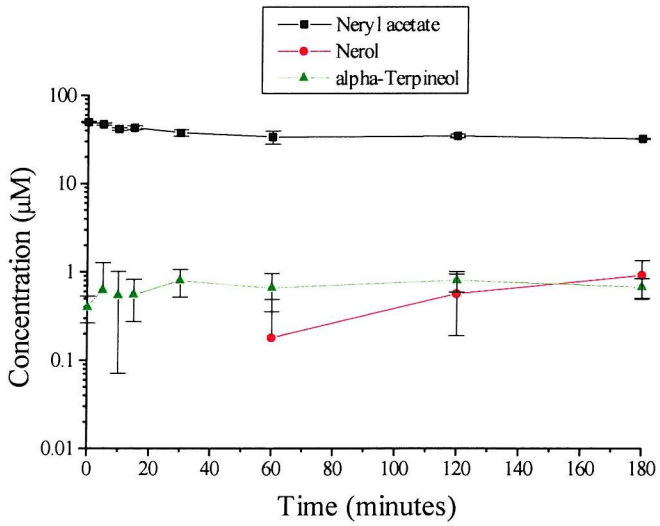


Figure 3.12: 50 μM geranyl acetate incubated in artificial pancreatic fluid 37°C for 180 minutes (mean ± SD) **A**. Artificial pancreatic fluid not containing pancreatin. **B**. Artificial pancreatic fluid containing pancreatin.

A.



B.

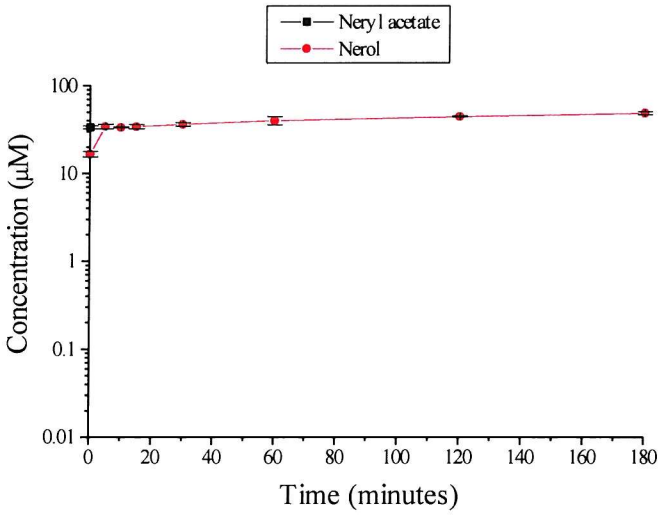
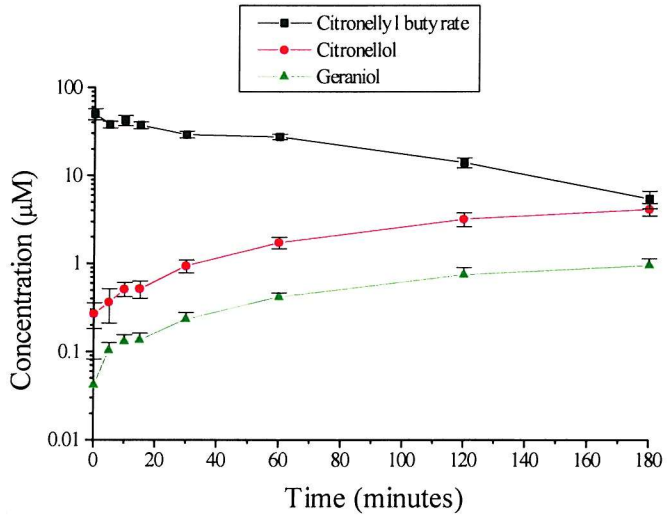


Figure 3.13: 50 μM neryl acetate incubated in artificial pancreatic fluid 37°C for 180 minutes (mean ± SD) **A**. Artificial pancreatic fluid not containing pancreatin. **B**. Artificial pancreatic fluid containing pancreatin.

A.



B.

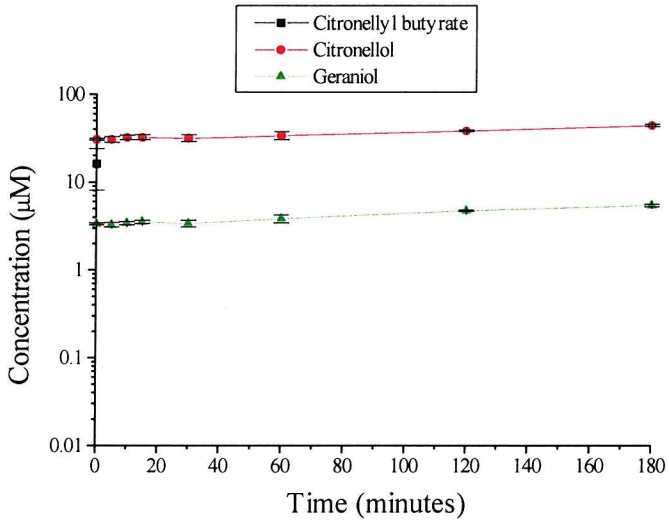


Figure 3.14: 50 μM citronellyl butyrate incubated in artificial pancreatic fluid 37°C for 180 minutes (mean ± SD) **A**. Artificial pancreatic fluid not containing pancreatin. **B**. Artificial pancreatic fluid containing pancreatin.

3.2.2 *Investigations into the Enzyme Characteristics and Stereoselective Hydrolysis of Linalyl Butyrate by Artificial Pancreatic Fluid*

The metabolism of linalyl butyrate by artificial pancreatic fluid (as described in table 3.1) was found to show slight stereoselectivity, with preference over the initial 5 minutes of incubations for (S)-linalyl butyrate, as illustrated in figure 3.15 and table 3.2.

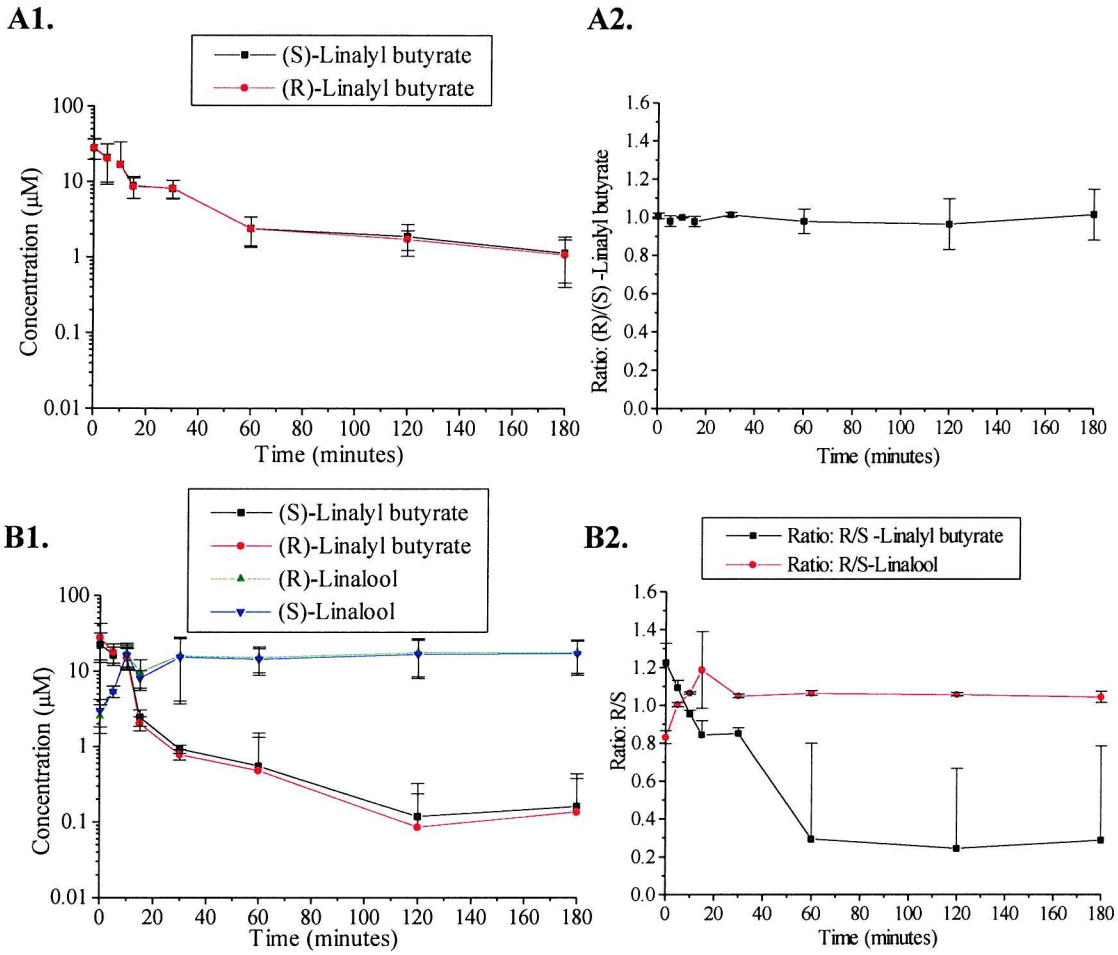


Figure 3.15: Stereoselective metabolism of 50 µM linalyl butyrate incubated in artificial pancreatic fluid over 180 minutes at 37°C (mean ± SD) **A.** Incubations not containing pancreatin (**A1** concentration versus time, **A2** ratio: R/S versus time) **B.** Incubations containing pancreatin (**B1** concentration versus time, **B2** ratio: R/S versus time). Initial ratio (R)/(S) –linalyl butyrate = 1:1.

Figure 3.16 and table 3.2 illustrate the results of incubations containing 200 µM linalyl butyrate in artificial pancreatic fluid, with an additional set of incubations containing 100 µM paraoxon (see 2.2.7 for method development). These

investigations attempted to better characterise the stereoselectivity described above (figure 3.15), and to determine the contribution of A- and B-esterases to the hydrolysis of linalyl butyrate by porcine pancreatin, in an attempt to identify possible stereoselective characteristics of different esterase types. No appreciable A-esterase activity was detected, and all hydrolytic metabolism was due to the action of B-esterase activity (including stereoselectivity during the first minutes of the incubations which was consistent with that demonstrated using 50 μM substrate). These studies additionally demonstrated that the S-isomer of linalyl butyrate was slightly favoured during hydrolysis which occurred via entirely non-biological means in aqueous solution.

As no appreciable A-esterase metabolism of linalyl butyrate was detected in artificial pancreatic fluid (based upon an analysis of the quantity of product remaining at the final time points, no more than 0.8 % of artificial pancreatic fluid linalyl butyrate esterase activity remained following inhibition with paraoxon), planned investigations which were to attempt to identify inhibitors of A-esterase activity were not undertaken. However, further to very limited information[69; 128] that known serine protease inhibitors may differentially inhibit serine-esterase isoenzymes, investigations were undertaken utilising the protease inhibitor phenylmethylsulfonyl fluoride (see 2.2.7 for method development). The results of these investigations are shown in table 3.2. No statistically significant differences were observed in the rates of metabolism between incubations containing and not containing PMSF, and the initial stereoselectivity for (S)-linalyl butyrate observed in previous studies (illustrated in figure 3.16B2) was apparent in incubations containing pancreatin in the presence and absence of PMSF.

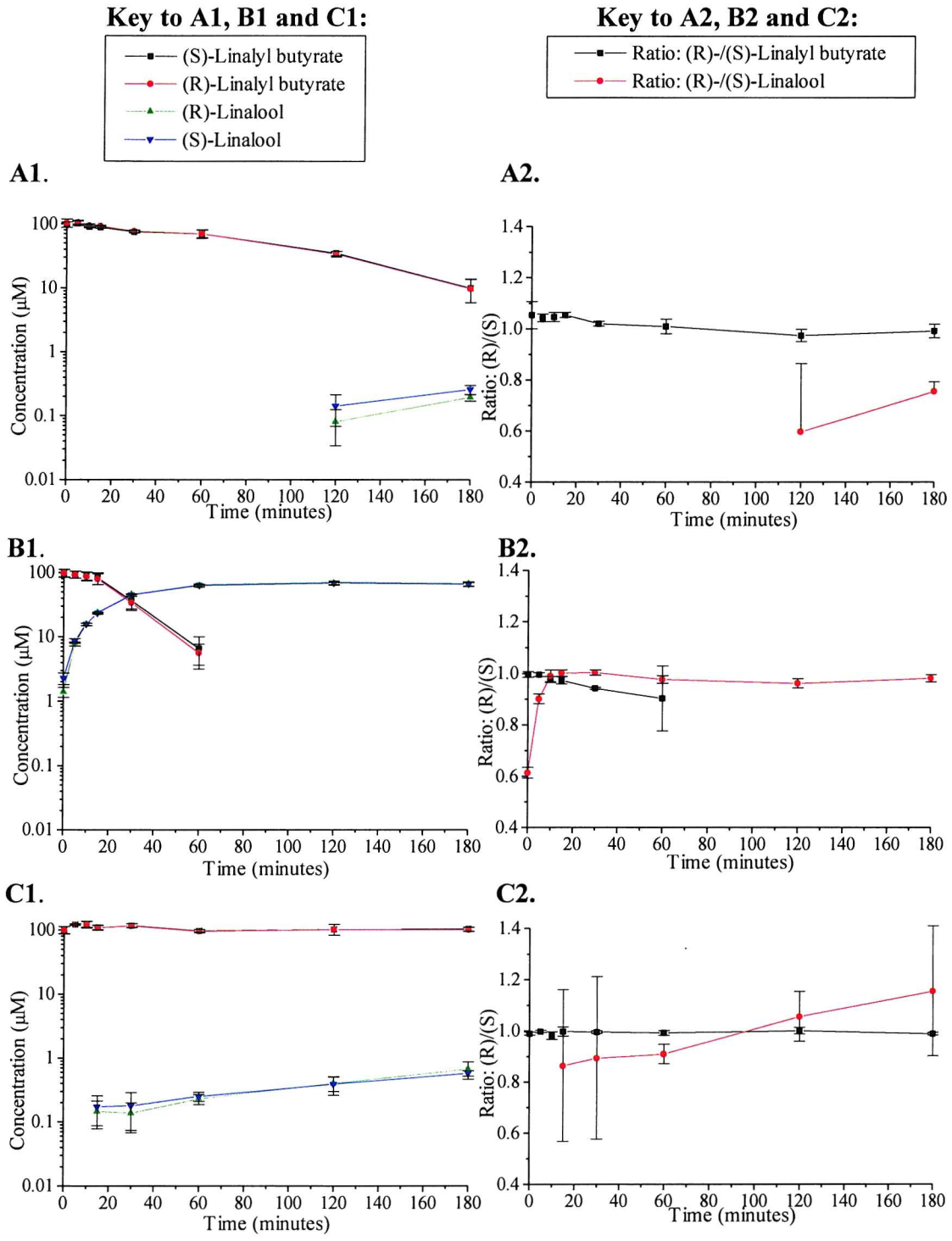


Figure 3.16: Stereoselective metabolism of 200 µM linalyl butyrate incubated in artificial pancreatic fluid (mean ± SD). **A.** Incubations not containing pancreatin (**A1** concentration versus time, **A2** ratio R/S versus time) **B.** Incubations containing pancreatin (**B1** concentration versus time, **B2** ratio R/S versus time) **C.** Incubations containing pancreatin and 100 µM paraoxon (**C1** concentration versus time, **C2** ratio R/S versus time). Initial ratio (R)/(S) –linalyl butyrate = 1:1.

Investigation and Compound	**Control (no pancreatin, no inhibitor)**	**Test (with pancreatin, no inhibitor)**	**Test + Inhibitor (with pancreatin, with inhibitor)**
50 μM substrate			
(R)-Linalyl butyrate	40 ± 5	5.0 ± 0.5	-
(S)-Linalyl butyrate	41 ± 6	5.6 ± 0.6	-
(R)-Linalool	-	0.32 ± 0.01*	-
(S)-Linalool	-	0.24 ± 0.00*	-
200 μM substrate +/- 100 μM paraoxon			
(R)-Linalyl butyrate	56 ± 10	14 ± 1	11000 ± 17000
(S)-Linalyl butyrate	58 ± 10	15 ± 2	11000 ± 17000
(R)-Linalool	0.017 ± 0.009	0.43 ± 0.02*	0.0088 ± 0.0024
(S)-Linalool	0.011 ± 0.006	0.31 ± 0.02*	0.0069 ± 0.0008
200 μM substrate +/- 100 μM PMSF			
(R)-Linalyl butyrate	78 ± 6	18 ± 2	16 ± 1
(S)-Linalyl butyrate	92 ± 9	17 ± 1	15 ± 1
(R)-Linalool	-	0.43 ± 0.11*	0.33 ± 0.10*
(S)-Linalool	-	0.32 ± 0.13*	0.23 ± 0.12*

Table 3.2: Reaction rates [disappearance of substrates = $t_{½}$ (minutes) ± SD, and rate of formation of alkyl products = k (minutes^{-1}) ± SD] of 50 μM and 200 μM linalyl butyrate incubated in artificial pancreatic fluid with and without 100 μM paraoxon or phenylmethylsulfonyl fluoride. Incubations performed at 37°C for 180 minutes (120 minutes in the case of PMSF investigations). * - rate based upon analysis of the first three time-points only.

The initial stereoselectivity of artificial pancreatic fluid for (S)-linalyl butyrate appeared entirely due to processes which occurred before the first time-point (0.1 minutes), subsequent to which the rate of metabolism of (R)-linalyl butyrate, as measured by the rate of formation of metabolites, was always moderately more rapid than the S-stereoisomer (although statistically significant differences were not observed). This process was most clearly observed in respect of incubations containing 50 μM substrate were between 0 and 0.1 minutes incubation the stereoisomer ratio: R/S for linalyl butyrate increased to approximately 1.2, falling to 0.85 over the subsequent 15 minutes (see figure 3.15B2). The greatest difference in stereoselectivity was observed in incubations of 200 μM linalyl butyrate were between 0 and 0.1 minutes incubation, the S-isomer was favoured as a substrate

approximately 1.7 times more than the R-isomer (as measured by the formation of alkyl products).

3.2.3 *Hydrolysis of Cinnamyl / Cinnamate Esters by Artificial Gastrointestinal Fluids*

The hydrolysis of esters in artificial human gastric fluid was shown to be pH-catalysed and not enzyme mediated. As such, the hydrolysis of cinnamyl esters in artificial gastric fluid was not studied except in the case of cinnamyl propionate the results of which are given in table 3.3. This investigation was undertaken to enable a comparison between the hydrolysis of cinnamyl propionate (and furfuryl propionate) in solutions of pH 1.2 and rat gastric contents homogenates pH 2.5 (see Chapter 5). The hydrolysis by artificial pancreatic fluid of a series of 9 esters of cinnamyl alcohol or cinnamic acid, and the methyl ester of anthranilic acid were investigated, the results of which are given in table 3.4.

	$t_{½}$ (minutes) ± SD with identified metabolites	
	pH 7.0	**pH 1.2**
Cinnamyl propionate	∞ No metabolites	170 ± 60 ca

Table 3.3: Cinnamyl propionate incubated in acidic media (artificial gastric fluid not containing pepsin) for 180 minutes 37°C (disappearance of substrate = $t_{½}$), ca = cinnamyl alcohol.

Cinnamyl propionate was more resistant to acid-catalysed hydrolysis than comparable esters of monoterpenoid alcohols, although it was more readily hydrolysed than furfuryl propionate (see 3.2.4 below).

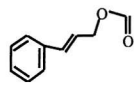
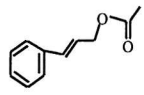
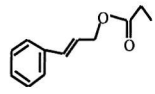
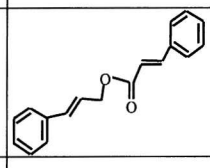
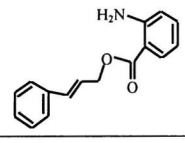
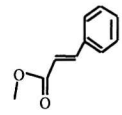
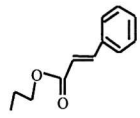
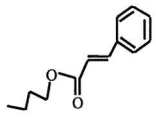
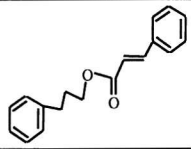
Compound	Structure	Solubility H_2O, 37°C (mM)	Artificial Pancreatic Fluid $t_{½}$ (minutes) ± SD with identified metabolites	
			No Pancreatin	With Pancreatin
Cinnamyl formate		8.57 ± 2.05	55 ± 2 ca	<0.01 ca
Cinnamyl acetate		2.15 ± 0.11	21000 ± 34000 No metabolites	<0.01 ca
Cinnamyl propionate		0.84 ± 0.21	∞ No metabolites	<0.01 ca
Cinnamyl cinnamate		6.10 ± 1.06	850 ± 50 No metabolites	0.04 ± 0.01 ca
Cinnamyl anthranilate		27.42 ± 5.58	4200 ± 4800 No metabolites	780 ± 780 No metabolites
Methyl cinnamate		1.65 ± 0.84	∞ nd	14 ± 0 nd
Propyl cinnamate		0.22 ± 0.07	1100 ± 200 nd	1.4 ± 0.1 nd
Butyl cinnamate		0.20 ± 0.04	180 ± 50 nd	0.04 ± 0.00 nd
3-Phenylpropyl cinnamate		5.83 ± 2.24	1200 ± 600 No metabolites	0.06 ± 0.02 ppa
Methyl anthranilate		20.65 ± 6.87	∞ nd	∞ nd

Table 3.4: Esters of cinnamyl alcohol, cinnamic acid and the methyl ester of anthranilic acid incubated in artificial pancreatic fluid for 180 minutes, 37°C (disappearance of substrate = $t_{½}$). ca = cinnamyl alcohol, ppa = phenylpropyl alcohol, nd = alcohol hydrolysis product not detectable with the methods used.

All esters of cinnamyl alcohol were rapidly hydrolysed by artificial pancreatic fluid, except cinnamyl anthranilate. In the case of esters of cinnamyl alcohol which have linear acyl moieties, hydrolysis was too rapid to enable quantification. Cinnamyl cinnamate was hydrolysed less rapidly than esters with linear acyl moieties.

Hydrolytic enzymes in porcine pancreatin were observed to prefer ester substrates with alkyl moiety carbon chains greater than 4 atoms in size, this is demonstrated in the hydrolytic rates of methyl, propyl and butyl cinnamate. There was no observable hydrolysis of methyl anthranilate. These results illustrate the distinct substrate specificities lent by the alkyl and acyl moieties, for example, cinnamyl formate was hydrolysed by pancreatin at least 1400 times more rapidly than methyl cinnamate.

No statistically significant difference was seen in the rates of hydrolysis of cinnamyl cinnamate and 3-phenylpropyl cinnamate, as such, structural rigidity lent by the double bond in the propyl chain of the alkyl moiety did not have a measurable impact upon hydrolytic rate.

3.2.4 Hydrolysis of Furfuryl Esters by Artificial Gastrointestinal Fluids

The hydrolysis of furfuryl propionate in artificial gastric fluid not containing pepsin was studied, the results of which are given in table 3.5. The hydrolysis by artificial pancreatic fluid of a series of 4 esters of furfuryl alcohol were investigated, the results of which are given in table 3.6.

	$t_{½}$ (minutes) ± SD	
	pH 7.0	**pH 1.2**
Furfuryl propionate	∞	390 ± 120

Table 3.5: Furfuryl propionate incubated in acidic media (artificial gastric fluid not containing pepsin) for 180 minutes, 37°C (disappearance of substrate = $t_{½}$).

Furfuryl propionate was hydrolysed in an acidic environment less rapidly than comparable esters of monoterpenoid alcohols or cinnamyl propionate.

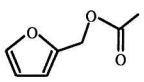
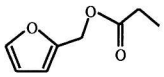
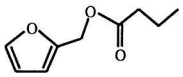
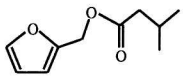
Compound	Structure	Solubility H_2O, 37°C (mM)	$t_{½}$ (minutes) ± SD Artificial Pancreatic Fluid	
			No Pancreatin	With Pancreatin
Furfuryl acetate		10.27 ± 1.34	480 ± 290	<0.01
Furfuryl propionate		2.16 ± 0.42	∞	<0.01
Furfuryl butyrate		0.85 ± 0.53	∞	<0.01
Furfuryl 3-methyl butanoate		0.46 ± 0.29	700 ± 320	5.1 ± 0.4

Table 3.6: Esters of furfuryl alcohol incubated in artificial pancreatic fluid for 180 minutes, 37°C (disappearance of substrate = $t_{½}$).

All esters of furfuryl alcohol were rapidly hydrolysed by porcine pancreatin, however, furfuryl 3-methyl butanoate was hydrolysed at least 500 times less rapidly than the other furfuryl esters investigated.

3.3 Discussion

3.3.1 Effect of Artificial Gastric Fluid on Esters

Monoterpenoid esters were not observed to undergo enzymic hydrolysis in artificial human gastric fluid due to the action of pepsin. A comparison of the rates of acid-catalysed hydrolysis of a series of monoterpenoid esters is given in figure 3.1. The stability of the monoterpenoid esters in acidic solution (pH 1.2) in comparison to neural controls decreased in the order:

citronellyl esters > neryl esters ≥ terpinyl esters ≥ geranyl esters > linalyl esters

In all cases, an increasing number of carbon atoms in the acyl group, resulted in a trend towards increasing stability in acidic solution. In the case of the largest series investigated, an approximate 1000 fold difference was observed in the rate of hydrolysis of linalyl octanoate in comparison to linalyl formate. Both cinnamyl propionate and furfuryl propionate were found to be less prone to hydrolysis in an acidic environment than comparable monoterpenoid esters investigated. However, cinnamyl propionate underwent more rapid hydrolysis than furfuryl propionate.

Figure 3.17 illustrates the proposed mechanism of acid-catalysed hydrolysis of monoterpene esters, with the example of linalyl esters. Following protonation in an acidic environment, the functional acyl carbon atom undergoes nucleophilic attack by the lone electron pair of H_2O, a series of structural rearrangements via transient intermediates subsequently leads to the molecule being split, thus producing the alcohol and carboxylic acid products. In an acidic environment further protonation of monoterpene alcohols such as linalool leads to the loss of the hydroxyl group and formation of a carbocation intermediate, this may rearrange to form secondary monoterpene products following hydrogenation. As monoterpene alcohols were found to undergo unusual acid-catalysed isomerisations, this was the subject of further study described in Chapter 4.

Hydrocarbon groups are 'electron donating' when bonded to more electronegative elements such as oxygen. Presumably, the presence of electron donating hydrocarbon groups forming the acyl moiety helps prevent hydrogenation of intermediate 1 and/or decreases the stability of intermediate 2, thus esters with larger acyl moieties are less prone to acid-catalysed hydrolysis than esters with a smaller acyl moiety. Linalyl cinnamate is more acid-labile than would be predicted for an analogue with a linear acyl moiety, this may be due to electron delocalisation in the benzene ring reducing electron donating capacity. Furthermore, the presence of *pi* bonds two carbon atoms distant from the ester bond may lead to stabilisation of intermediate 3 via charge delocalisation. These may also be the reasons why cinnamyl propionate was observed to undergo more rapid hydrolysis than furfuryl propionate. Branched chain hydrocarbons possess a greater capacity to donate electronegative charge than their

linear analogues, thus terpinyl and citronellyl esters of branched chain acids were more stable than their linear isomers. However, linalyl isovalerate was more acid-labile than anticipated from the series of linalyl esters investigated. Perhaps the extra electron charge available adds stability to intermediate 3 which more than off-sets the instability lent to intermediate 2 (or perhaps has an effect in encouraging initial protonation), this hypothesis is more salient when considered in conjunction with the following.

Linalyl esters were more prone to acid-catalysed hydrolysis than the other monoterpene esters investigated. This was probably due to the stabilisation of intermediate 3 by the presence of a methyl group and allyl group in addition to the main body of the monoterpene, all donating electronegative charge towards the functional alkyl carbon atom. However, delocalisation of *pi*-electrons may also assist in this stabilisation. The ester bond of the less acid-labile geranyl and neryl esters is positioned on an allyl group, and presumably the stabilisation lent by charge delocalisation in response to the formation of the oxonium tri-hydroxy compound (intermediate 3), is sufficient to stabilise this intermediate, although possibly to a smaller degree than in the case of linalyl esters (which has additional electronegative charge-donating carbon chains). Linalool, geraniol and nerol will undergo reduction in acidic media to form a carbocation intermediate and subsequent isomerisation product(s) (as further investigated in Chapter 4), and this may also assist in promoting ester hydrolysis. The ester bond of terpinyl esters is situated on an atom which may receive electronegative charge from two methyl groups and the main body of the compound. As such, a similar degree of intermediate 3 stabilisation may occur in the case of terpinyl esters as it did in the case of geranyl and neryl esters. However, α-terpineol was found to be more stable in an acidic environment than linalool, geraniol or nerol and this may be related to the stability of terpinyl esters in acidic media. Citronellyl esters were remarkably resistant to acid-catalysed hydrolysis, no electron donating species are present to stabilise positively charged intermediates save the main body of the compound itself. Furthermore, citronellol has no *pi*-electrons adjacent to the functional group, and is unlikely to form a stable carbocation intermediate or isomerisation product(s) in acidic media.

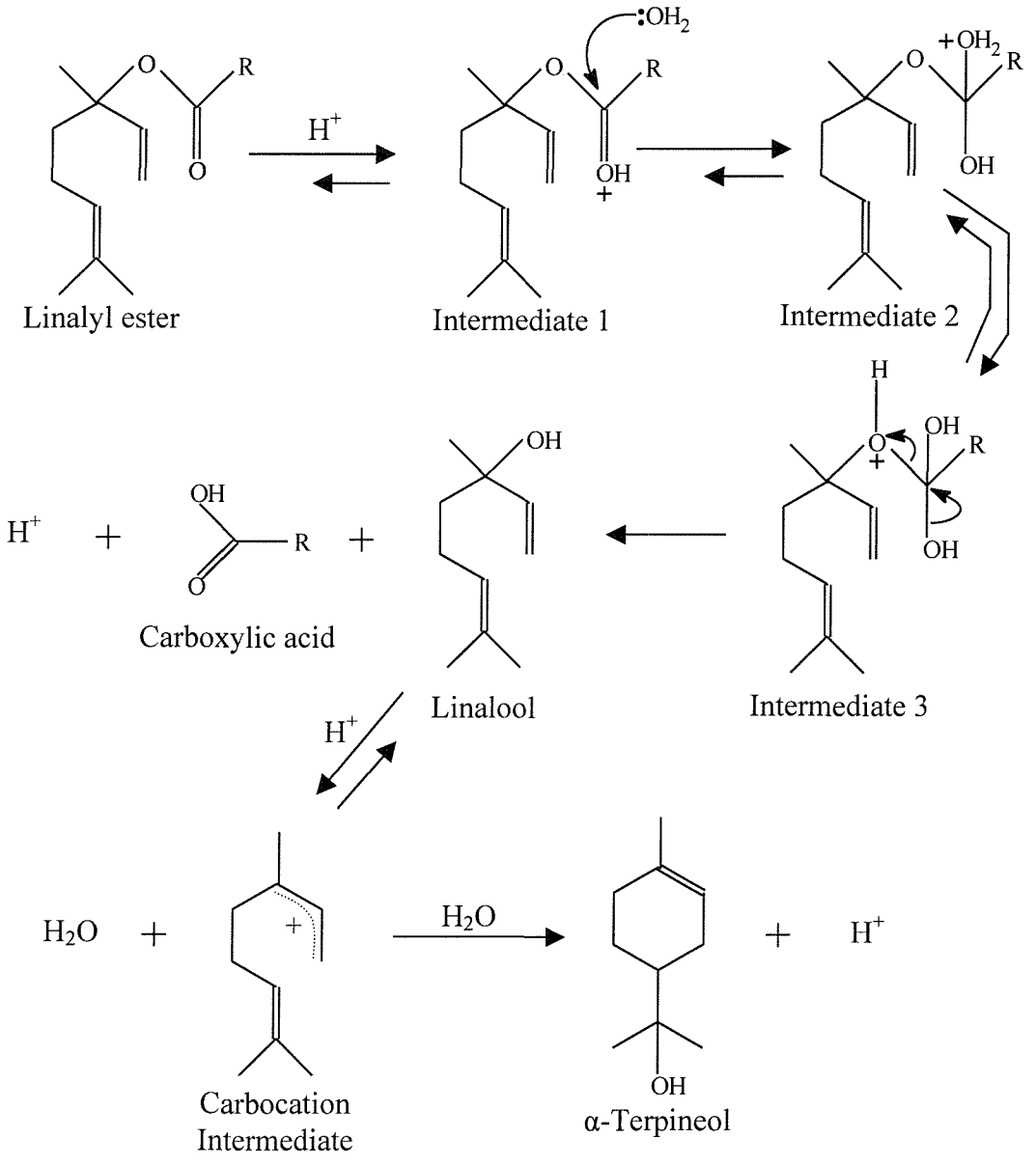


Figure 3.17: Postulated mechanism of acid-catalysed hydrolysis of linalyl esters. Curved arrows represent the movement of electrons.

Terpinyl and citronellyl esters underwent acid-catalysed hydrolysis to produce the parent alcohol only, except in the case of terpinyl formate; a limited proportion of which lost the methanoic acid group entirely (an acyl moiety which possesses an overall δ^- charge) to form limonene or it s isomer terpinolene. Neryl esters primarily formed α-terpineol, geranyl esters primarily formed linalool, and linalyl esters

primarily formed linalool and α-terpineol (although additional minor products also resulted). The structural rearrangement of monoterpenoid alcohols in acidic solutions is further discussed in Chapter 4.

It has been demonstrated that monoterpenoid alcohols are stable in an aqueous environment at neutral pH (see 4.3.1). Esters with small acyl carbon chains showed some degree of instability in a neutral aqueous environment, nevertheless, with all monoterpenoid ester substrates studied the alkyl products formed were identical to those formed in acidic solution. Importantly however, in contrast to incubations of monoterpenoid esters in acidic solution (in which the primary product is the parent alcohol which subsequently undergoes acid-catalysed rearrangement), in incubations at neutral pH all alcohol products appeared to be formed concomitantly with the parent alcohol being the major or second major product. Clearly the mechanism of hydrolysis was different to that given in figure 3.17, and figure 3.18 illustrates the proposed mechanism at neutral pH, using the example of linalyl esters.

Figure 3.18 demonstrates that the hydrolysis of monoterpenoid esters at neutral pH concomitantly produces alcohols which have been demonstrated to be a product of acid-catalysed rearrangement due to the formation of charge-separated intermediates and the stability of the carbocation intermediate. In a neutral environment, the ratio of products due to ester hydrolysis is constant, and as described in 4.3.4, can be used as a marker to identify the presence of hydrolysis as a result of esterase activity which produces only the parent alcohol via a mechanism described in 1.2.2.

It is interesting to note the differences in hydrolytic rate between monoterpenoid esters in neutral artificial gastric fluid (gastric fluid control) and pancreatic fluid without pancreatin (pancreatic fluid control). Essentially the former is a solution containing 34 mM NaCl whereas the latter is a saturated solution of sodium hydrogen phosphate salts. Monoterpenoid esters generally showed longer half-lives in gastric fluid controls than they did in pancreatic fluid controls (although this was not clear in the case of linalyl esters). The large number of ions in pancreatic fluid controls may have stabilised charged intermediates so that hydrolysis was more energetically favourable.

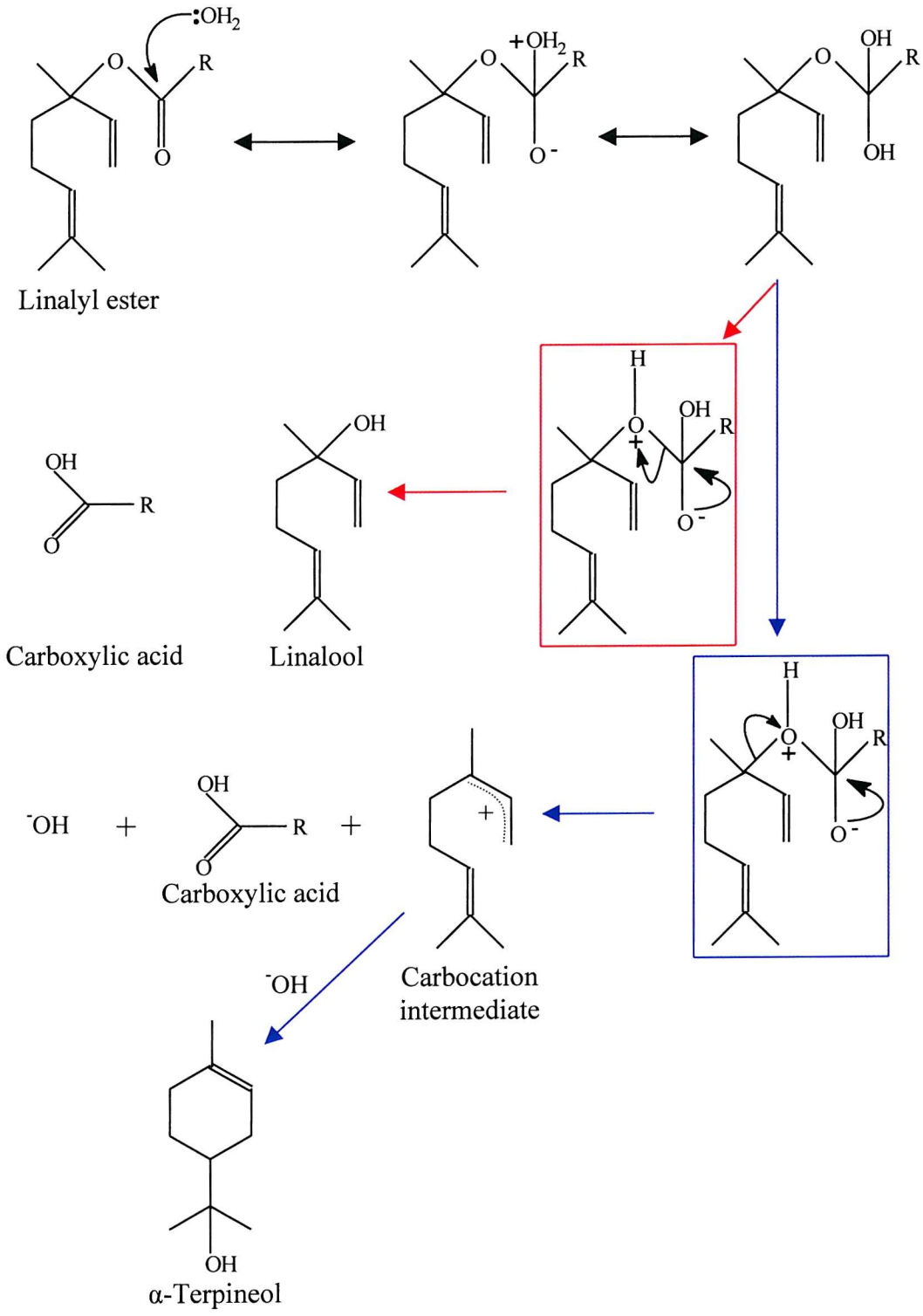


Figure 3.18: Proposed mechanism of hydrolysis of linalyl esters in neutral solution. Charge-separated intermediates are indicated by boxes, major route = red, minor route = blue. Curved arrows represent the movement of electrons.

3.3.2 *Effect of Artificial Pancreatic Fluid on Esters*

The evaluation of the fate of methyl anthranilate and esters of monoterpene alcohols, cinnamyl alcohol, cinnamic acid and furfuryl alcohol in a model of human pancreatic fluid was undertaken in order to identify possible differences between compounds in pre-absorption hydrolytic fate. Furthermore, as pancreatic fluid contains a range of esterases and lipases (see 1.2.1.1.2), the activities of which are at present essentially undistinguishable from that attributed to tissue carboxylesterases, these investigations were conducted as a screening exercise to identify compounds which would be used in further *ex-vivo* and *in-vivo* studies (see Chapters 5 & 6). These additional studies were aimed partly at assessing the suitability of artificial pancreatic fluid as a predictive model of tissue esterase activity.

The substrates investigated in artificial gastrointestinal fluids were selected for study to enable data to be gathered regarding the effects upon hydrolytic rates attributed to defined structural features of both the alkyl and acyl moieties of food flavouring esters. It is interesting to note that in the case of monoterpenoid esters which were found to be poor substrates for enzymic hydrolysis, an analysis of the products formed was often insightful as enzymic hydrolysis produced the parent alcohol only, whereas during non-enzymic hydrolysis a constant ratio of related alkyl products was produced (as described above).

Effects upon hydrolytic rates which were due to the structure of the alkyl moiety were distinct in that the rates of hydrolysis by porcine pancreatin were observed to decrease in the order:

$$\text{Furfuryl} \approx \text{Cinnamyl} > \text{Citronellyl} > \text{Geranyl} \approx \text{Neryl} > \text{Linalyl} > \text{Terpinyl}$$

Figure 3.19 illustrates that the structural features in this sequence which are most probably determinant in the rate of hydrolysis, is primarily steric hindrance around the ester bond by additional carbon chains attached to the functional alkyl carbon atom, and secondarily the accessibility of the ester bond as a result of the macro-configuration (folding pattern) of the compound.

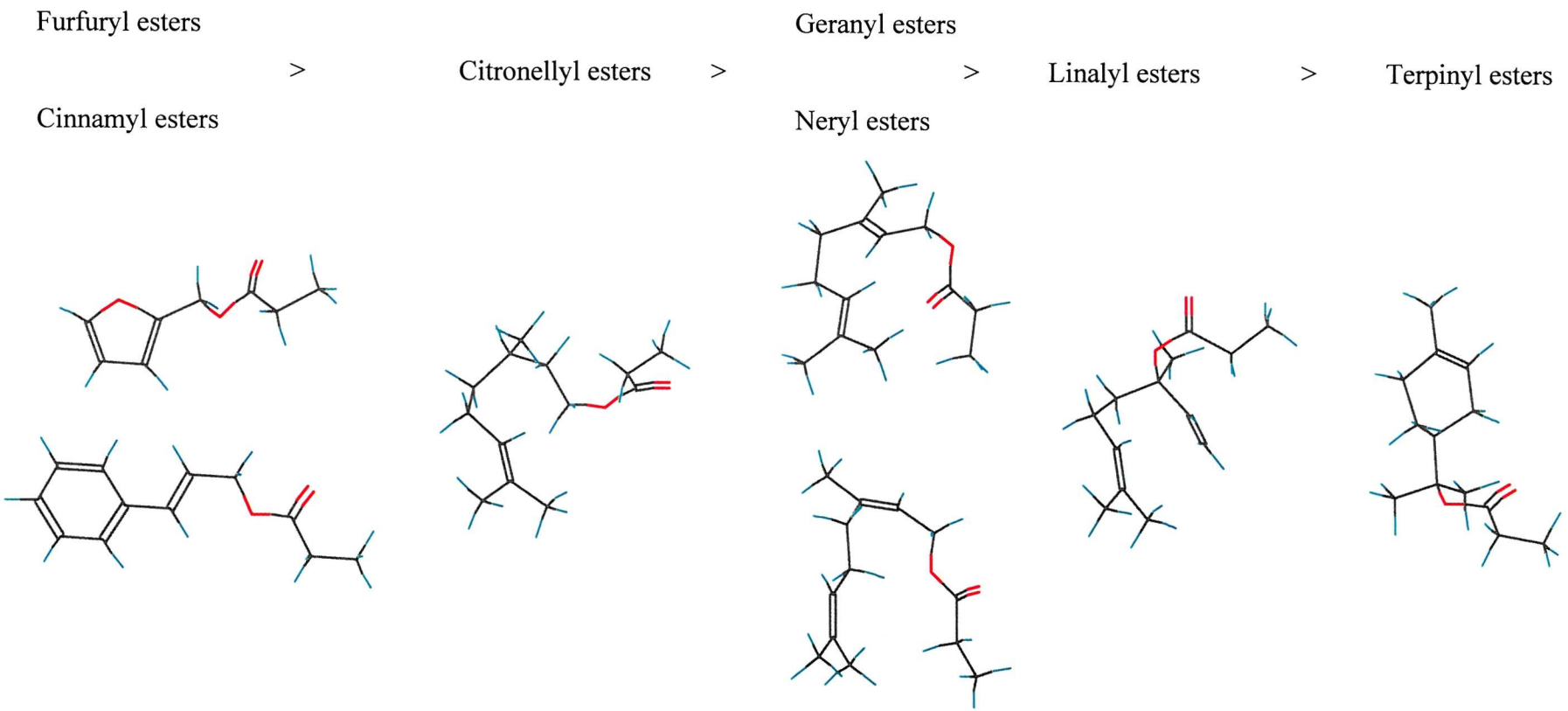


Figure 3.19: Wire frame model illustrating the observed sequence of alkyl group specificity in respect of hydrolysis rates in artificial pancreatic fluid. Esters of propionic acid are shown in their lowest energy states, stereoenantiomers (citronellyl, linalyl and terpinyl propionate) are shown in their *rectus* configuration. Black = carbon, blue = hydrogen, red = oxygen.

Table 3.7 is a complete comparison between the esters investigated in respect of metabolic differences attributable to the alkyl moieties.

	Citronellyl	**Geranyl / Neryl**	**Linalyl**	**Terpinyl**
Furfuryl / Cinnamyl	>1.2 (7) max: >2.2 min: 1.2	>5.4 (6) max: >5.4 min: N/A	>14 (8) max: >1900 min: 14	>17000 (4) max: >17000 min: N/A
Citronellyl	-	2.4 ± 1.6 (7) max: 4.7 min: 0.84	5700 ± 5900 (4) max:14000 min: 460	7300 ± 2200 (4) max: 10000 min: 5800
Geranyl / Neryl	-	-	1800 ± 1300 (6) max: 3600 min: 420	4200 ± 2700 (7) max: 7700 min: 1200
Linalyl	-	-	-	6.8 ± 10.9 (4) max: 23 min: 0.42

Table 3.7: Average differences in the rates of hydrolysis in artificial pancreatic fluid between esters belonging to different alkyl groups. Figures in red indicate the extent to which the substrates in the vertical column are metabolised faster than the substrates in the horizontal row. Numbers in parenthesis indicate the number of comparisons made, maximum and minimum values are indicated. Calculated by comparing the metabolic rates (k, reaction rates in artificial pancreatic fluid – reaction rates in controls) of substrates from different alkyl structural groups but which have the same acyl moieties [which included formate, acetate, propionate, butyrate, pentanoate (valerate), hexanoate, octanoate (caprylate), isobutyrate, 3-methyl butanoate (isovalerate), benzoate, cinnamate and anthranilate]. The majority of furfuryl / cinnamyl esters were hydrolysed too rapidly to enable the quantification of rates, N/A = calculation of minimum difference not possible.

Figure 3.19 illustrates that furfuryl and cinnamyl esters have their ester bonds in a linear open structured position. However, despite the presence of a furan ring bonded to the functional alkyl carbon atom in the case of furfuryl esters, and a prop-2-ene benzene group bonded to the functional carbon atom in the case of esters of cinnamyl alcohol, hydrolysis by pancreatin was at least 1.2 times more rapid than it was for citronellyl esters (the most rapidly hydrolysed group of monoterpenoid esters investigated), and over 17,000 times more rapid than comparable terpinyl esters (the least rapidly hydrolysed group of monoterpenoid esters investigated). Citronellyl esters have a less linear more closed branched chain structure than furfuryl or

cinnamyl esters although the ester bond and acyl moiety are relatively open. The same is true for geranyl and neryl esters, however they were hydrolysed approximately 2 times less rapidly than esters of citronellol. Geranyl and neryl esters have a double bond one carbon atom distant from the ester bond, and presumably this restricts rotational flexibility, and as such may moderately affect the fit of the substrate into the enzymic active site. Linalyl esters have a methyl and an allyl group attached to the functional alkyl carbon atom and this clearly had a strong impact on the rate of hydrolysis, these compounds were metabolised on average over 1000 times less rapidly than esters of geraniol or nerol. Terpinyl esters have two methyl groups bonded to the functional alkyl carbon atom and were hydrolysed on average over 5 times less rapidly than linalyl esters (and up to 10,000 times less rapidly than comparable esters of citronellol). That two methyl groups should reduce the rate of hydrolysis more than a methyl and an allyl group is presumably due to the fit into the catalytic site, and/or destabilisation of the first tetrahedral catalytic intermediate (see 1.2.2). However, terpinyl esters also have a six membered ring bonded to the functional alkyl carbon atom. The five membered ring of furfuryl esters was shown to have no measurable negative effect upon hydrolytic rates, however this may not be true in the case of a six membered ring so close to the ester bond.

Investigations using esters of cinnamic acid demonstrated that alkyl groups with more than 4 carbon atoms were up to 350 times more readily hydrolysed than esters with fewer alkyl carbon atoms. For esters with alkyl moieties larger than 4 carbon atoms which do not have steric hindrance around the ester bond, the acyl moiety appeared to be most important in determining catalytic rate.

The observed differences in substrate specificity lent by the acyl moiety resulted in trends which were consistent irrespective of the alkyl species. These trends could be separated into two distinct types; substrate specificity a result of the length of linear acyl carbon chains, and effects due to branched and/or aromatic acyl carbon chains.

Substrate specificity as a result of linear acyl carbon chain length was shown to have an effect of up to approximately 17 fold. Butyric acid esters were the preferred substrates, although monoterpenoid esters with an acyl carbon chain-length longer than six atoms showed increasing reaction rates. Esters of acetic and pentanoic acid

were poor substrates in comparison to esters of formic and propionic acid (although monoterpenoid formic acid esters were less stable in an aqueous environment than the other esters investigated). Substrates with branched acyl carbon chains were hydrolysed at least 30 fold slower than linear chained analogues, and esters with aromatic acyl moieties were particularly poor substrates. Within this group, esters of anthranilic acid, which have an amino group on the benzene ring, were notable as the slowest hydrolysed. These results are consistent with the available published data (discussed in 1.3 and 3.1) in which citronellyl acetate was shown to be hydrolysed more rapidly than citronellyl phenylacetate by artificial pancreatic fluid, and methyl anthranilate was shown to be particularly resistant to hydrolysis[247].

The results are also in agreement with the limited quantity of published data concerning carboxylesterases, as discussed in 1.2, which suggest that the preferred acyl carbon chain length of esterases is either 4 or longer than 6, with esters of pentanoic acid being particularly poor substrates[44; 67]. Furthermore, the published data indicates that an alkyl chain length of at least 4 is the preferred substrate size[16]. It is generally recognised that the acyl group is more important in determining catalytic reactivity than the alkyl group, which appears more important in determining enzymic affinity[41; 42], and that V_{max} is more variable than K_M[43]. Esters of branched chain alcohols and acids have been found to be poorer substrates than compounds with straight-chains, with hydrolytic rates decreasing the closer the branch is to the ester bond. Acyl carbon chains containing double bonds have also been shown to usually be poorer substrates than corresponding chains without double bonds[44; 63].

Figure 1.2 illustrates the proposed catalytic mechanism of B-esterases, according to this scheme it is likely that esters of alcohols which possess groups attached to the functional carbon atom are poorer substrates than primary alcohols due to steric hindrance preventing the initial nucleophilic attack and formation of the first tetrahedral intermediate (a principal determinant of enzyme affinity). However, results demonstrate that for esters with alkyl groups larger than 4 carbon atoms, which do not have chain branching proximal to the ester bond, the acyl moiety is most important in determining catalytic rate. It is possible that branched chain and cyclic acyl groups may present a poor fit to the catalytic groove, although they may also hinder the formation of the first tetrahedral intermediate. It is additionally likely however that

branched or bulky acyl moieties may sterically hinder the formation of the intermediate acyl-enzyme complex, or may be more slowly de-acylated from the enzyme than linear analogues.

The enzymic preference of substrates with an alkyl moiety larger than 4 carbon atoms in size is presumably a function of catalytic site fit, although it is possible that esters with larger alkyl and acyl chains may result in more stable tetrahedral intermediates due to the electron donating properties of carbon atoms when bonded to more electronegative elements. As described in 1.2.1.1.2, esters of pentanoic acid have previously been found to be poor substrates for pancreatic lipase. The reason for this was suggested to be related to the orientation of the ester at the oil / water interface of micelles[54]. The same substrate specificity has been demonstrated in incubations of solubilised substrate, and thus this explanation has now been disproved. The reason why butyric acid esters are favoured substrates rather than pentanoic acid esters (and acetic acid esters) is presumably a function of catalytic site fit.

As discussed in 1.2.1.1.2, pancreatic fluid contains three lipolytic enzymes; cholesterol / carboxyl ester hydrolase, phospholipase A_2, and pancreatic lipase (two forms of which have been isolated)[35; 50-52]. The hydrolysis of monoterpenoid esters by pancreatin was found to be due to B-esterases, and enzymic activity was shown to posses a limited degree of initial catalytic preference for (S)-linalyl butyrate. This suggests that an esterase present in pancreatin has a high affinity and low capacity for (S)-linalyl butyrate compared to (R)-linalyl butyrate, or implicates the action of more than one esterase enzyme. Studies with the serine-protease inhibitor phenylmethylsulphonyl fluoride failed to separate esterase activities. Pancreatic lipase has been shown to hold no stereoselectivity in respect of the hydrolysis of fatty acid esters[59], and as such it is possible to speculate that linalyl butyrate hydrolysis was due to the action of both pancreatic lipase and carboxyl ester hydrolase with the latter resulting in the observed stereoselectivity. Whether it was the activity of one or more individual enzymes which resulted in the stereoselectivity is unknown, but the nature of the selectivity indicates that it was the formation of the first tetrahedral intermediate enzyme substrate complex (k_1, see 1.2.2) and not the rate of subsequent reactions (k_2 and k_3) which determined the stereoselectivity. Figure 3.20 is a representation of the structures of (R)- and (S)-linalyl butyrate, and illustrates that the

ester bond of (S)-linalyl butyrate is more accessible than it is on the R-isomer (which may help explain the observation that (S)-linalyl butyrate appears to more readily undergo non-enzymic hydrolysis in a neutral aqueous environment than the R-isomer). Stereoselectivity in the hydrolysis of linalyl butyrate is as such an additional indication as to the importance of steric hindrance around the ester bond in determining the hydrolytic rate. It is also further evidence that the wide substrate specificities observed between esters of monoterpene alcohols which have differing degrees of steric hindrance around the ester bond, is due to inhibition of nucleophilic attack by esterases (differences in k_1; enzyme affinity).

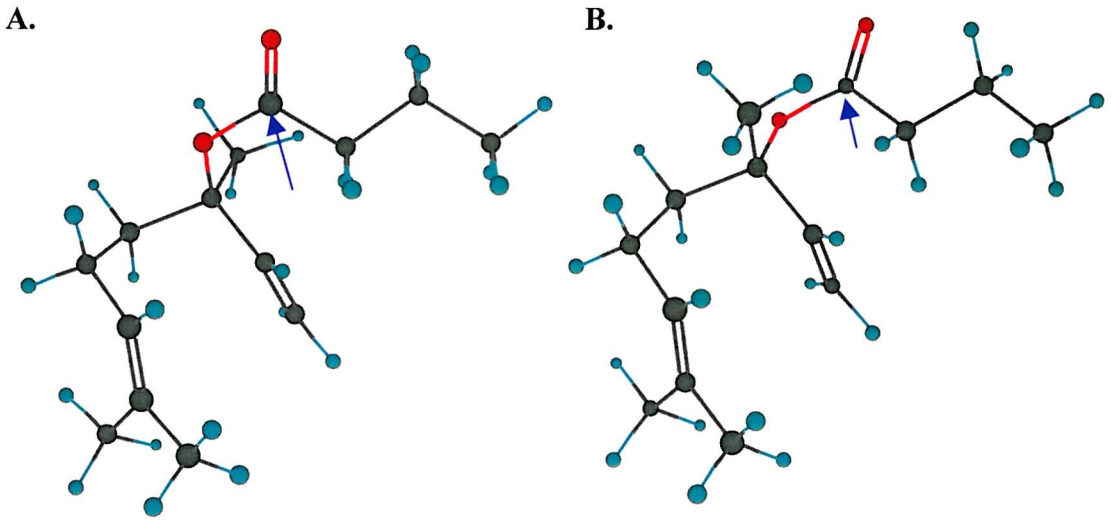


Figure 3.20: Ball and stick model of **A**. (R)-linalyl butyrate and **B**. (S)-linalyl butyrate. Structures are depicted in their minimum energy states. Sites of nucleophilic attack necessary for hydrolysis are indicated by blue arrows. Carbon atoms = black, oxygen atoms = red, hydrogen atoms = blue.

In summarising the available data, selected esters of furfuryl, cinnamyl / cinnamate and monoterpenoid alcohols illustrated distinct trends in their hydrolysis by porcine pancreatin. These trends are associated with the following structural criteria which had varying degrees of negative influence on hydrolysis rates, and which are ranked below in order of decreasing importance:

1. **Major Influence** - Steric hindrance around the ester bond

Steric hindrance of catalysis by esterases (nucleophilic attack) due to the presence of hydrocarbon groups bonded to carbon atoms directly adjacent to the ester bond, was found to have a major impact upon hydrolytic rates. This was further demonstrated by the identification of stereoselectivity in the case of linalyl esters (which have a chiral centre adjacent to the ester bond).

2. **Medium to Major Influence** - Branched / aromatic groups

Carbon chain branching (distal to the ester bond) and the presence of aromatic or cyclic structures were not shown to have an important effect upon hydrolytic rates when they were present as part of the alkyl moiety. However, such structures did have a substantial impact upon hydrolytic rates when they were present as part of the acyl moiety, aromatic structures had the greatest effect.

3. **Minor to Medium Influence** - Linear length of groups and unsaturation

The trend in substrate specificity resulting from the linear length of the acyl moiety of esters, was found to be consistent irrespective of the structure of the alkyl moiety. The linear length of the alkyl moiety was found to be of importance only if the number of carbon atoms was less than 4.

Double bonds proximal to the ester bond were found to have negligible impact upon hydrolytic rates in the case of cinnamyl esters, but resulted in a possibly minor impact in the case of geranyl / neryl esters.

The relationships identified between the rates of hydrolysis of selected food flavouring esters, and the rationales for these relationships, formed the basis of further studies using *in vitro* (see Chapter 5) and *in vivo* (see Chapter 6) systems to build and validate a hydrolytic model. Furthermore, to provide data relevant to the safety evaluation of monoterpenoid compounds, including investigations of the structural isomerisations identified in artificial gastrointestinal fluids, the chemistry, absorption and metabolism of monoterpene alcohols was studied (see Chapter 4).

CHAPTER 4

IN VITRO INVESTIGATIONS INTO THE METABOLISM OF MONOTERPENE ALCOHOLS

4.0 *In Vitro* Investigations into the Metabolism of Monoterpene Alcohols

4.1 Introduction

4.1.1 Background

In 1980 a group Acceptable Daily Intake (ADI) value was set of 0-0.5 mg/kg body weight for geranyl acetate and the monoterpenes citral (which consists of geranial and it's *cis*-isomer neral), citronellol and linalool. This was based mainly upon data from a chronic carcinogenicity bioassay performed on a mixture of geranyl acetate and citronellyl acetate[257; 273-275], in conjunction with recognised data upon linalool and linalyl acetate concerning absorption, hydrolysis, metabolism, low acute toxicities and lack of adverse effects in short-term dietary studies[276]. Specific ADI values have not been assigned by JECFA to any other monoterpenoid compound. JECFA now use a safety evaluation protocol (see Appendix I A.5.1) which assigns (or does not assign) flavouring compounds to be of 'no safety concern' under current conditions of use[277]. This is in essence similar to the approach which has traditionally been applied by FEMA under the GRAS legislative provisions[278] (see Appendix I A.4.3).

Monoterpene compounds are present to some degree in all plant species (and many animal species), and are now mainly manufactured via synthetic means. They form the basis of the essential oil industry, and besides the flavour / fragrance industry are commonly used for medicinal, anti- bacterial / viral / fungal, insecticidal, industrial manufacturing, cleaning and solvent purposes[264; 279; 280]. Terpenes are synthesised in plants through the coupling of Δ^2- and Δ^3- isopentyl pyrophosphate molecules (isoprene units), and belong to a very large family of compounds which includes camomile oil (3 isoprene units), most fat soluble vitamins (4 isoprene units), and rubber (1000's of isoprene units)[281; 282].

Terpenoid alcohols and related esters occur naturally in a wide variety of foods, including fruits, spices and tea[283], the exposure to linalool and α-terpineol from their natural occurrence in food has been estimated to be greater than 11 and 3 times,

respectively, the total combined intake from their use and use of their esters as flavouring substances[7; 284].

The total annual production of terpenoid alcohols and related esters is approximately 58,100 kg in Europe and 15,200 kg in the USA. Linalool, linalyl acetate, α-terpineol and terpinyl acetate account for approximately 95 % of the total annual volume in the USA and 96 % in Europe[274]. Based on the reported annual volumes, the total estimated daily *per capita* ('esters only') intake (see Appendix I A.4.3.2) of linalool from the use of linalool and related esters as flavouring substances is approximately 81 μg / kg body weight / day in Europe and 21 μg / kg body weight / day in the USA. Similarly, the total estimated daily per capita (esters only) intake of α-terpineol is approximately 71 μg / kg body weight / day in Europe and 23 μg / kg body weight / day in the USA[274]. In the unlikely event that all monoterpene alcohols and esters were consumed simultaneously on a daily basis, the estimated combined intake would exceed the human intake threshold for class I substances, defined according to the new safety evaluation decision tree protocol of JECFA (see A.5.1).

4.1.2 Toxicological Data

Monoterpene alcohols are known to cause local irritation[285], and limonene has been extensively studied as a promoter of trans-dermal drug absorption[286]. Essential oils derived from citrus plants have been studied for their cancer chemopreventative and chemotherapeutic properties[287], and have been employed for millennia for their psychopharmacological (particularly sedative) effects[282]. Linalool has recently been shown to inhibit glutamate binding in the rat cerebral cortex[288], and (R)-linalool appears to be a more effective sedative than (S)-linalool[289].

A number of monoterpene compounds, including geraniol and citral, have been found to possess a variable teratogenic effect on chick embryos[290], and citral has been shown to induce reproductive failure but not malformations in rats (following the intraperitoneal administration of 300 mg/kg body weight on the day of pro-oestrus for 6 consecutive cycles)[291]. An explanation suggested for these effects is that following

high dose exposure interaction may occur between the lipid soluble unsaturated carbonyl compounds and the lipid constituents of cell membranes[292].

The acute oral toxicities of linalool and α-terpineol are low. Reported oral LD_{50} values for linalool are 2200 mg / kg body weight for the mouse[251] and 2790 for the rat[252]. Reported oral LD_{50} values for α-terpineol are 2830 mg / kg body weight for the mouse[253] and 4300 for the rat[254].

A group of 19 acyclic terpenes, including linalool, geraniol, nerol and citronellol, have been evaluated for possible metabolic activation by cytochromes P-450 1A1, 1A2 and 2E1 using computational enzymic spatial mapping techniques[293]. Findings indicated that the compounds were unlikely to be activated by the enzymes studied.

In a 90 day feeding study a 50:50 mixture of linalool and citronellol was fed to male and female rats at a dose level of approximately 50 mg / kg body weight / day, and no statistically significant differences between test and control groups were observed in haematological or clinical chemistry parameters. Histopathology revealed no dose-related lesions[255].

Menthol is an unsaturated monoterpenoid alcohol which bears a close structural relationship to α-terpineol. Menthol feeding studies[294] [13 weeks (mice) and 103 weeks (rats and mice)] demonstrated a slight increase in interstitial nephritis and perivascular lymphoid hyperplasia at doses above 600 mg / kg body weight / day, with no adverse effects at levels approximately 10,000 times the estimated total daily *per capita* intake (eaters only) from all monoterpenoid flavouring compounds combined.

4.1.3 Metabolic Data

The chemistry of the economically important monoterpene alcohols has been investigated by organic chemists who have described the conversion of linalool to geraniol, nerol and α-terpineol via a proposed unusually stable carbocation intermediate[264; 267; 295]. It has been suggested that in an acidic environment citronellol

may cyclise to form a cyclohexane or cycloheptanone-type compound[264; 265; 267; 296; 297].

Terpenoid alcohols which are ingested, or are formed via hydrolysis of an ester, are presumed to be rapidly absorbed from the gastrointestinal tract, however this presumption is based upon data available for the monoterpene aldehyde citral[298; 299].

In humans and animals, terpenoid tertiary alcohols have been shown to be primarily conjugated with glucuronic acid and to be excreted in the urine and faeces[300-303]. Terpenoid alcohols with unsaturation may undergo allylic oxidation to form polar diol metabolites which may be excreted free or conjugated. If the diol contains a primary alcohol function, it may undergo further oxidation to the corresponding carboxylic acid[300; 303; 304]. Non-tertiary monoterpenoid alcohols (for example geraniol) may also undergo oxidation by alcohol dehydrogenase and subsequently form the corresponding carboxylic acid[305; 306].

The metabolic fate of linalool has been studies in mammals. Linalool labelled with ^{14}C was orally administered to male Wistar rats at a single dose of 500 mg / kg body weight. The majority (55 %) of the radioactivity was excreted in the urine as the glucuronic acid conjugate, while 23 % was excreted as CO_2 in expired air, and 15 % was excreted in faeces within 72 hours of dose administration. Only 3 % of the radioactivity was detected in tissues after 72 hours, with 0.5 % in the gut, 0.8 % in the skin and 1.2 % in the skeletal muscle[301]. Reduced metabolites such as dihydro- and tetrahydrolinalool have been identified in the urine after administration of a single dose of linalool to rats[307].

In a study by Chadha and Madyastha, male rats (160-200g) were given a daily oral dose of 800 mg / kg body weight linalool for 20 days which was excreted as urinary metabolites indicative of cytochrome-P-450 allylic oxidation (further evidence for which was the observed enzyme induction discussed in 4.1.3.1). These included the metabolites hydroxylinalool and carboxylinalool which showed selectivity for oxidation at the 8- position[306] (this study failed however to detect reduced metabolites). Chadha and Madyastha have also identified that geraniol undergoes oxidation in rats at the 8- position[306], as does *l*-menthol[308] (which may subsequently

form an epoxide due to the proximity of the alcohol moiety of *l*-menthol to the 8-position). Furthermore, the aldehydes citronellal and citral have been shown to undergo selective carboxylation at this position in rabbits[309], as has citral in rats[310].

The available data generally suggest that glucuronic acid conjugation and excretion is the primary route of metabolism of linalool, allylic oxidation becoming an important pathway only after repeated dosing. Most branched chain alcohols are oxidised rapidly to their corresponding aldehydes by alcohol dehydrogenase, the rate of oxidation increasing with the presence of a double bond[311]. A metabolite (3,7-dimethyl-3-hydroxy-6-octenoic acid) of the aldehyde geranial has been demonstrated in the urine of rats following dosing with geraniol[306], however on the balance of evidence it would appear that the tertiary alcohol linalool is not a substrate for alcohol dehydrogenase. It has been suggested that the biotransformation of the diol metabolite of linalool to the corresponding aldehyde via the action of NAD^+-dependent alcohol dehydrogenase would be inhibited due to the 'bulky' nature of the compound[312]. Citral is less sterically hindered around the functional group than linalool, and it has been found that this aldehyde readily undergoes reduction to the corresponding alcohol by rat hepatic cytosolic alcohol dehydrogenase. However, citral is a poor substrate for aldehyde dehydrogenase, and has in fact been shown to be an inhibitor of rat microsomal aldehyde dehydrogenase activity[313]. However the fact that the major urinary metabolites of citral in rats (the main route of elimination[298]) consists of several bi-carboxylic acid compounds[310] is suggestive of the action of both alcohol and aldehyde dehydrogenase enzymes subsequent to oxidation by cytochrome P-450. Therefore it is possible that monoterpene aldehydes undergo an alcohol dehydrogenase-mediated reduction, or metabolism via other pathways, prior to (or as well as) oxidation by aldehyde dehydrogenase. As linalool appears to be a poor substrate for alcohol dehydrogenase whereas geraniol and citral are probably good substrates, it is clear that care should be taken when making predictions concerning the metabolism of monoterpenes.

In a repeated dose study by Madyastha and Srivastava, male IISc rats (185-225g) were orally administered α-terpineol at a dose of 600 mg / kg body weight for 20 days. Oxidation of the allylic methyl group yielded the corresponding carboxylic acid

which, to a small extent, was hydrogenated to yield the corresponding saturated carboxylic acid. Test animals in this study also demonstrated an increased hepatic microsomal cytochrome P-450 content and activity of NADPH-cytochrome c reductase[304] (see 4.1.3.1 below) suggesting that oxidation is mediated by cytochrome P-450. Following the inadvertent, or intentional ingestion by humans of pine oil disinfectant containing α-terpineol, a triol metabolite has additionally been reported[300], which is presumably formed via the epoxidation of α-terpineol followed by hydrolysis. It has however been suggested that α-terpineol would undergo metabolism like linalool, primarily by glucuronic acid conjugation and excretion in the urine[306]. The cyclic monoterpenoid alcohols *l*-menthol and *trans*-sobrerol, which are structurally related to α-terpineol, have been shown to undergo two principal routes of metabolism; hydroxylation (allylic hydroxylation in the case of the unsaturated *trans*-sobrerol) and conjugation of the alcohol functions with glucuronic acid[303; 308; 314; 315].

4.1.3.1 Enzyme Induction

In the study by Chadha and Madyastha (discussed above), cytochrome P-450 activity in hepatic microsomes increased approximately 50 % after three days' linalool treatment, returning back to control values after only six days[306]. This work is tentatively supported by Roffey *et al* who have subsequently shown that the oral administration to male rats (140-170g) of either citral or linalool at a dose of 1.5 g / kg body weight / day, with enzyme concentrations measured at a single necropsy following 5 days, induced both peroxisome proliferation and cytochrome P-450 4A1 in the case of citral, whereas linalool induced peroxisome proliferation but did not induce this cytochrome isoenzyme[316]. Parke *et al* orally dosed 4 week-old male Wistar rats with 500 mg linalool / kg body weight / day and saw a biphasic response. The concentration of hepatic cytochrome P-450 was reduced after 7 days and increased after 30 days[302]. Citral has been shown to induce its own metabolism in rats, although the pathway was not identified[298]. Further, Madyastha and Srivastava (as discussed above) have more recently shown that the structural isomer of linalool, α-terpineol, when dosed to 185-225 g male rats at 600 mg / kg body weight / day leads to a large

increase in hepatic cytochrome P-450 concentration after 1 day (peaking at 204 % after 2 days), this gradually decreasing again towards control levels[304].

The available data indicates that monoterpenoid compounds can stimulate a rapid and transient induction of cytochrome P-450 activity. It may be postulated that the biphasic initial reduction of hepatic cytochrome P-450 concentration of approximately 20 %, seen in the investigations of Parke *et al*[302], are related to the young age of the animals employed in the study. Rat hepatic cytochrome P-450 concentration reaching its peak at about 4 weeks of age, following which it steadily declines with age (in the study of Parke *et al* the 4 week-old animals employed expressed a total of 0.85 ± 0.10 nmol cytochrome P-450/mg microsomal protein, where as the adult animals employed by Chadha and Madyasthra[306] had a concentration of 0.56 ± 0.04 nmol/mg microsomal protein. The reduction in cytochrome P-450 concentration observed by Parke *et al* equated to approximately 0.17 nmol/mg microsomal protein). As such, it may be that with a larger initial amount of cytochrome P-450, any inhibitory effects of the substrate may be more pronounced.

It has been shown that rat hepatic microsomal glucuronyl transferase (uridine diphosphate-glucuronosyltransferase) activity towards monoterpenoid alcohols is inducible by phenobarbital. In guinea pigs this induction is selective for monoterpenoid alcohols when compared to other typical phenobarbital-induced activities such as those for the conjugation of morphine[317].

4.1.4 Study Objectives

Following investigations into the hydrolysis of monoterpenoid esters in artificial gastrointestinal fluids (see Chapter 3), it was determined to further investigate the observed structural isomerisations of monoterpenoid alcohols in solution, and to study the metabolism of the important food flavouring compound linalool.

A number of *in-vitro* studies were conducted to investigate the extent of metabolism of the parent alcohols of monoterpene esters which could occur at various sites following ingestion. The studies presented below include an examination of the

behaviour of linalool and citronellol in artificial gastric fluids made to model both the human and rat gastric environments. The stereoselectivity of linalool rearrangement in an acidic environment was investigated, as was the effects of artificial gastric fluid upon the multiple products of such rearrangement. Furthermore, the effects of artificial pancreatic fluid upon linalool and citronellol were examined. Investigations of the rate of absorption of linalool across isolated inverted rat intestinal preparations were undertaken, as were studies of the metabolism of linalool by preparations of rat stomach contents, intestinal contents, intestinal mucosa, blood and liver. These were complemented by more detailed study of Phase I (cytochrome P-450 oxidation) and Phase II (glucuronic acid and sulphate conjugation) metabolism of linalool by both rat intestinal and hepatic preparations. Stereoselective differences in such metabolism were investigated.

4.2 Methods

4.2.1 Investigations into Intestinal Absorption

4.2.1.1 Method Development

Tissue study of everted (inverted) rat small intestine was undertaken in order to evaluate the intestinal absorption of flavouring compounds. This method was developed and refined through a series of 9 trial investigations.

Initial studies illustrated difficulties in preparing inverted intestine, extracting samples from the lumen (serosal side) of the intestines, and the rapid evaporation of substrate from the carbogen-gassed organ baths. The first two problems were overcome, however loss of volatile substrate due to evaporation from the organ baths remained a problem.

It would be anticipated that two main processes would be occurring in the incubation system; the loss of substrate from the organ bath, and the equilibrium of the substrate concentration in the intestinal lumen with that in the organ bath. If the concentration of substrate is larger in the organ bath than it is in the intestinal lumen, a ratio of the concentration of substrate in the intestinal lumen compared to the organ bath (L/B)

will discount for the loss of substrate from the organ bath, and allow a determination of the rate of absorption. However, this initial period may be followed by a period when the concentrations in the intestinal lumen and the organ bath are equal, and subsequently where substrate moves to the organ bath from the less open system of the intestinal lumen. Depending upon the rate of transfer of the substrate from the intestinal lumen to the organ bath and the loss from the organ bath by evaporation, this may be represented by a plateau or a continuous increase in the ratio L/B (to levels above 1). In early studies the latter effect was observed, but by decreasing the rate of gassing and increasing the size of bubbles (to a level above the minimum considered necessary for the maintenance of the intestinal samples[318]) so that the loss of substrate from the baths was minimal, and substrate concentration in the baths was always higher than that in the intestinal lumens over the 3 hour time course, interpretable results were obtained.

4.2.1.2 Experimental Procedure

3 Male Wistar albino rats (250-300 g) were used per experiment. Animals were killed by cervical dislocation following which the abdominal cavity was opened and 20 cm of small intestine proximal to the stomach was removed to pre-warmed (37°C) and carbogen gassed Krebs solution (NaCl 121.49 mM, KCl 4.69 mM, KH_2PO_4 1.18 mM, $MgSO_4.7H_2O$ 1.18 mM, $NaHCO_3$ 25.00 mM, glucose 11.10 mM, $CaCl_2.2H_2O$ 2.52 mM). Intestinal samples were cleaned by rinsing through with Krebs solution using a 5 ml syringe. Samples were then inverted by placing over a glass rod of diameter equal to the internal diameter of the intestine, followed by drawing off the intestine onto a second rod such that inversion occurred.

One end of the inverted intestine samples was doubled-over and tied tightly and a 5 g weight attached. 10 cm of cannula tubing of external diameter 0.75 mm was placed inside the inverted intestine (the tubing having numerous holes along it's final 3 cm for ease of fluid sampling), and the open end of the intestinal loop was tied securely around the cannula head. Inverted intestinal samples were checked for tears/holes by gently pressurising the lumen with Krebs solution using a 5 ml syringe, this was then withdrawn and 4 ml of fresh Krebs solution introduced.

Each inverted intestinal sample was placed in a pre-warmed (37°C) 50 ml organ bath containing pre-gassed Krebs solution with 1.3 mM linalool (time = 0 mins). Care was taken to keep the cannulated end of the intestinal samples above the organ bath contents. Carbogen was gassed through each organ bath at a rate of 10 ml/min through a capillary tube. One organ bath was maintained as a control with no intestinal sample present.

At set time points (0, 5, 10, 15, 30, 60, 120, 180 minutes) 0.25 ml samples were taken from the cannulated intestinal lumen and the organ baths by calibrated micro-syringe. Care was taken not to leave any fluid in the cannula following each sampling point by introducing a small dead-space of air. Samples were immediately extracted with 1 volume of CHX/THN. Samples were stored for no more than 2 weeks at -20°C following which they were defrosted at 21°C and analysed by gas chromatography.

4.2.2 The Metabolism of Linalool in Rat Liver & Intestinal Tissue, by Cytochrome P-450 Oxidation, and Glucuronic Acid and Sulphate Conjugation

Experiments were conducted in triplicate in-order to investigate the metabolism of linalool in rat intestinal and liver preparations, with and without metabolic activation systems for cytochrome P-450 oxidation, and glucuronic acid and sulphate conjugation. The triplicate investigations were undertaken using tissues from 3 different animals [male Wistar albino rats (250-300 g)], each triplicate set of incubations was conducted concurrently.

Experimental procedures were developed from a combination of information from a number of sources[272; 319-325].

4.2.2.1 Tissue Preparation

The preparation of rat liver homogenates was as described previously (see 2.2.6.1). Rat small intestine homogenate was prepared by removing 60 cm of small intestine (proximal to the stomach), and cutting into 10 cm sections. Using a 5 ml syringe filled with ice cold tissue buffer the intestinal contents were washed out and the intestinal sections kept on ice in pre-weighed vessels prior to homogenisation.

From the pre-weighed vessels, the weight of the liver and intestinal samples was ascertained. The amount of tissue sample to be added to ice-cold tissue buffer to result in 25 % (w/v) homogenates, was calculated. The remaining tissue samples were frozen in buffer for use at a later date in the preparation of standard curves and total protein determinations. Homogenisation was as previously described (see 2.2.6.1)

All incubations were conducted at 37°C in a shaking water bath, using pre-warmed incubation flasks and buffers. All reactions were initiated by the addition of substrate.

4.2.2.2 Investigations into the Effect of Cytochrome P-450 Enzyme Systems on Linalool Metabolism

In order to investigate the effect of cytochrome P-450 enzymes upon the metabolism of linalool in rat liver and intestinal tissues, 4 incubations were undertaken per experiment, with all experiments performed in triplicate. The first two incubations were undertaken to ascertain the activity of the cytochrome P-450 system, and the second two to investigate the metabolism of linalool with and without metabolic activation systems.

Incubation 1 consisted of a total of 2 ml of: 7-ethoxycoumarin 0.05 mM (from a dimethyl sulphoxide stock solution so that the final concentration of dimethyl sulphoxide was not more than 0.5 % v/v in the final incubation mix); β-nicotinamide adenine dinuncleotide phosphate 0.75 mM (NADP, stock solution made fresh prior to each incubation in tris-HCl 50 mM pH 7.8); glucose-6-phosphate 7.5 mM; glucose-6-phosphate dehydrogenase 1.8 Units / ml; magnesium sulphate 5 mM; tris-HCl 50 mM (pH 7.8); and 5 % (w/v) tissue.

Incubation 2 was as incubation 1 with the exception that no metabolic activation system was present (NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase).

Incubation 3 consisted of a total of 10 ml of: linalool 130 μM (from a solubilised stock solution in tris-HCl 50 mM pH 7.8); NADP 0.75 mM; glucose-6-phosphate 7.5 mM; glucose-6-phosphate dehydrogenase 1.8 Units / ml; magnesium sulphate 5 mM; Tris-HCl 50 mM (pH 7.8); and 5 % (w/v) of tissue.

Incubation 4 was as incubation 3 with the exception that no metabolic activation system was present (NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase).

4.2.2.3 Investigations into the Effect of Glucuronic Acid Conjugation on Linalool Metabolism

In order to investigate the effect of glucuronic acid conjugation upon the metabolism of linalool in rat liver and intestinal tissues, 4 incubations were undertaken per experiment, with all experiments performed in triplicate. The first two incubations were undertaken to ascertain the activity of the glucuronic acid conjugation enzyme system, and the second two to investigate the metabolism of linalool with and without metabolic activation systems.

Incubation 1 consisted of a total of 2 ml of: 7-hydroxycoumarin 0.05 mM (from a dimethyl sulphoxide stock solution, so that the final concentration of dimethyl sulphoxide was not more than 0.5 % v/v in the final incubation mix); uridine 5'-diphosphoglucuronic acid 4 mM (UDPGA, made up fresh prior to every experiment in tris-maleate 0.25 M pH 7.4); magnesium sulphate 5 mM; tris-maleate 0.25 M (pH 7.4); and 5 % (w/v) tissue.

Incubation 2 was as incubation 1, except no metabolic activation system was present (UDPGA).

Incubation 3 consisted of a total of 10 ml of: linalool 130 μM (from a stock in tris-maleate 0.25 M pH 7.4); UDPGA 4 mM; magnesium sulphate 5 mM; tris-maleate 0.25 M (pH 7.4); and 5 % (w/v) tissue.

Incubation 4 was as incubation 3, except that no metabolic activation system was present (UDPGA).

4.2.2.4 Investigation into the Effect of Sulphate Conjugation on the Metabolism of Linalool.

In order to investigate the effect of sulphate conjugation upon the metabolism of linalool in rat liver and intestinal tissues, 4 incubations were undertaken per experiment, with all experiments performed in triplicate. The first two incubations were undertaken to ascertain the activity of the sulphate conjugation enzyme system, and the second two to investigate the metabolism of linalool with and without metabolic activation systems.

Incubation 1 consisted of a total of 2 ml of: 7-hydroxycoumarin 0.05 mM (from a dimethyl sulphoxide stock solution, so that the final concentration of dimethyl sulphoxide was not more than 0.5 % v/v in the final incubation mix); 3'-phosphoadenosine-5'-phosphosulphate 0.1 mM (PAPS, made up fresh prior to each experiment in tris-HCl 0.1 M pH 7.5); magnesium sulphate 5 mM; tris-HCl 0.1 M (pH 7.5); and 5 % (w/v) tissue.

Incubation 2 was as incubation 1, except that no metabolic activation system was present (PAPS).

Incubation 3 consisted of a total of 10 ml of: linalool 130 μM (from a stock in tris-HCl 0.1 M pH 7.5); PAPS 0.1 mM; magnesium sulphate 5 mM; tris-HCl 0.1 M (pH 7.5); and 5 % (w/v) tissue.

Incubation 4 was as incubation 3, except that no metabolic activation system was present (PAPS).

4.2.2.5 Sampling

Incubations 1 & 2 from all the above metabolic investigations were incubated for 60 minutes at 37°C in 10 ml glass tubes in a shaking water bath, and the reaction stopped with addition of 0.5 volume 10% (w/v) trichloroacetic acid followed by vortexing and immediate freezing on dry ice in light-proof vessels.

Incubations 3 & 4 from all the above metabolic investigations were incubated for 180 minutes at 37°C in sealed flasks in a shaking water bath, with 1 ml samples taken at 0.1, 5, 10, 15, 30, 60, 120 and 180 minutes to 10 ml glass tubes containing 1 ml CHX/THN, samples being immediately frozen on dry ice.

Following the final time-point, 1 ml of remaining incubation solutions was immediately frozen on dry ice (known as terminal samples) for investigations at a later date on the effect of glucuronidase and sulphatase enzymes.

All samples were stored frozen at -20°C for a maximum of two weeks following which samples were centrifuged at 6030 g for 10 minutes prior to gas chromatographic analysis.

4.2.2.6 Analysis

Incubations 1 & 2: Samples were defrosted and centrifuged at 6030 g for 10 mins. 100 ul was diluted with 900 ul of 0.154 M KCl buffer containing 50 mM tris-HCl (pH 7.4). Then 0.25 ml 4 M HCl was added, followed by extraction for 20 minutes in 6 ml chloroform. A 5 ml aliquot of the chloroform was extracted with 3 ml 0.5 M glycine-NaOH (pH 10.5) for 10 mins, and fluorescence of the aqueous layer determined by fluorimetry using a Jenway 6200 fluorimeter; excitation 380 nm, emission 452 nm. Standards of 7-hydroxycoumarin were prepared to enable the quantitation of results.

In the case of the P-450 investigations an increase in fluorescence indicated activity, (with the formation of 7-hydroxycoumarin from 7-ethoxycoumarin). In the case of the glucuronic acid and sulphate conjugation investigations a decrease in fluorescence indicated activity (with the conjugation of 7-hydroxycoumarin).

Tubes 3 & 4: Linalool concentration and possible metabolite formation were assayed via gas chromatography (see 2.2.1).

To determine and quantify conjugates of linalool in the terminal samples, 100 ul aliquots were diluted with 0.5 volume of 0.5 M sodium acetate buffer (pH 5.0), containing either 5000 U/ml β-glucuronidase, or 250 U/ml sulphatase and 17 mM D-saccharic acid 1,4-lactone (present in-order to inhibit any glucuronidase activity of the sulphatase). After incubation for 16 hours at 37°C, free linalool was determined by gas chromatographic analysis.

4.3 Results

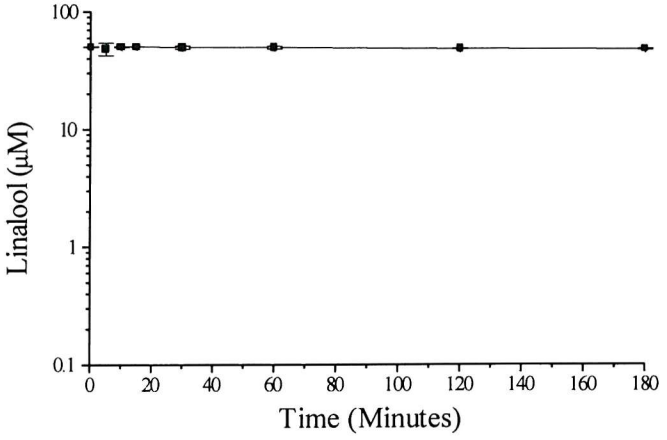
4.3.1 Effect of Artificial Gastric Fluid upon Monoterpenoid Alcohols

Table 4.1 illustrates the reaction constants for linalool and citronellol in artificial gastric fluid. In artificial gastric fluid containing pepsin, linalool and citronellol exhibited the same behaviour as in artificial gastric fluid which did not contain pepsin. As illustrated in figure 4.1, linalool was found to undergo acid-catalysed rearrangement with first-order kinetics, to primarily form α-terpineol, with geraniol and some nerol. As illustrated in figure 4.2, citronellol was found to be unstable in an acidic environment (with first-order kinetics), although reaction products were not detected, despite studies using different extraction and GC conditions.

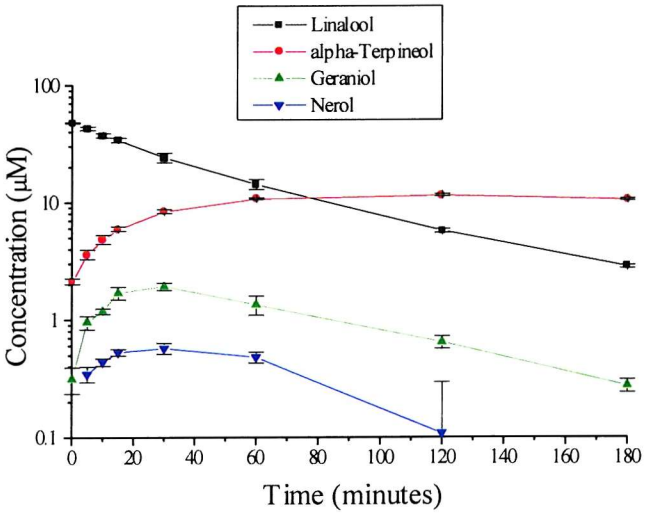
	No Pepsin pH 7.0	**No Pepsin pH 1.2**	**With Pepsin pH 1.2**
Linalool	2100 ± 600	44 ± 1	47 ± 1
Citronellol	2200 ± 1200	30 ± 1	35 ± 1

Table 4.1: Reaction of 50 μM linalool and citronellol in artificial gastric fluid at 37°C over 180 minutes. $T_{½}$ (minutes) ± standard deviation.

A.



B.



C.

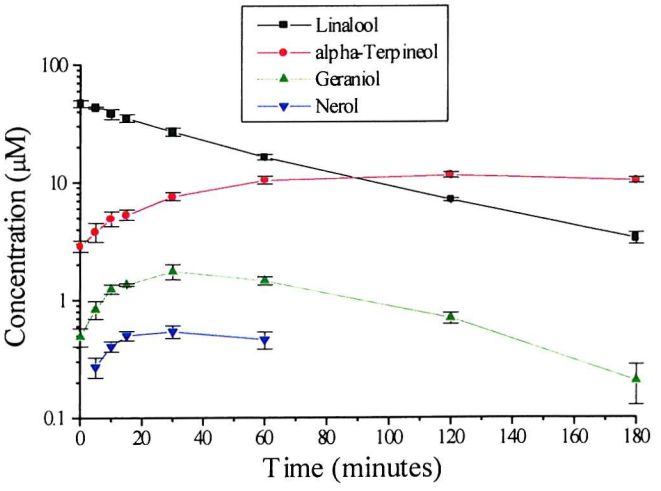
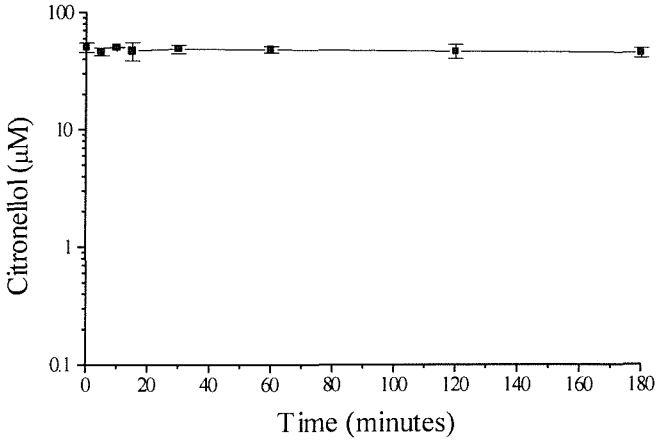
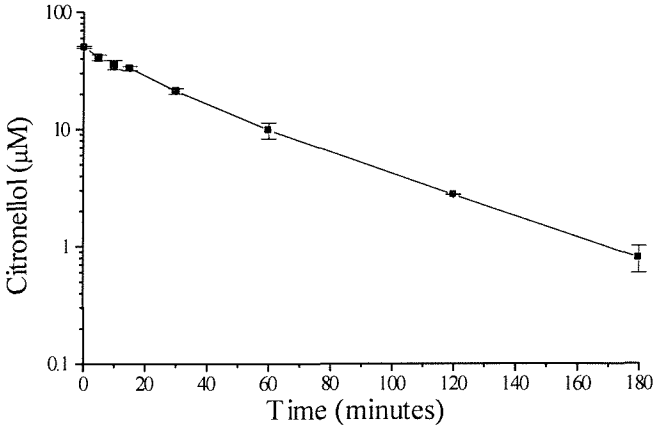


Figure 4.1: 50 μM linalool in artificial gastric fluid (mean ± SD). **A**. Linalool in artificial gastric fluid not containing pepsin pH 7.0. **B**. Linalool in artificial gastric fluid not containing pepsin pH 1.2. **C**. Linalool in artificial gastric fluid containing pepsin pH 1.2.

A.



B.



C.

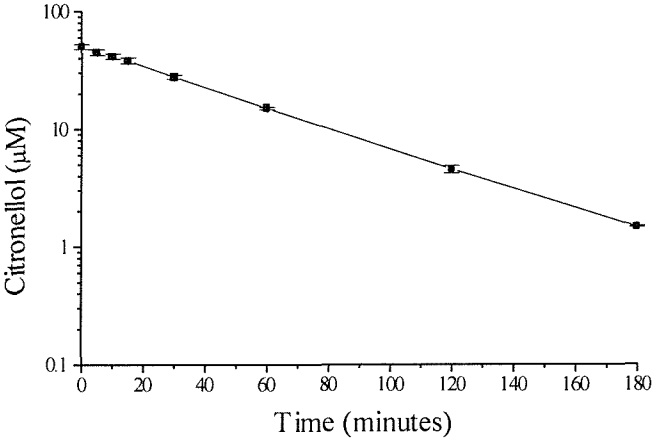


Figure 4.2: 50 μM citronellol in artificial gastric fluid (mean ± SD). **A**. Citronellol in artificial gastric fluid not containing pepsin pH 7.0. **B**. Citronellol in artificial gastric fluid not containing pepsin pH 1.2. **C**. Citronellol in artificial gastric fluid containing pepsin pH 1.2.

In order to assess the rate of acid-catalysed rearrangement of linalool in incubation systems intended to model the rat gastric environment (as opposed to the human model above), linalool was incubated in artificial gastric fluid pH 2.5, the results of which are illustrated in table 4.2. These incubations did not contain pepsin as the above investigations illustrated the lack of any significant effect additional to acid-catalysed rearrangement. The rate of disappearance of linalool in artificial gastric fluid at pH 2.5 was significantly faster ($P<0.05$) than the pH 7.0 control and significantly slower [by approximately 20 times ($P<0.05$)] than incubations at pH 1.2. The only product detected during the incubations at pH 2.5 was α-terpineol which at no time during the incubations increased in concentration to a level above 1 μM (which represented no more than 2 % of the total monoterpene alcohol present).

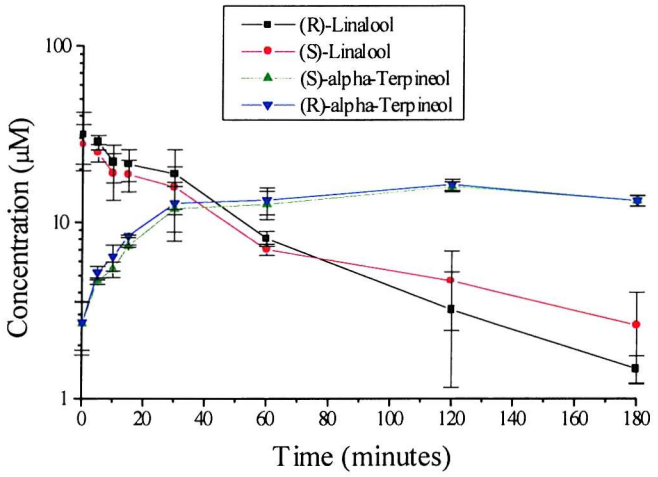
	pH 7.0	**pH 2.5**
Linalool	5600 ± 6300	950 ± 240

Table 4.2: Reaction of 50 μM linalool in artificial gastric fluid not containing pepsin at 37°C over 180 minutes. $T_{½}$ (minutes) ± standard deviation.

4.3.1.1 Stereoselectivity of Acid-Catalysed Linalool Rearrangement

An analysis of linalool and α-terpineol stereoisomers showed no substantial enantiomeric preference during the acid-catalysed rearrangement of linalool, as illustrated in Figure 4.3. However, although the enantiomeric ratio for linalool remained reasonably constant over 60 minutes, a slight enantiomeric preference could be possibly detected from the data. The chiral centre of α-terpineol is not directly related to the chiral centre of linalool, but is formed by cyclisation of linalool. Although the standard deviations are considerable, figure 4.3B demonstrates that at 0.1 minutes there was approximately 14 % more (S)-α-terpineol produced than (R)-α-terpineol.

A.



B.

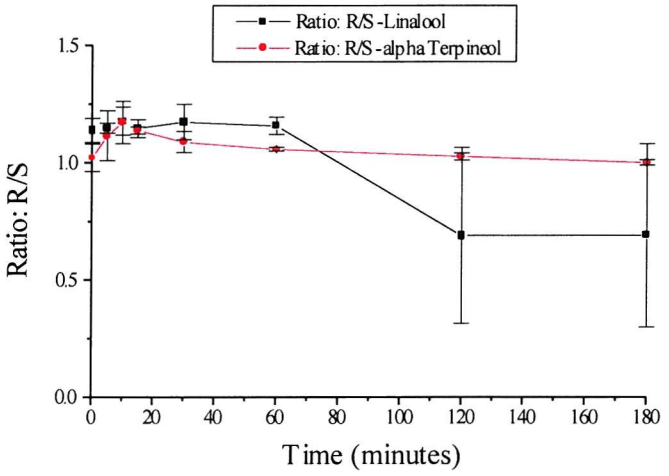


Figure 4.3: Enantiomeric preference during the acid-catalysed rearrangement of linalool in artificial gastric fluid (mean ± SD). **A.** Concentrations of linalool and α-terpineol stereoisomers. **B.** Ratio (R)-/(S)- linalool and α-terpineol (initial ratio (R)-/(S)- linalool = 1.1:1).

4.3.1.2 Effect of Artificial Gastric Fluid upon the Linalool Acid-Catalysed Rearrangement Products; α-Terpineol, Geraniol and Nerol.

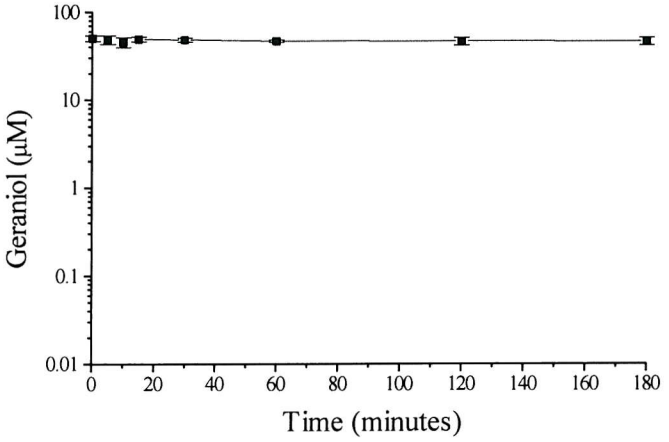
To investigate the possible further metabolism of the linalool acid-catalysed rearrangement products α-terpineol, geraniol and nerol, these compounds were incubated in artificial gastric fluid not containing pepsin, pH 7.0 and pH 1.2 at 37°C over 180 minutes. The results which are illustrated in table 4.3, demonstrate acid catalysed loss of all 3 substrates. No reaction products were identified in the case

of incubations containing α-terpineol substrate. However, geraniol was found to rearrange in acid, to initially form predominantly linalool with smaller quantities of α-terpineol as illustrated in figure 4.4. Furthermore, nerol was found to undergo acid-catalysed rearrangement to initially form predominantly α-terpineol, with smaller quantities of linalool, as illustrated in figure 4.5. In both the geraniol and nerol investigations, the linalool metabolite underwent acid-catalysed rearrangement to form α-terpineol (as previously demonstrated in 4.3.1 above).

	pH 7.0	**pH 1.2**
α-Terpineol	1000 ± 100	200 ± 20
Geraniol	4000 ± 4900	31 ± 1
Nerol	1500 ± 800	18 ± 1

Table 4.3: Reaction of 50 μM α-terpineol, geraniol and nerol in artificial gastric fluid not containing pepsin at 37°C over 180 minutes. $T_{½}$ (minutes) ± standard deviation.

A.



B.

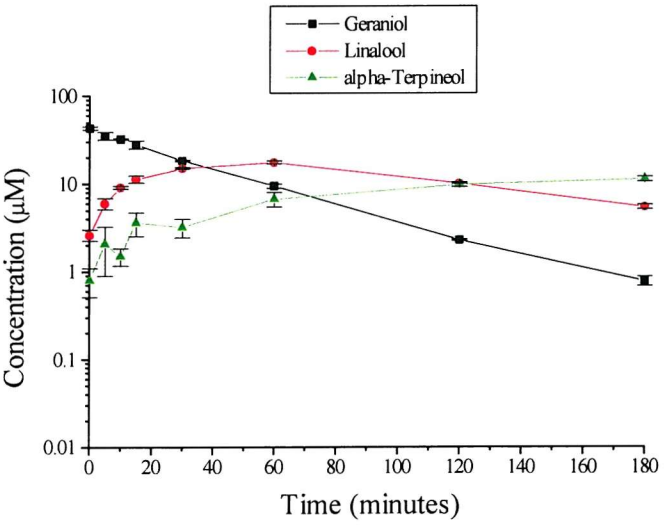
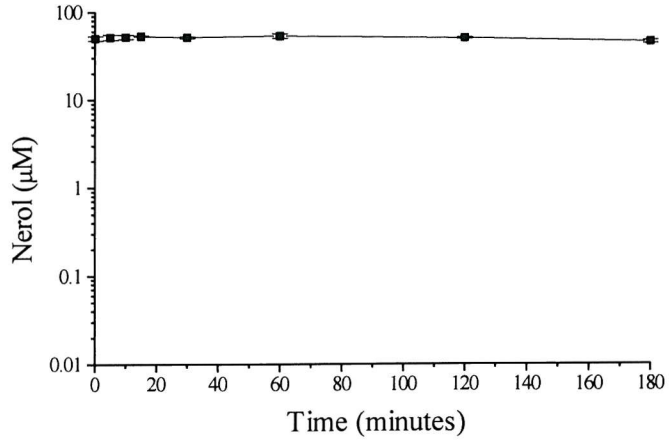


Figure 4.4: 50 μM geraniol in artificial gastric fluid not containing pepsin (mean ± SD). **A**. Geraniol in artificial gastric fluid pH 7.0. **B**. Geraniol in artificial gastric fluid pH 1.2.

A.



B.

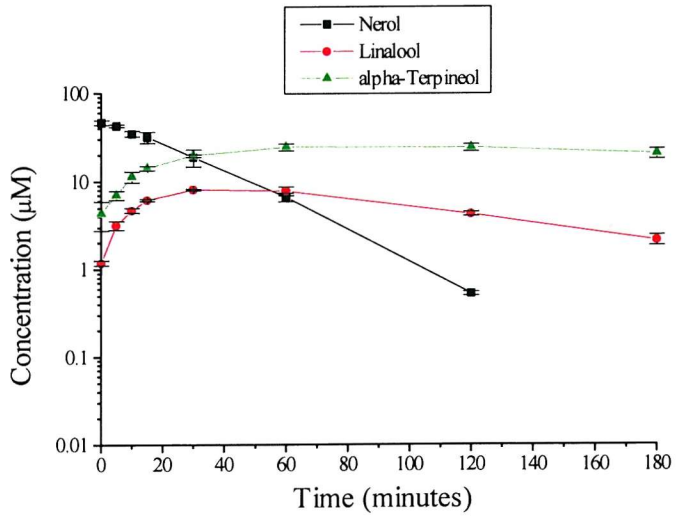


Figure 4.5: 50 μM nerol in artificial gastric fluid not containing pepsin (mean ± SD). **A**. Nerol in artificial gastric fluid pH 7.0. **B**. Nerol in artificial gastric fluid pH 1.2.

4.3.2 Effect of Artificial Pancreatic Fluid upon Linalool and Citronellol

As illustrated by table 4.4, no metabolism of linalool or citronellol in artificial pancreatic fluid was observed, furthermore, no reaction products were identified.

	Not Containing Pancreatin	**Containing Pancreatin**
Linalool	1500 ± 300	2500 ± 600
Citronellol	7000 ± 7100	6600 ± 7300

Table 4.4: Reaction of linalool and citronellol in artificial pancreatic fluid. $T_{½}$ (minutes) ± standard deviation.

4.3.3 Intestinal Absorption of Linalool

Tissue culture of inverted rat small intestine was undertaken in order to evaluate the intestinal absorption of linalool. This method was developed and refined through a series of 9 trial investigations as described in 4.2.1.

Figure 4.6 illustrates the absorption of linalool across rat inverted intestine a process which occurred with an initial rate of 0.09 ± 0.04 mmoles/second or 2.76 ± 1.29 μmoles/second/cm^2 (with an initial concentration of linalool in the organ baths of 1.3 mM). It would be anticipated that esters of linalool with lipophilic non-polar acyl moieties would demonstrate more rapid movement across the gut wall.

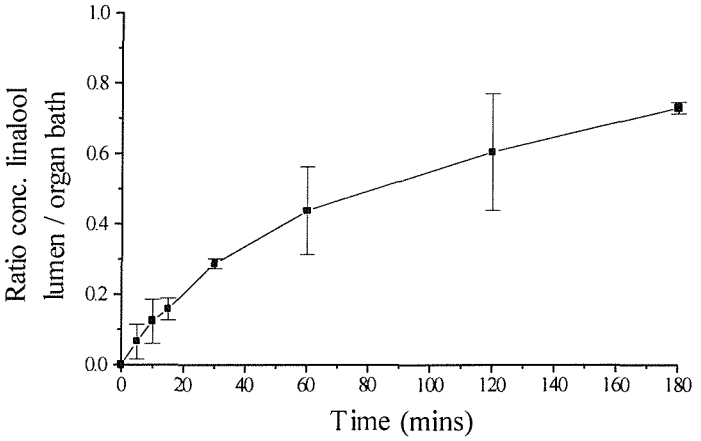


Figure 4.6: Ratio of linalool concentration within the lumen of inverted rat intestines compared with the concentration in the bathing buffer of the organ baths (mean ± SD). Tissue samples were maintained at 37°C with carbogen gassing over 3 hour time course.

Further investigations, using inverted rat intestine, into the rate of absorption and metabolism of linalyl acetate and butyrate did not yield interpretable results despite several attempts. This was because of the rapid loss of substrates from organ bath solutions (esters of linalool are generally less water soluble than linalool itself, and therefore lower concentrations of substrate were required than were possible for linalool).

4.3.4 Metabolism of Linalool by Rat Blood, Hepatic and Gastrointestinal Preparations

Table 4.5 illustrates the results of incubations of 6.5 mM linalool with homogenates of rat stomach contents, intestinal contents, intestinal mucosa, blood and liver. No statistically significant differences (at $P<0.01$) were observed for the disappearance of linalool in comparison to controls, and no metabolic products were observed. However, the incubation of linalool in blood homogenates did show a statistically significant difference ($P<0.1$) in comparison to controls.

The protein concentrations of the homogenates varied quite substantially, presumably due mainly to the variance in homogenate concentrations utilised. Examination of the concentration / time graphs for individual incubations did not correlate the rate of

disappearance of linalool with homogenate, or protein, concentration (this being a potential indication that linalool disappearance was not caused by enzymic action). Therefore the large standard deviations for the kinetic parameters given in table 4.5 are most likely a function of experimental inaccuracy given that linalool half lives were at least 4.5 times longer than the 3 hour incubations employed.

Tissue	Homogenate concentration (% w/v ± SD) (protein concentration [mg/ml ± SD])	Linalool $t_{1/2}$ (minutes ± SD)	Statistical confidence of difference from control (*P*)
Control (buffer only)	-	2400 ± 700	-
Stomach contents	15.4 ± 8.2 (8.5 ± 6.2)	11000 ± 17000	NS
Intestinal contents	10.6 ± 7.7 (3.0 ± 2.2)	1300 ± 1500	NS
Intestinal mucosa	5.7 ± 3.1 (7.6 ± 3.4)	1500 ± 1000	NS
Blood	7.3 ± 3.2 (21.0 ± 7.1)	810 ± 830	<0.1
Liver	36.4 ±10.7 (78.2 ± 20.3)	11000 ± 17000	NS

Table 4.5: Metabolism of 6.5 mM linalool by homogenates of rat stomach contents, intestinal contents, intestinal mucosa, blood and liver, over a 3 hour time course at 37°C. NS = no statistically significant difference.

4.3.5 Oxidation of Linalool by Rat Intestinal and Hepatic Cytochrome P-450

Table 4.6 illustrates the rate of metabolism of linalool by the cytochrome P-450 systems of rat intestinal and hepatic preparations. No statistically significant difference in the rates of disappearance of linalool was seen between incubations of intestinal homogenate containing and not containing activation system. The rates of

disappearance of linalool in hepatic preparations were significantly different between incubations containing and not containing activation system ($P<0.05$), this is illustrated in figure 4.7.

Initial investigations identified the requirement of glucose-6-phosphate dehydrogenase. In the absence of glucose-6-phosphate dehydrogenase from the activation system, approximately only 50 % of the linalool present was metabolised by the hepatic preparations within 15 minutes (with 64 ± 12 μM remaining of 130 μM) at an initial rate of $t_{½}$ = 11 ± 1 minutes (standard not containing activation system $t_{½}$ = 850 ± 260 minutes), following which no further metabolism took place. In order to investigate if this cessation of metabolism was influenced by the linalool concentration, and was not solely due to the lack of glucose-6-phosphate dehydrogenase, a further series of investigations were conducted utilising 65 μM as the initial concentration of linalool. These investigations illustrated complete metabolism of linalool within 15 minutes at a rate of $t_{½}$ = 8 ± 8 minutes (standard not containing activation system $t_{½}$ = 1700 ± 1400 minutes). When a substrate concentration of 130 μM was used with a metabolic activation system which did contain glucose-6-phosphate dehydrogenase, virtually complete metabolism of linalool was observed (see figure 4.7). This demonstrated the requirement for glucose-6-phosphate dehydrogenase in the incubation system when the linalool concentration was higher than 65 μM.

	Linalool $t_{1/2}$ (minutes ± SD)	
Tissue	**Not containing cytochrome P-450 activation system**	**Containing cytochrome P-450 activation system**
Small Intestine Homogenate	1200 ± 500	890 ± 390
Liver Homogenate	1500 ± 690	4 ± 2

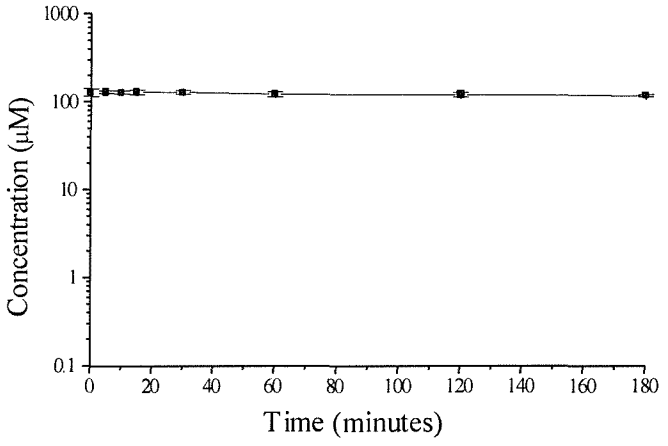
Table 4.6: Metabolism of 130 μM linalool by the cytochrome P-450 mixed function oxidase system of rat small intestinal homogenate (5 % w/v, 7.92 ± 1.56 mg/ml protein), and liver homogenate (5 % w/v, 16.33 ± 1.51 mg/ml protein). Reaction rates have been calculated using the initial linear portions of $\log^{10}$ concentration versus time graphs.

Hydrolysis of samples, taken at 180 minutes from intestinal and hepatic incubations containing and not containing activation system, with ß-glucuronidase or sulphatase were found to release no additional free linalool, indicating that the disappearance of linalool was not due to conjugation with glucuronic acid or sulphate. Table 4.7 illustrates that the intestinal and liver homogenate samples employed in all the investigations had cytochrome P-450 activity in the presence of an activation system, as measured by the oxidation of 7-ethoxycoumarin to form 7-hydroxycoumarin.

Incubation Type	**nmoles 7-hydroxycoumarin produced/min/mg protein; Difference between incubations containing activation system - incubations not containing activation system (incubations not containing activation system given in parenthesis)**
5 % Intestinal Homogenate	Intestine A = 0.05 (0.24) Intestine B = 0.04 (0.50) Intestine C = 0.07 (0.48)
5 % Liver Homogenate	
Investigations containing glucose-6-phosphate dehydrogenase	Liver A = 0.17 (0.58) Liver B = 0.34 (0.44) Liver C = 0.39 (0.48)
Investigations not containing glucose-6-phosphate dehydrogenase	Liver A = 0.48 (0.36) Liver B = 0.13 (0.25) Liver C = 0.52 (0.31)
Investigations not containing glucose-6-phosphate dehydrogenase (reduced substrate concentration experiments)	Liver A = 0.15 (0.53) Liver B = 0.17 (0.52) Liver C = 0.21 (0.57)

Table 4.7: Metabolism of 7-ethoxycoumarin to 7-hydroxycoumarin by the tissue homogenates employed in the investigations of the metabolism of linalool by rat intestinal and hepatic cytochrome P-450.

A.



B.

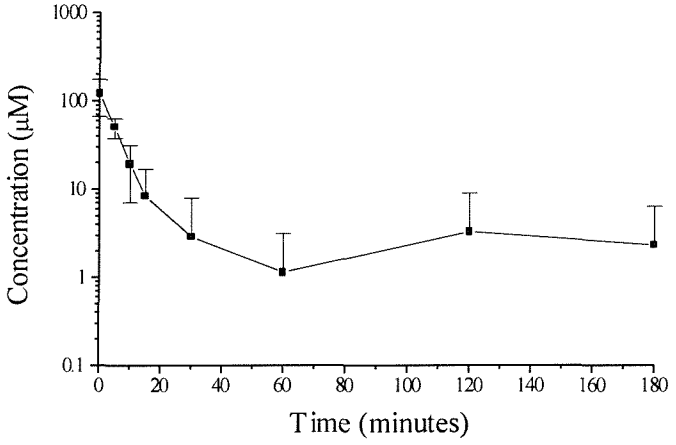
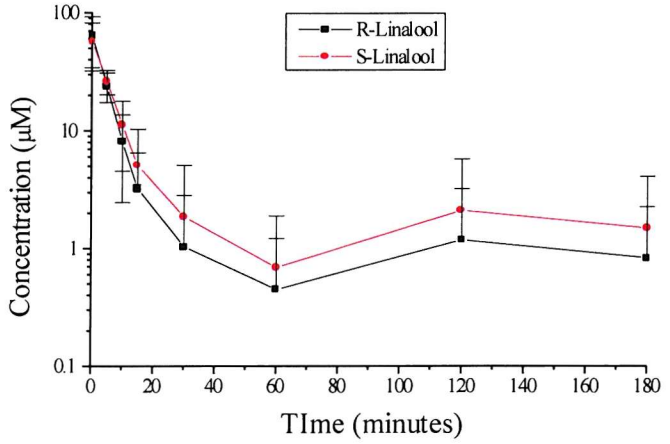


Figure 4.7: Hepatic cytochrome P-450-mediated metabolism of 130 μM linalool (mean ± SD). **A.** Incubations not containing cytochrome P-450 activation system. **B.** Incubations containing cytochrome P-450 activation system.

4.3.5.1 Stereoselectivity of Linalool Oxidation by Rat Hepatic Cytochrome P-450

The metabolism of a racemic mixture of (R)- and (S)- linalool by rat hepatic cytochrome P-450 (the results illustrated in figure 4.7) was found to be stereoselective for (R)-linalool, as illustrated in figure 4.8. In comparison of the rates of disappearance of (R)- and (S)- linalool no statistically significant difference was observed. In the incubation system employed, (R)-linalool $t_{½}$ = 4 ± 2 minutes, and (S)-linalool $t_{½}$ = 5 ± 2 minutes. However, calculation of the isomeric ratios, as illustrated in figure 4.8B demonstrates the clear preferential metabolism of (R)-linalool.

A.



B.

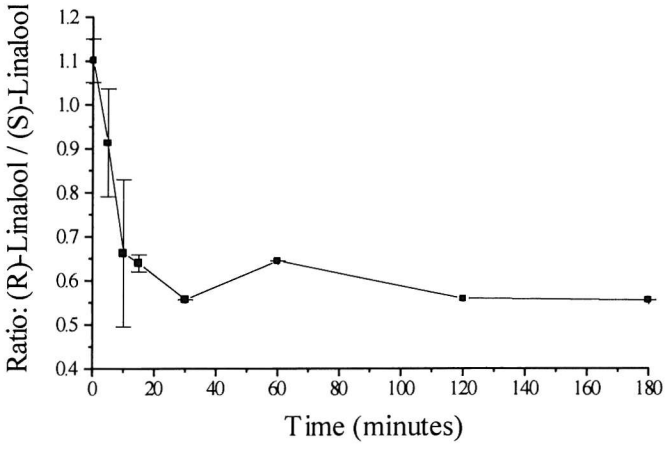


Figure 4.8: Enantiomeric preference during the metabolism of 130 µM racemic linalool by rat hepatic cytochrome P-450 (mean ± SD). **A.** Concentrations of linalool stereoisomers. **B.** Ratio (R)-/(S)- linalool (initial ratio (R)-/(S)- linalool = 1.1:1).

4.3.6 Sulphate Conjugation of Linalool by Rat Intestinal and Liver Preparations

As illustrated by table 4.8, No significant difference in the rates of disappearance of linalool was seen between incubations of intestinal and hepatic homogenates containing or not containing sulphate conjugation activation system. Although a small difference was observed between intestinal incubations containing and not containing activation system ($P<0.2$), the difference did not reliably demonstrate conjugation activity.

	Linalool $t_{1/2}$ (minutes ± SD)	
Tissue	**Not containing sulphate conjugation activation system**	**Containing sulphate conjugation activation system**
Small Intestine Homogenate	1200 ± 900	620 ± 90
Liver Homogenate	1100 ± 200	1400 ± 500

Table 4.8: Metabolism of 130 μM linalool by the sulphate conjugation system of rat small intestinal homogenate (5 % w/v, 7.92 ± 1.56 mg/ml protein), and liver homogenate (5 % w/v, 17.81 ± 0.93 mg/ml protein).

Hydrolysis of samples, taken at 180 minutes from intestinal and hepatic incubations containing and not containing activation system, with ß-glucuronidase or sulphatase was found to release no additional free linalool, confirming the lack of conjugation. Table 4.9 illustrates that the intestinal and liver homogenate samples employed in all incubations had sulphate conjugating activity in the presence of an activation system, as measured by the loss of fluorescence of 7-hydroxycoumarin.

Incubation Type	nmoles 7-hydroxycoumarin conjugated / min / mg protein Difference between incubations containing activation system – incubations not containing activation system (incubations not containing activation system given in parenthesis)
5 % Intestinal Homogenate	Intestine A = 6.27 (10.03) Intestine B = 7.70 (27.20) Intestine C = 2.13 (16.03)
5 % Liver homogenate	Liver A = 10.00 (8.93) Liver B = 9.14 (10.10) Liver C = 10.02 (6.98)

Table 4.9: Conjugation of 7-hydroxycoumarin by the tissue homogenates employed in the investigations of the metabolism of linalool by rat intestinal and hepatic sulphate conjugation systems.

4.3.7 Glucuronic Acid Conjugation of Linalool by Rat Liver and Intestinal Preparations

As illustrated in table 4.10, no significant difference in the rates of disappearance of linalool was seen between incubations of intestinal homogenate containing or not containing glucuronic acid conjugation activation system. The disappearance of linalool in hepatic preparations was significantly different between incubations containing and not containing activation system ($P<0.05$), this is illustrated in figure 4.9.

	Linalool $t_{1/2}$ (minutes ± SD)	
Tissue	**Not containing glucuronic acid conjugation activation system**	**Containing glucuronic acid conjugation activation system**
Small Intestine Homogenate	990 ± 610	2400 ± 3800
Liver Homogenate	1500 ± 620	23 ± 9

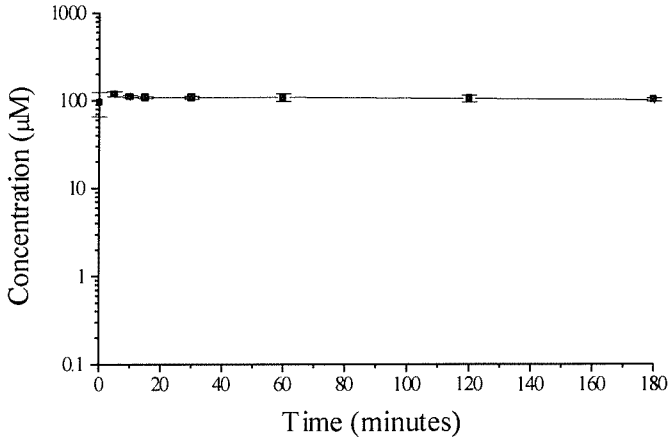
Table 4.10: Metabolism of 130 μM linalool by the glucuronic acid conjugation system of rat small intestinal homogenate (5 % w/v, 7.92 ± 1.56 mg/ml protein), and liver homogenate (5 % w/v, 17.81 ± 0.93 mg/ml protein).

Hydrolysis of samples, taken at 180 minutes from intestinal and hepatic incubations containing and not containing activation system, with sulphatase was found to release no additional free linalool. Hydrolysis of aliquots of the same samples with ß-glucuronidase did not release any additional free linalool in the case of intestinal and hepatic incubations not containing activation system, and in the case of intestinal incubations containing activation system. However, hydrolysis with ß-glucuronidase did release 70.4 ± 5.8 % of the metabolised linalool in the case of hepatic incubations containing activation system. This confirmed that the disappearance of linalool in hepatic incubations containing glucuronidation activation system was due to metabolic conjugation with glucuronic acid. Table 4.11 illustrates that the intestinal and liver homogenate samples utilised in all incubations had glucuronic acid conjugating activity in the presence of an activation system, as measured by the loss of fluorescence of 7-hydroxycoumarin.

Incubation Type	**nmoles 7-hydroxycoumarin conjugated / min / mg protein** **Difference between incubations containing activation system – incubations not containing activation system (incubations not containing activation system given in parenthesis)**
5 % Intestinal Homogenate	Intestine A = 13.49 (15.61) Intestine B = 15.00 (33.60) Intestine C = 9.03 (23.60)
5 % Liver homogenate	Liver A = 15.67 (2.98) Liver B = 13.60 (5.40) Liver C = 13.23 (6.37)

Table 4.11: Conjugation of 7-hydroxycoumarin by the tissue homogenates employed in the investigations of the metabolism of linalool by rat intestinal and hepatic glucuronic acid conjugation systems.

A.



B.

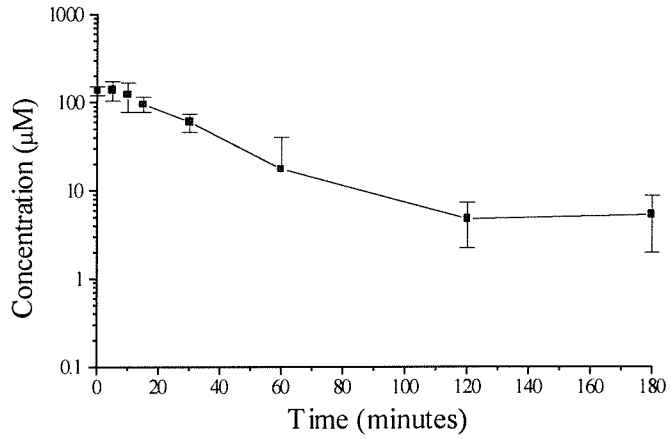


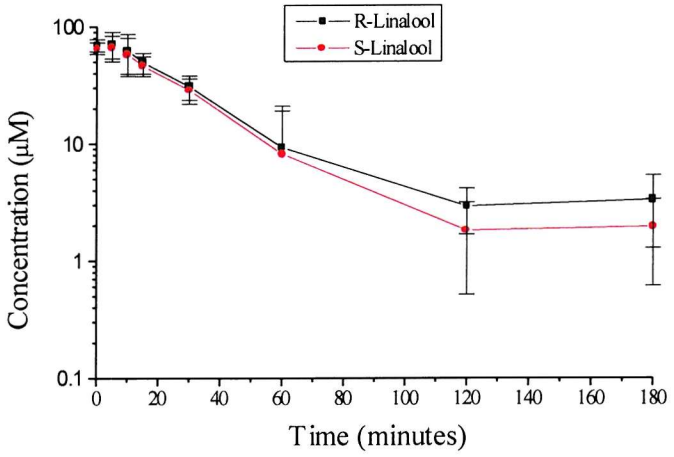
Figure 4.9: Hepatic glucuronic acid conjugation of 130 μM linalool (mean ± SD). **A.** Incubations not containing glucuronic acid conjugation activation system. **B.** Incubations containing glucuronic acid conjugation activation system.

4.3.7.1 Stereoselectivity of Linalool Glucuronic Acid Conjugation by Rat Hepatic Preparations

The metabolism of a racemic mixture of (R)- and (S)- linalool by rat hepatic glucuronic acid conjugation (the results illustrated in 4.3.7) was found not to be stereoselective, as demonstrated by figure 4.10. In comparison of the rates of disappearance of (R)- and (S)- linalool no statistically significant difference was

observed. In the incubation system employed, (R)-linalool $t_{½}$ = 23 ± 9 minutes, and (S)-linalool $t_{½}$ = 23 ± 9 minutes.

A.



B.

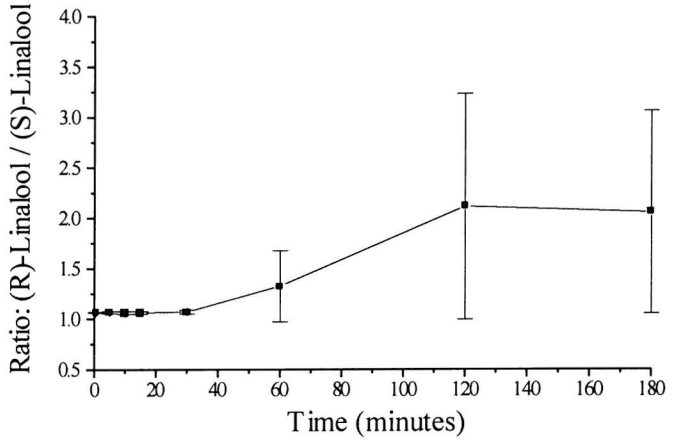


Figure 4.10: Enantiomeric preference during the metabolism of 130 μM racemic linalool by rat hepatic glucuronic acid conjugation (mean ± SD). **A.** Concentrations of linalool stereoisomers. **B.** Ratio (R)-/(S)- linalool (initial ratio (R)-/(S)- linalool = 1.1:1).

4.4 Discussion

Studies into the chemistry, absorption and metabolism of monoterpenoid alcohols (particularly linalool) were undertaken to investigate the structural isomerisations identified in incubations of related esters in artificial gastrointestinal fluids, and to provide additional data valuable for the safety evaluation of this important group of food flavouring additives. Following the initial 'screen' of ester substrates in artificial gastrointestinal fluids (Chapter 3), these studies also provided a wider knowledge of monoterpenoid alcohol metabolism prior to tissue homogenate and *in vivo* ester hydrolysis investigations (Chapters 5 and 6 respectively).

4.4.1 Monoterpene Alcohols in Artificial Gastrointestinal Fluids

Pepsin was observed to have no effect upon linalool or citronellol. However, both compounds were unstable in an acidic environment (pH 1.2). The citronellol reaction product(s) were not detected although organic chemistry publications have suggested that citronellol may undergo acid-catalysed rearrangement to form compounds such as tetramethyl cyclohexane or possibly trimethyl cycloheptanone[264; 265; 267]. Based upon experimental data, a scheme is proposed for the behaviour of linalool and it's products in gastric fluid (pH 1.2), which is illustrated in figure 4.11. The final product of linalool, geraniol and nerol rearrangement in an acidic environment is α-terpineol.

Due to the unusual stability of the carbocation intermediates, linalool, geraniol and nerol readily undergo acid-catalysed rearrangement. The stability of the alicyclic compound α-terpineol at pH 1.2 was approximately 10 times the stability of all other monoterpenoid alcohols investigated (and partly for this reason α-terpineol was observed to be the final product of all acid-catalysed rearrangement reactions). However, the rate of loss of α-terpineol from incubations at pH 1.2 was substantially faster than the rate of loss from incubations at pH 7.0 ($P<0.05$). It is possible to speculate that if incubations had been conducted for a longer period of time, terpinolene and limonene may have been detected as reduction products, following which more saturated products may form.

It is interesting to note, that the fact that geraniol is a minor product of linalool rearrangement whilst nerol is only a trace product indicates that it is substantially more energetically favourable for the primary carbocation intermediate to cyclise and form α-terpineol than it is for this intermediate to form nerol. Additionally, it may be less energetically favourable to form the *cis*-isomer nerol, than it is to form the *trans*-isomer geraniol.

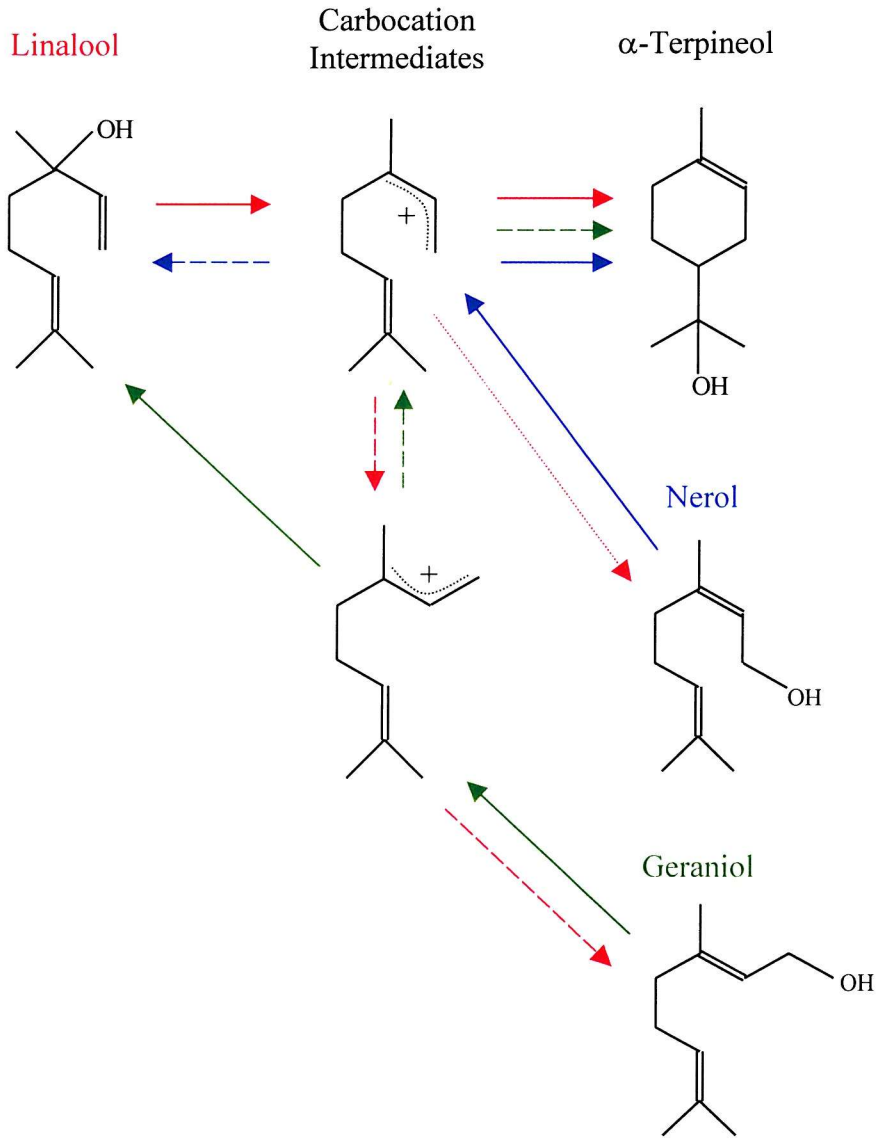


Figure 4.11: Proposed scheme for the behaviour of linalool in acidic gastric fluid. Red arrows = linalool, green arrows = geraniol, blue arrows = nerol. Solid arrows indicate major routes, dashed arrows indicate minor routes, dotted arrows indicate trace routes.

All the monoterpene alcohols investigated were stable in aqueous solution at neutral pH. Investigations into the stability of linalool in aqueous solution at pH 2.5 demonstrated an approximate 20 fold increase in stability in comparison to incubations conducted at pH 1.2. The stability of linalool in rat stomach is as such likely to be significantly greater than in human stomach. The structural rearrangements of monoterpenoid alcohols in biological media have not previously been studied.

Although acid-catalysed structural isomerisations are likely to be slow in the rat gastric environment, it is interesting to speculate that the cytochrome P-450 induction observed following dosage with linalool, as described in 4.1.3.1, may be due more to α-terpineol than it is to linalool, as the data suggests that α-terpineol is a more potent enzyme inducer than linalool.

As the loss of the hydroxy group (by electrophilic addition) from the chiral centre of linalool results in the formation of a carbocation intermediate, the enantiomeric specificity of acid-catalysed rearrangement was investigated. No stereoselectivity in the hydrolysis of linalool or the formation of α-terpineol was observed.

The hydroxy group of linalool is possibly sterically hindered by the presence of a methyl group and an allyl group on the carbon atom to which the functional group is bonded, the hydroxy group of citronellol has no such possible steric hindrance. The lack of structural rearrangement or metabolism of linalool and citronellol in artificial pancreatic fluid illustrates that monoterpenoid alcohols are likely to be absorbed intact into the gastrointestinal mucosa.

4.4.2 Intestinal Absorption of Linalool

The permeability coefficient of non-ionised solutes driven by a concentration difference through an intestinal membrane may be calculated using the equation[326]:

$$n = AP(c_o - c_i)$$

Were n = net flux (mol/s), A = intestinal surface area (cm^2), P = permeability coefficient (cm/s), c_o = initial substrate concentration in the organ bath (M) and c_i = initial substrate concentration in the intestinal lumen (M). This equation requires that the concentration of substrate in the water bath remains constant, as this was not the case, net flux may be calculated from the initial linear portion of the concentration versus time graph for substrate concentration in the intestinal lumen. The permeability coefficient includes such parameters as the partition coefficient of the substrate between oil/water, and the thickness of the permeable membrane.

The permeability coefficient of linalool across rat intestine, in the experimental model employed, was $2.12x10^{-3} \pm 0.99x10^{-3}$ cm/s. As the permeability coefficient of pure unstirred water to solutes is always between $1x10^{-4}$ and $1x10^{-3}$ cm/s[327], it is clear that rat small intestine presents little barrier to concentration driven permeation by linalool. Esters of linalool which possess greater lipophilicity than the parent alcohol would be anticipated to illustrate higher permeability coefficients than linalool.

4.4.3 Metabolism of Linalool by Rat Tissue Homogenates

No statistically significant differences were observed in the rates of disappearance of linalool between control incubations and incubations containing homogenates of rat stomach contents, intestinal contents, intestinal mucosa, and liver. The disappearance of linalool from incubations containing rat blood homogenate was slightly more rapid ($P<0.1$) than from control incubations. The balance of evidence indicates that linalool is a poor substrate for alcohol dehydrogenase (see 4.1.3).

No metabolism of linalool by rat intestinal cytochrome P-450, glucuronic acid or sulphate conjugation activity was identified. No linalool sulphate conjugation activity was detected in rat liver. Rat hepatic cytochrome P-450 was shown to very readily oxidise linalool with a distinct stereoselective preference for (R)-linalool. Furthermore, rat liver preparations were shown to possess a strong capacity to conjugate linalool with glucuronic acid, glucuronyl transferase(s) showing no stereoselective preference. The observations of differences in stereoselective metabolism could be interpreted in terms of the more substrate-selective nature of

cytochrome P-450 isoenzymes in comparison to glucuronyl transferases, and may also indicate that hepatic oxidation of linalool is mainly a function of a single cytochrome P-450 isoenzyme. This may be supported by the observed lack of oxidation in intestinal preparations, as members of the 3A subfamily are the predominant forms of cytochrome P-450s found in intestinal tissue[328].

It is interesting that the 8-position is the common site of hydroxylation of monoterpenoid alcohols and aldehydes (see 4.1.3), in view of the observed distinct (R)-linalool stereoselectivity, as the chiral centre is at the opposite end of the small molecule. Presumably, linalool may be a good substrate for probing the mechanism of specific cytochrome P-450 isoenzymes.

Some discrepancy exists in the information produced for safety assessment purposes over the precise location of the 8 carbon atom-position of monoterpenoid alcohols[9]. Figure 4.12 illustrates the nomenclature of linalool according to the International Union of Pure and Applied Chemistry[329].

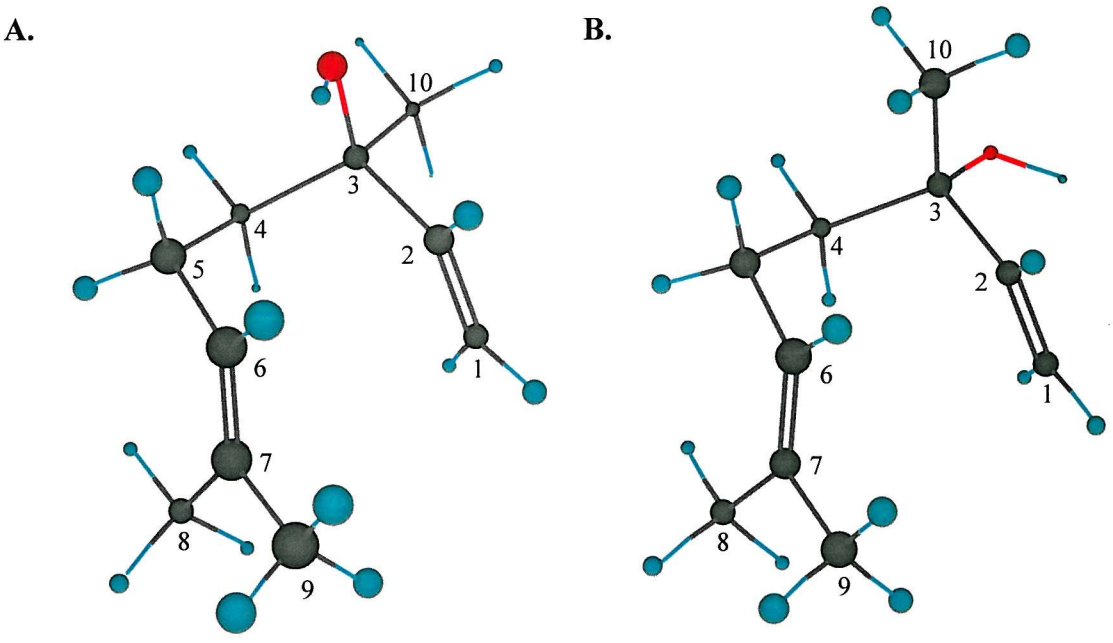


Figure 4.12: Ball and stick model of **A.** (R)-linalool and **B.** (S)-linalool. Structures are depicted in their minimum energy states. Carbon number designations are indicated. Carbon atoms = black, oxygen atoms = red, hydrogen atoms = blue. Rotational movement of the covalent bonds between carbon atoms 3 – 6 and 3 – 2 will result in movement of elements of the 2-methylhept-2-ene group and allyl group respectively in relation to the hydroxy group.

These investigations demonstrate the ready cytochrome P-450 oxidation and glucuronic acid conjugation of linalool by rat hepatic preparations. These results should be examined in light of the currently accepted view of linalool metabolism, as described in 4.1.3 (which has been used for safety evaluation purposes).

The currently accepted view of linalool metabolism is based upon *in vivo* studies and states that linalool conjugation with glucuronic acid and subsequent excretion is the primary route of metabolism, with oxidation becoming important only following repeated animal dosing. That cytochrome P-450-mediated oxidation occurs via allylic hydroxylation to form polar diols which may be excreted free or conjugated, and also that further oxidation may occur to the corresponding carboxylic acid. These suggestions have been based mainly upon the work of Chadha and Madyasthra[306] and Parke *et al*[301; 302]. Furthermore, linalool and related monoterpenoid compounds have been shown to readily induce the hepatic cytochrome P-450 mixed-function oxidase system (see 4.1.3.1).

In high dose *in vivo* studies, glucuronidation and excretion via urine and bile is clearly the major route of clearance. However, in the investigations presented in this report, the rate of NADPH-dependent metabolism of linalool is considerable in view of the 5 % liver tissue employed, and that the animals had not been pre-treated with any hepatic enzyme-inducing substances.

Based on the structure of linalool and knowledge of rat hepatic cytochrome P-450 isoenzyme substrate specificities, it is probable that the 2E1 isoenzyme is the determinant protein[330]. This is supported by the considerable cytochrome P-450 stereoselectivity for (R)-linalool, possibly indicating the action of a single isoenzyme, and the lack of oxidation by intestinal tissue (in which the 3A family of isoenzymes are the predominant forms of cytochrome P-450 found[328]). It is extremely interesting to note that the cytochrome P-450 isoenzyme 2E1 is most active at low concentrations of substrate[330]. In all the investigations previously cited on the *in vivo* metabolism of linalool, dosage was by direct administration to the stomach of the lipophilic substrate in single daily doses of between 500 and 1500 mg / kg body weight[301; 302; 306; 316]. As

such, for periods of time it is likely that the plasma concentration of linalool would be in excess of the 130 μM (0.02 mg/ml) employed in the incubations described in this report. The high capacity glucuronic acid conjugation system is clearly important in the high dose conditions of the cited literature, but perhaps the metabolism and pharmacokinetics of linalool metabolism is somewhat different in its low dosage use as a food flavouring compound. The *in vitro* experiments described in this report indicate the possibly greater prominence that oxidation plays in the metabolism of low doses of linalool. However, it may be erroneous to extrapolate such *in vitro* experiments to *in vivo* pharmacokinetics without further investigations of the dose dependency of metabolism.

There is very limited data available concerning the human metabolism of monoterpenoid alcohols, however the ingestion of a mixture containing α-terpineol has been shown to result in metabolites indicative of the production of epoxides (which were identified as triol metabolites and not as compounds indicative of glutathione conjugation)[300]. Limonene is a non-hydroxylated structural analogue of α-terpineol, the metabolism of which has been extensively studied as it is a male rat-specific renal tumorigen[331-334], promoter of trans-dermal drug absorption[286; 335-338], and a potential chemotherapeutic agent[287; 339]. The metabolism of limonene has been found to be similar in rats and humans, and results in the formation of 1,2-epoxides (8,9–epoxide has also been demonstrated but is more readily hydrolysed to the respective diol in comparison to the 1,2-epoxide)[334; 340]. Allylic oxidation at a comparable position has also been demonstrated following the administration of *trans*-sobrerol to rats, dogs and humans[303; 315]. Furthermore, the hydroxylation of the monoterpene alcohol *l*-menthol in rats by cytochrome P-450 has been shown to result in the formation of an epoxide, due to the conformational proximity of the two hydroxyl moieties[308]. As such, to enable the safety evaluation process it is necessary to more fully investigate the metabolism of monoterpenoid alcohols particularly those which have unsaturated cyclic structures or have their hydroxyl moiety proximal to the common site of cytochrome P-450 –mediated oxidation.

CHAPTER 5

HYDROLYSIS OF LINALYL, CINNAMYL AND FURFURYL ESTERS IN HOMOGENATES OF RAT AND HUMAN TISSUE AND RAT GASTROINTESTINAL PREPARATIONS

5.0 Hydrolysis of Linalyl, Cinnamyl and Furfuryl Esters in Homogenates of Rat and Human Tissue and Rat Gastrointestinal Preparations

5.1 Introduction

5.1.1 Background

As discussed in 1.4 and 3.1.1, for the purposes of safety evaluation, it has been assumed that esters would be readily hydrolysed to their component alcohols and carboxylic acids[250]. However, as described in 1.3, there exists little data to support such predictions, furthermore as described in 1.2, mammalian esterases are poorly characterised.

5.1.2 Study Objectives

Ester compounds were selected for study based upon the metabolic trends identified in the results of investigations with artificial gastrointestinal fluids described in Chapter 3. Studies were undertaken using these substrates in order to evaluate differences in the characteristics of hydrolysis of monoterpenoid, cinnamyl and furfuryl esters in preparations of rat and human tissue, toward aiding the construction of a model of flavouring ester metabolism. Compounds were selected which were found to undergo rapid or slow hydrolysis in artificial pancreatic fluid in comparison to other members of the same structural group, and such that they would best enable a comparison of the metabolic characteristics lent by both the alkyl and acyl ester moieties.

Investigations were also undertaken into the hydrolysis of esters in homogenates of rat gastric and intestinal contents to allow a comparison with artificial gastrointestinal fluids.

The quantity of linalool, linalyl acetate, α-terpineol and terpinyl acetate produced for use as food additives represent approximately 96 % of all monoterpenoid esters

produced[341]. As such, additional investigations into the fate of linalyl acetate following ingestion were undertaken.

In an attempt to characterise the esterase enzyme(s) present in rat liver, investigations were conducted using known serine-esterase and serine-protease inhibitors. The stereoselective characteristics of linalyl ester hydrolysis by preparations of whole rat liver and by putative serine-esterase isoenzymes were investigated.

5.2 Methods

5.2.1 Investigations with Rat and Human Tissue Homogenates

Experimental protocols were conducted as described in 2.2.6, with the following exceptions in respect to the investigations given in 5.3.2 below:

- The same sets of tissue (derived from the same three animals) were used in metabolic investigations in the case of all the substrates employed, unless otherwise stated, with incubations conducted concurrently. In consequence, intra-individual and experimental variation were minimised.

- This approach necessitated the use of a lower incubation volume, which was 10 ml in all cases, with 1 ml samples withdrawn at set time points to 0.5 ml CHX/THN.

- Rat gastric contents homogenates were prepared in tissue buffer pH 2.5.

5.2.1.1 Human Blood

Human blood (5 ml) was collected at the time of approximately 10.00 am from the brachial vein of 3 volunteers, all of whom were non-smoking Caucasian males aged between 20 and 28 years of age (20, 26 and 28). Homogenates were prepared as

described in 2.2.6.1. As with all other tissue investigations, experimental procedures were commenced immediately following tissue isolation.

5.2.1.2 Human Liver

Samples of human liver tissue (5 – 25 g) were collected, with informed consent, from patients (Caucasian males aged 48, 59 and 63 years, smoking history unknown) undergoing biopsy during procedures related to the treatment of colo-rectal cancer (lobe of origin of tissue unknown). No evidence of metastasis was detected in the areas of tissue used in these investigations, and hepatocellular carcinoma was known not to be present. Homogenates were prepared as described in 2.2.6.1. Experimental procedures were commenced no longer than 3¼ hours following surgical removal of tissue from patients, during which time samples were stored on ice in tissue buffer.

5.3 Results

5.3.1 Linalyl Acetate

Linalyl acetate is poorly metabolised by artificial pancreatic fluid, and is inherently unstable in an aqueous environment, undergoing hydrolysis to form linalool and α-terpineol (see table 3.1), products which are stable in a neutral or alkaline environment (see 4.3.1 and 4.3.2). Table 5.1 illustrates the results of incubations of 50 μM linalyl acetate in homogenates of rat stomach contents, intestinal contents, intestinal mucosa, blood and liver.

Only incubations in 2.5 % liver homogenate resulted in a statistically significant increase in hydrolytic rate in comparison to controls, although incubations in 0.3 % intestinal mucosa showed an 80 % probability of statistical difference to controls (P=0.2). As discussed in 3.3.2, the ratios of the products linalool / α-terpineol indicate the reaction mechanism as enzymic hydrolysis results in the production of a greater proportion of linalool rather than α-terpineol which is a product of linalyl ester instability in neutral / alkaline conditions (additionally being a product of linalool

instability in acidic conditions). The ratio of products for incubations of linalyl acetate in artificial pancreatic fluid (as described in table 3.1) was 1.73 ± 0.23 in the case of incubations not containing pancreatin and 4.26 ± 0.23 in the case of incubations containing pancreatin. In comparing the ratios linalool / α-terpineol described in table 5.1 below, all the homogenates investigated showed statistically significant differences to controls except for homogenates of stomach contents.

Incubations of linalyl acetate in stomach contents homogenate showed a statistically significant decrease ($P<0.1$) in the rate of loss of the substrate in comparison to control incubations, and no significant difference in the ratio of products linalool / α-terpineol. This indicated that no metabolism occurred and that the lipophilic substrate was probably sequestered by the partially digested rat dietary material present.

A comparison of hydrolysis rates between homogenate types is complicated by differences in homogenate concentration. Normalising reaction rates by expressing disappearance of linalyl acetate or rate of formation of linalool as moles/min/mg protein would not yield useful data due to the high percentage of non-esterase protein present in the incubations. It is also not practical to express reaction rates as moles/min/g tissue as this would not provide a useful platform to compare between homogenates. This is because of the difference in homogenate concentrations between incubations, which affects the evaporation rate of the substrate, in conjunction with the generally very small degree of enzymic hydrolysis occurring (particularly when compared to spontaneous hydrolysis due to ester instability). However, an indication as to the most active homogenate can be gained by comparing the ratio of products / homogenate concentration. As such enzymic hydrolytic activity was seen to decrease between rat tissue and intestinal homogenates in the order: liver > intestinal mucosa > blood > intestinal contents.

Tissue	Homogenate conc. (% w/v) (protein concentration [mg/ml])	Linalyl acetate $t_{1/2}$ (minutes)	*P*-value	Final ratio of products: linalool / α-terpineol
Control (buffer)	-	130 ± 20	-	1.78 ± 0.16
Stomach contents	0.8 ± 0.5 (0.4 ± 0.3)	250 ± 90	<0.1	1.70 ± 0.16
Intestinal contents	0.4 ± 0.1 (0.31 ± 0.19)	130 ± 10	NS	2.11 ± 0.11
Intestinal mucosa	0.3 ± 0.0 (0.42 ± 0.07)	110 ± 20	=0.2	4.70 ± 0.71
Blood	0.5 ± 0.1 (1.40 ± 0.38)	140 ± 20	NS	2.76 ± 0.21
Liver	2.5 ± 0.0 (5.13 ± 0.36)	55 ± 14	<0.01	No α-terpineol produced

Table 5.1: Metabolism of 50 μM linalyl acetate by homogenates of rat stomach contents, intestinal contents, intestinal mucosa, blood and liver, over 180 minutes at 37°C. Homogenate and protein concentrations, half lives and product ratios are expressed ± SD. *P*-values represent confidence of probability of difference from controls. NS = no statistically significant difference. Number in red indicates a statistical difference based upon a longer half-life in homogenates in comparison to controls.

5.3.2 *Investigations into the Hydrolysis and Stereoselective Metabolism of Selected Linalyl, Cinnamyl and Furfuryl Esters by Rat Gastric and Intestinal Contents, Rat Intestinal Mucosa, Rat and Human Blood, and Rat and Human Hepatic Preparations*

To investigate differences in the hydrolytic metabolism of flavouring esters in 5 % homogenates of rat gastric and intestinal contents, rat intestinal mucosa, rat and human blood and rat and human liver, a number of compounds were selected for

study. In the case of gastrointestinal and blood investigations the substrates employed were linalyl propionate, linalyl cinnamate, cinnamyl cinnamate and furfuryl propionate (which showed hydrolysis half-lives of 34 ± 0, 500 ± 40, 0.04 ± 0.01 and <0.01 minutes respectively in artificial pancreatic fluid). In the case of liver investigations cinnamyl propionate was employed as an additional substrate (which showed a hydrolysis half-life of <0.01 minutes in artificial pancreatic fluid).

Compounds were selected for study, from the collection available, based upon the results of investigations with artificial gastrointestinal fluids, to best enable a comparison of the metabolic characteristics related to both the alkyl and acyl ester moieties.

5.3.2.1 Rat Gastric Contents

As illustrated in table 5.2, 5 % homogenates of rat gastric contents (protein concentration = 2.62 ± 0.93 mg/ml) showed no hydrolytic capacity towards linalyl propionate, linalyl cinnamate, cinnamyl cinnamate or furfuryl propionate.

Substrate	Compound Assayed	Control (no gastric contents)	Test (5 % gastric contents)
Linalyl propionate	Linalyl propionate	25 ± 1	160 ± 120
	Linalool	0.062 ± 0.012	0.0099 ± 0.0021
	α-Terpineol	0.045 ± 0.005	0.012 ± 0.007
Linalyl cinnamate	Linalyl cinnamate	19 ± 4	∞
	Linalool	0.014 ± 0.002	None produced
Cinnamyl cinnamate	Cinnamyl cinnamate	∞	∞
	Cinnamyl alcohol	None produced	None produced
Furfuryl propionate	Furfuryl propionate	270 ± 80	∞

Table 5.2: Reaction rates [disappearance of esters = $t_{½}$ (minutes ± SD) and rate of formation of identified alcohol products = k ($minutes^{-1}$ ± SD)] of 50 μM linalyl propionate, linalyl cinnamate, cinnamyl cinnamate and furfuryl propionate incubated in 5 % rat gastric contents homogenate pH 2.5. Incubations performed at 37°C over 180 minutes.

5.3.2.2 Rat Intestinal Contents

As demonstrated in table 5.3 and figure 5.1, linalyl propionate and linalyl cinnamate did not undergo measurable hydrolysis in 5 % rat intestinal contents homogenate (protein concentration = 3.72 ± 0.65 mg/ml). Stereoselective analysis of linalyl propionate disappearance failed to demonstrate any stereoselective differences, further indicating the lack of enzymic hydrolysis (examination of linalyl propionate stereoselectivity was based upon an analysis of the alkyl product linalool, due to chromatographic difficulties in resolving linalyl propionate stereoisomers).

Statistically significant increases were observed in the rates of disappearance of cinnamyl cinnamate (and in the formation of cinnamyl alcohol) and furfuryl propionate in homogenate incubations in comparison to controls. Figure 5.1 illustrates the nmole rate of hydrolysis, the rate of loss of furfuryl propionate was found to be slightly more rapid than the rate of hydrolysis of cinnamyl propionate.

Substrate	Compound Assayed	Control (no intestinal contents)	Test (5 % intestinal contents)
Linalyl propionate	Linalyl propionate	26 ± 4	40 ± 20
	Linalool	0.036 ± 0.000	None produced
Linalyl cinnamate	Linalyl cinnamate	14 ± 3	∞
	Linalool	0.0094 ± 0.0027	None produced
Cinnamyl cinnamate	Cinnamyl cinnamate	∞	56 ± 14
	Cinnamyl alcohol	None produced	0.0089 ± 0.0030
Furfuryl propionate	Furfuryl propionate	570 ± 90	10 ± 3

Table 5.3: Reaction rates [disappearance of esters = $t_{½}$ (minutes ± SD) and rate of formation of identified alcohol products = k ($minutes^{-1}$ ± SD)] of 50 µM linalyl propionate, linalyl cinnamate, cinnamyl cinnamate and furfuryl propionate incubated in 5 % rat intestinal contents homogenate. Incubations performed at 37°C over 180 minutes.

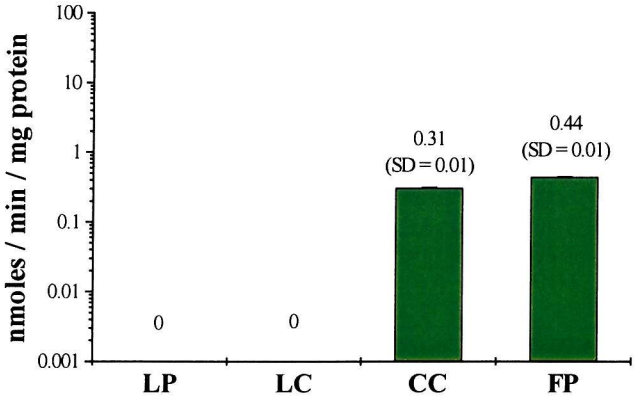


Figure 5.1: Hydrolysis rates of selected linalyl, cinnamyl and furfuryl esters incubated in 5 % rat intestinal contents homogenate (protein concentration = 3.72 ± 0.65 mg/ml). Rates are expressed as nmoles of substrate hydrolysed (-controls) / minute / mg protein ± SD. LP = linalyl propionate, LC = linalyl cinnamate, CC = cinnamyl cinnamate, FP = furfuryl propionate.

5.3.2.3 Rat Intestinal Mucosa

Investigations with 5 % rat intestinal mucosa (protein concentration = 8.02 ± 0.83 mg/ml) demonstrated clear biphasic metabolism. In all cases there was an initial rapid metabolism of the substrates within the first 5 minutes of incubations, which was followed over the subsequent 175 minutes by a period in which there was significantly less metabolism (following the final time-points, at least 40 % of substrates remained intact, except in the case of furfuryl propionate which was completely metabolised within 10 minutes). The cessation of metabolism was possibly due to the action of proteases present in the intestinal mucosa destroying the enzyme activity. However, if this was not the case then rat intestinal mucosa was seen to posses a low affinity / high capacity enzyme system but little activity in respect of a high affinity system.

Linalyl propionate was observed to undergo rapid initial metabolism up to 5 minutes incubation, at which time substrate concentration was approximately 20 μM, as illustrated in table 5.4 and figure 5.2. Although results were inconclusive, some possible stereoselectivity was observed for (R)-linalyl propionate as illustrated in figure 5.2.

Time-Frame And Compound	**Control (no intestinal mucosa)**	**Test (5 % intestinal mucosa)**
0.1 – 180 minutes		
Linalyl propionate	100 ± 10	-
Linalool	0.0091 ± 0.0005	-
0.1 – 5 minutes (at 5 minutes substrate = 21.12 ± 7.04 μM)		
Linalyl propionate	-	5.5 ± 2.3
Linalool	-	0.22 ± 0.10
5 – 180 minutes		
Linalyl propionate	-	69 ± 13
Linalool	-	0.011 ± 0.002

Table 5.4: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD) and rate of formation of alcohol = k ($minutes^{-1}$ ± SD)] of 50 μM linalyl propionate incubated in 5 % rat intestinal mucosa homogenate. Incubations performed at 37°C over 180 minutes. Reaction rates are expressed based upon biphasic kinetics for incubations in intestinal mucosa homogenates.

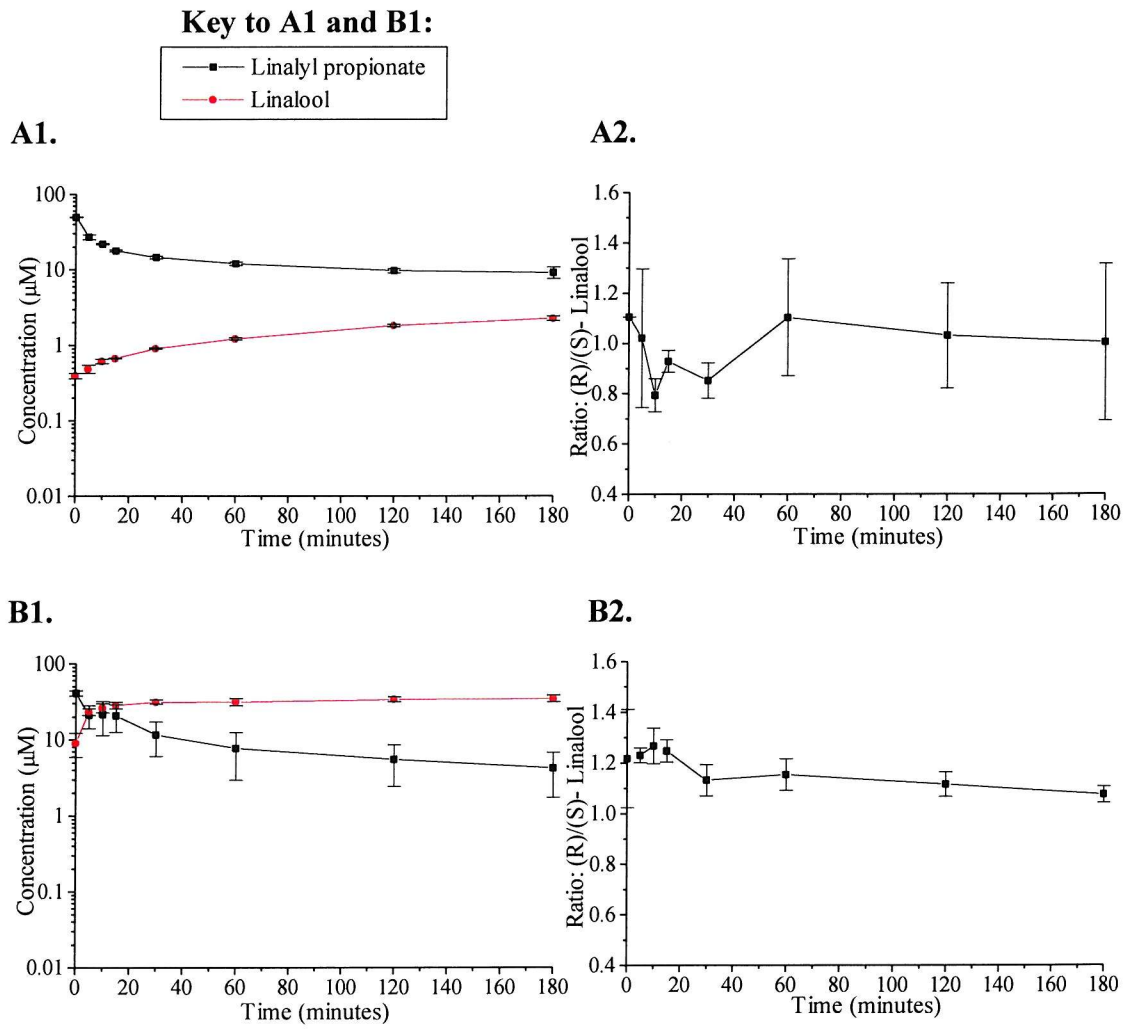


Figure 5.2: Metabolism of 50 µM linalyl propionate incubated in 5 % rat intestinal mucosa homogenate over 180 minutes at 37°C (mean ± SD) **A.** Incubations not containing intestinal mucosa (**A1** concentration versus time, **A2** ratio: (R)/(S)- linalool versus time) **B.** Incubations containing intestinal mucosa (**B1** concentration versus time, **B2** ratio: (R)/(S)- linalool versus time). Initial ratio (R)/(S)-linalyl propionate = 1:1.

Linalyl cinnamate underwent less pronounced rapid initial metabolism up to 0.1 minutes incubation, a time at which the substrate concentration was approximately 30 µM, as illustrated in table 5.5.

Time-Frame and Compound	Control (no intestinal mucosa)	Test (5 % intestinal mucosa)
0.1 – 180 minutes		
Linalyl cinnamate	43 ± 6	-
Linalool	0.0046 ± 0.0003	-
0 – 0.1 minutes ◊ **(at 0.1 minutes substrate = 32.15 ± 8.62 μM)**		
Linalyl cinnamate	-	0.20 ± 0.12
Linalool	-	*
0.1 – 180 minutes		
Linalyl cinnamate	-	53 ± 8
Linalool	-	0.020 ± 0.009

Table 5.5: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD) and rate of formation of alcohol = k (minutes^{-1} ± SD)] of 50 μM linalyl cinnamate incubated in 5 % rat intestinal mucosa homogenate. Incubations performed at 37°C over 180 minutes. Reaction rates are expressed based upon biphasic kinetics for incubations in intestinal mucosa homogenates. ◊ - Reaction rates based upon the assumption of 50 μM substrate present at time = 0 minutes. * - Calculation of rate constant based upon log^{10} concentration versus time graphs not possible.

Cinnamyl cinnamate underwent rapid initial metabolism up to 0.1 minutes incubation following which no further metabolism took place and substrate concentration remained stable at approximately 40 μM, as illustrated in table 5.6.

Time-Frame and Compound	Control (no intestinal mucosa)	Test (5 % intestinal mucosa)
0.1 – 180 minutes		
Cinnamyl cinnamate	93 ± 4	-
Cinnamyl alcohol	None produced	-
0 – 0.1 minutes ◊ **(at 0.1 minutes substrate = 37.63 ± 13.44 μM)**		
Cinnamyl cinnamate	-	0.52 ± 0.43
Cinnamyl alcohol	-	*
0.1 – 180 minutes		
Cinnamyl cinnamate	-	4000 ± 1800
Cinnamyl alcohol	-	0.0010 ± 0.0010

Table 5.6: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD) and rate of formation of alcohol = k (minutes^{-1} ± SD)] of 50 μM cinnamyl cinnamate incubated in 5 % rat intestinal mucosa homogenate. Incubations performed at 37°C over 180 minutes. Reaction rates are expressed based upon biphasic kinetics for incubations in intestinal mucosa homogenates. ◊ - Reaction rates based upon the assumption of 50 μM substrate present at time = 0 minutes. * - Calculation of rate constant based upon log^{10} concentration versus time graphs not possible.

Furfuryl propionate was very rapidly and completely metabolised within 10 minutes incubation, as illustrated in table 5.7.

Time-Frame and Compound	**Control (no intestinal mucosa)**	**Test (5 % intestinal mucosa)**
0.1 – 180 minutes		
Furfuryl propionate	920 ± 160	0.013 ± 0.001 ◊

Table 5.7: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD)] of 50 µM furfuryl propionate incubated in 5 % rat intestinal mucosa homogenate. Incubations performed at 37°C over 180 minutes. Substrate was completely hydrolysed in test incubations within 10 minutes. ◊ - Reaction rate based upon single time-point, and the assumption of 50 µM substrate present at time = 0 minutes.

For comparative purposes, as in 5.3.2.2 above, metabolic rates have additionally been calculated based upon the quantities of substrate and product in incubation mixtures at specific time-points. Figure 5.3 illustrates a comparative histogram of the rates of metabolism of all the four substrates investigated in this study in rat intestinal mucosa. In the case of linalyl and cinnamyl esters, rates are expressed as the nmoles of alkyl product formed (-controls) / minute / mg protein, and in the case of furfuryl propionate (the parent alcohol of which is not detectable under the method employed), as the nmoles of substrate hydrolysed (-controls) / minute / mg protein. This approach has been taken due to the following criteria (which refer only to the construction of comparative histograms):

- the presence of organic material in test incubations may sequester lipophilic substrates such that the rate of loss of substrates due to evaporation into the head-space may be less in test incubations than it is in control incubations. For substrates which are poorly metabolised by the tissue in test incubations (less than 0.25 nmoles of alkyl product formed / minute / mg protein), this sequestration effect may mask the small amount of enzyme-mediated hydrolysis possibly occurring. In these cases, an assessment of reaction rates is based on an analysis of the formation of the parent alcohol. This is not the case for furfuryl propionate which is very rapidly hydrolysed in test incubations and has a long half-life in control incubations (see table 5.7).

- In all instances the mean reaction rate of triplicate control incubations is subtracted from the calculated reaction rates for individual test incubations.

- The amounts of product formed within the first 15 minutes of the incubation periods were used in the calculations for linalyl and cinnamyl esters, as following this period either the substrate had been completely hydrolysed or no further increase in product concentration occurred. The rate of hydrolysis of furfuryl propionate has been calculated based upon the first 0.1 minutes of incubations.

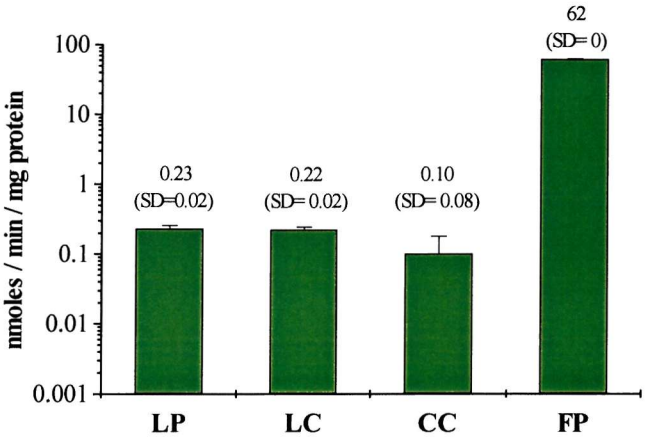


Figure 5.3: Hydrolysis rates of selected linalyl, cinnamyl and furfuryl esters incubated in 5 % rat intestinal mucosa homogenate (protein concentration = 8.02 ± 0.83 mg/ml). Rates are calculated over the period of the initial rapid metabolism only and are expressed as nmoles of alkyl product formed / minute / mg protein ± SD in the case of linalyl and cinnamyl esters, and as nmoles of substrate hydrolysed / minute / mg protein ± SD in the case of furfuryl propionate. LP = linalyl propionate, LC = linalyl cinnamate, CC = cinnamyl cinnamate, FP = furfuryl propionate.

5.3.2.4 Rat Blood & Human Blood

Investigations into the hydrolysis of 50 μM linalyl propionate, linalyl cinnamate, cinnamyl cinnamate and furfuryl propionate by homogenates of 5 % rat blood (protein concentration = 10.19 ± 0.36 mg/ml) and 5 % human blood (protein concentration = 11.97 ± 0.95 mg/ml), are illustrated in tables 5.8 to 5.11 respectively.

At least 1 μM of linalyl and cinnamyl esters were present following 180 minutes incubation in rat and human blood. Furfuryl propionate was completely hydrolysed within 5 minutes in rat blood homogenates and within 120 minutes incubation in human blood homogenates. As was the case in other studies, esters which were particularly poorly hydrolysed may demonstrate longer half-lives in test incubations in comparison to control incubations due to lower evaporative loss because of sequestration in organic material. In such cases, hydrolysis may be demonstrated by the formation of alkyl products.

The samples from the investigations of linalyl propionate incubated in rat blood homogenate were analysed with respect to assessing the stereoselective characteristics of hydrolysis. No stereoselective differences were observed in the quantity of (R)- or (S)- linalool produced, during the entire course of the incubations, samples from both control and test incubations contained equal quantities of both stereoisomers.

Blood Source And Compound	**Control (no blood)**	**Test (5 % blood)**
Rat Blood		
Linalyl Propionate	97 ± 6	45 ± 3
Linalool	0.0094 ± 0.0002	0.015 ± 0.000
Human Blood		
Linalyl propionate	100 ± 10	550 ± 250
Linalool	0.0097 ± 0.0007	0.0066 ± 0.0004

Table 5.8: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD) and rate of formation of alcohol = k ($minutes^{-1}$ ± SD)] of 50 μM linalyl propionate incubated in 5 % rat and human blood homogenates. Incubations performed at 37°C over 180 minutes.

Blood Source and Compound	Control (no blood)	Test (5 % blood)
Rat Blood		
Linalyl cinnamate	31 ± 10	440 ± 60
Linalool	0.0046 ± 0.0004	0.0098 ± 0.0007
Human Blood		
Linalyl cinnamate	45 ± 24	340 ± 90
Linalool	0.0042 ± 0.0002	0.0018 ± 0.0014

Table 5.9: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD) and rate of formation of alcohol = k (minutes^{-1} ± SD)] of 50 μM linalyl cinnamate incubated in 5 % rat and human blood homogenates. Incubations performed at 37°C over 180 minutes.

Blood Source And Compound	Control (no blood)	Test (5 % blood)
Rat Blood		
Cinnamyl cinnamate	87 ± 20	22 ± 9
Cinnamyl alcohol	(none produced)	0.043 ± 0.027
Human Blood		
Cinnamyl cinnamate	74 ± 21	∞
Cinnamyl alcohol	(none produced)	0.0042 ± 0.0018

Table 5.10: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD) and rate of formation of alcohol = k (minutes^{-1} ± SD)] of 50 μM cinnamyl cinnamate incubated in 5 % rat and human blood homogenates. Incubations performed at 37°C over 180 minutes.

Blood Source And Compound	Control (no blood)	Test (5 % blood)
Rat Blood		
Furfuryl propionate	1100 ± 400	0.01 ± 0.00 ◊
Human Blood		
Furfuryl propionate	880 ± 550	19 ± 2

Table 5.11: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD)] of 50 μM furfuryl propionate incubated in 5 % rat and human blood homogenates. Incubations performed at 37°C over 180 minutes. ◊ - Reaction rate based upon single time-point, and the assumption of 50 μM substrate present at time = 0 minutes.

For comparative purposes metabolic rates have additionally been calculated based upon the quantities of substrate and product in incubation mixtures at specific time-

points. Figure 5.4 illustrates a comparison of the rates of metabolism of all four substrates in both rat and human blood. Rates for linalyl and cinnamyl esters are expressed as nmoles of product formed (-controls) / minute / mg protein, rates for furfuryl propionate are expressed as nmoles of substrate hydrolysed (-controls) / minute / mg protein, in accordance with the protocol described in 5.3.2.3 above. Rat blood was observed to have substantially greater hydrolysing capacity than human blood. Cinnamyl cinnamate was hydrolysed approximately 7.5 times more rapidly in rat blood homogenate than human blood homogenate, and furfuryl propionate was hydrolysed approximately 770 times faster. No hydrolysis of the linalyl esters investigated was detected in human blood homogenates.

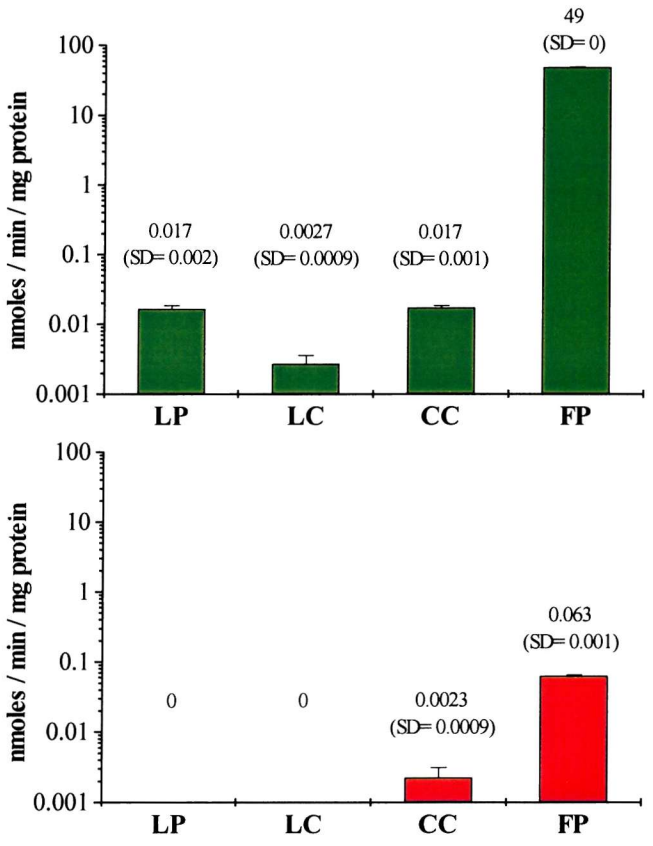


Figure 5.4: Hydrolysis rates of selected linalyl, cinnamyl and furfuryl esters incubated in: **A.** 5 % rat blood homogenates (protein concentration = 10.19 ± 0.36 mg/ml), and **B.** 5 % human blood homogenates (protein concentration = 11.97 ± 0.95 mg/ml). Rates expressed as nmoles of alkyl product formed / minute / mg protein ± SD in the case of linalyl and cinnamyl esters, and as nmoles of substrate hydrolysed / minute / mg protein ± SD in the case of furfuryl propionate. LP = linalyl propionate, LC = linalyl cinnamate, CC = cinnamyl cinnamate, FP = furfuryl propionate.

5.3.2.5 Rat Liver and Human Liver

Results of investigations into the metabolism of 50 μM linalyl propionate, linalyl cinnamate, cinnamyl propionate, cinnamyl cinnamate and furfuryl propionate by 5 % rat liver homogenates (protein concentration = 12.31 ± 0.22 mg/ml) are given in table 5.12. Hydrolysis of these substrates by 5 % human liver homogenates (protein concentration = 22.42 ± 3.91 mg/ml) are given in table 5.13.

In incubations of both rat and human hepatic preparations, substrates which had a half life which on average was longer than 0.1 minutes were demonstrated to undergo bi-phasic metabolism. As in the case of incubations containing rat intestinal mucosa, this consisted of an initial period of rapid metabolism which was followed by a period of less rapid hydrolysis.

When incubated in rat hepatic preparations, linalyl esters demonstrated biphasic kinetics with initial rapid metabolism. Linalyl propionate was completely hydrolysed within 30 minutes incubation, and metabolism of linalyl cinnamate ceased following 60 minutes incubation with 15.00 ± 6.04 μM of substrate remaining. Cinnamyl cinnamate was completely hydrolysed within 5 minutes incubation, cinnamyl propionate and furfuryl propionate were hydrolysed too rapidly to enable the quantification of reaction rates.

When incubated in human hepatic preparations, linalyl propionate and cinnamyl cinnamate were rapidly metabolised over a period of 0.1 minutes, following which no further metabolism was measured (16.01 ± 4.92 μM linalyl propionate and 34.58 ± 9.67 μM cinnamyl cinnamate was present at 0.1 minutes). No metabolism of linalyl cinnamate was measured, the hydrolysis of cinnamyl propionate and furfuryl propionate were too rapid to enable the quantification of reaction rates.

Substrate	Compound Assayed Time Frame	Control (no liver)	Test (5 % rat liver)
Linalyl propionate	**Linalyl propionate**		
	0.1 to 180 minutes	99 ± 3	-
	0 to 0.1 minutes	-	0.02 ± 0.00 ◊
	0.1 to 15 minutes	-	1.7 ± 0.3
	Linalool		
	0.1 to 180 minutes	0.0089 ± 0.0006	-
	0 to 0.1 minutes	-	*
	0.1 to 15 minutes	-	0.013 ± 0.006
Linalyl cinnamate	**Linalyl cinnamate**		
	0.1 to 180 minutes	23 ± 1	-
	0.1 to 10 minutes	-	22 ± 2
	10 to 60 minutes	-	59 ± 2
	Linalool		
	0.1 to 180 minutes	0.0050 ± 0.0008	-
	0.1 to 10 minutes	-	0.071 ± 0.010
	10 to 60 minutes	-	0.0073 ± 0.0036
Cinnamyl propionate	**Cinnamyl propionate**	∞	<0.01
	Cinnamyl alcohol	None produced	*
Cinnamyl cinnamate	**Cinnamyl cinnamate**	52 ± 11	0.04 ± 0.00 ◊
	Cinnamyl alcohol	None produced	*
Furfuryl propionate	**Furfuryl propionate**	1400 ± 900	<0.01

Table 5.12: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD) and rate of formation of alcohol = k ($minutes^{-1}$ ± SD)] of 50 µM selected linalyl, cinnamyl and furfuryl esters incubated in 5 % rat liver homogenates. Incubations performed at 37°C over 180 minutes. Incubation periods are indicated for substrates demonstrated to undergo biphasic metabolism. ◊ - Reaction rates based upon single time-points, and the assumption of 50 µM substrate present at time = 0 minutes. * - Calculation of rate constant based upon log^{10} concentration versus time graphs not possible.

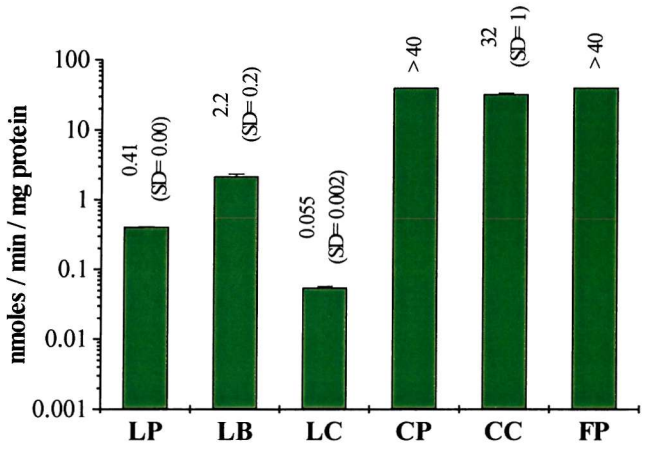
Substrate	Compound Assayed Time Frame	Control (no liver)	Test (5 % human liver)
Linalyl propionate	**Linalyl propionate**		
	0.1 to 180 minutes	64 ± 6	-
	0 to 0.1 minutes	-	0.06 ± 0.00 ◊
	0.1 to 180 minutes	-	40 ± 5
	Linalool		
	0.1 to 180 minutes	0.010 ± 0.001	-
	0 to 0.1 minutes	-	*
	0.1 to 180 minutes	-	None produced
Linalyl cinnamate	**Linalyl cinnamate**	23 ± 1	∞
	Linalool	0.0046 ± 0.0004	None produced
Cinnamyl propionate	**Cinnamyl propionate**	340 ± 160	<0.01
	Cinnamyl alcohol	None produced	*
Cinnamyl cinnamate	**Cinnamyl cinnamate**		
	0.1 to 180 minutes	76 ± 28	-
	0 to 0.1 minutes	-	0.13 ± 0.01 ◊
	0.1 to 180 minutes	-	∞
	Cinnamyl alcohol		
	0.1 to 180 minutes	None produced	-
	0 to 0.1 minutes	-	*
	0.1 to 180 minutes	-	None produced
Furfuryl propionate	**Furfuryl propionate**	∞	<0.01

Table 5.13: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD) and rate of formation of alcohol = k ($minutes^{-1}$ ± SD)] of 50 µM selected linalyl, cinnamyl and furfuryl esters incubated in 5 % human liver homogenates. Incubations performed at 37°C over 180 minutes. Incubation periods are indicated for substrates demonstrated to undergo biphasic metabolism. ◊ - Reaction rates based upon single time-points, and the assumption of 50 µM substrate present at time = 0 minutes. * - Calculation of rate constant based upon log^{10} concentration versus time graphs not possible.

For comparative purposes metabolic rates have additionally been calculated based upon the quantities of substrate and product in incubation mixtures at specific time-points. Figure 5.5 illustrates a comparison of the rates of metabolism of all five substrates in addition to linalyl butyrate (the investigations for which are given in section 5.3.3 below). Reaction rates are expressed as nmoles of substrate hydrolysed (-controls) / minute / mg protein except in the case of linalyl cinnamate which is

expressed as nmoles of product formed (-controls) / minute / mg protein in accordance with the criteria given in 5.3.2.3 above. It should be noted that calculations have been based upon time-points following which either the substrate had been completely hydrolysed or no further significant increase in product concentration occurred. As such, in the case of substrates demonstrated to undergo bi-phasic metabolism figure 5.5 is based upon the initial rapid phase of hydrolysis.

A.



B.

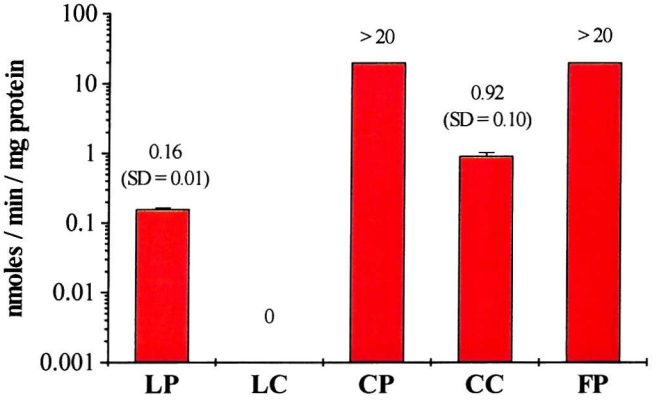


Figure 5.5: Hydrolysis rates of 50 μM linalyl propionate, linalyl cinnamate, cinnamyl propionate, cinnamyl cinnamate and furfuryl propionate incubated in **A.** 5 % rat liver homogenates (protein concentration = 12.31 ± 0.22 mg/ml), and **B.** 5 % human liver homogenates (protein concentration = 22.42 ± 3.91 mg/ml). Linalyl butyrate investigations in rat liver homogenates utilised 200 μM substrate and were conducted using a different set of rat liver tissue (homogenate protein concentration = 16.01 ± 1.10 mg/ml). Rates based on initial rapid phase of metabolism and are expressed as nmoles of substrate hydrolysed / minute / mg protein ± SD except in the case of linalyl cinnamate which is expressed as nmoles of alkyl product formed / minute / mg protein ± SD. LP = linalyl propionate, LB = linalyl butyrate, LC = linalyl cinnamate, CP = cinnamyl propionate, CC = cinnamyl cinnamate, FP = furfuryl propionate.

Rat liver was found to have a greater hydrolytic capacity than human liver. Cinnamyl cinnamate, linalyl propionate and linalyl cinnamate were hydrolysed approximately 35, 3, and at least twice as rapidly, respectively, by rat liver homogenates than they were by human liver homogenates.

5.3.3 Investigations into the Enzyme Characteristics and Stereoselective Metabolism of Selected Linalyl and Cinnamyl Esters by Rat Hepatic Preparations

To investigate the distinct bi-phasic hydrolysis of esters in preparations of mammalian liver, and in an attempt to further characterise the hepatic esterases hydrolysing food flavouring esters, a series of studies were undertaken using rat hepatic preparations (human liver was of limited supply). The activity and stereoselective characteristics of A- and B-esterases in rat liver were investigated and experiments were conducted using the B-esterase inhibitor paraoxon, and the serine protease inhibitor PMSF. The substrates employed were linalyl butyrate, linalyl cinnamate and cinnamyl cinnamate.

The hydrolysis of linalyl butyrate by 5 % rat liver homogenate showed a distinct initial stereoselectivity for (S)-linalyl butyrate, as illustrated in figure 5.6 and table 5.14. Based upon an analysis of the quantity of products remaining at the final time points, less than 2 % rat liver linalyl butyrate esterase activity remained following inhibition with 100 μM paraoxon. However, the limited activity which did remain demonstrated stereoselectivity for (S)-linalyl butyrate. The degree of inhibition by paraoxon was irrespective of the presence or absence of 0.1 mM Ca^{2+}, which is possibly required for A-esterase activity (0.1 mM $CaCl_2$ was a constituent of the buffer utilised in all other tissue homogenate incubations).

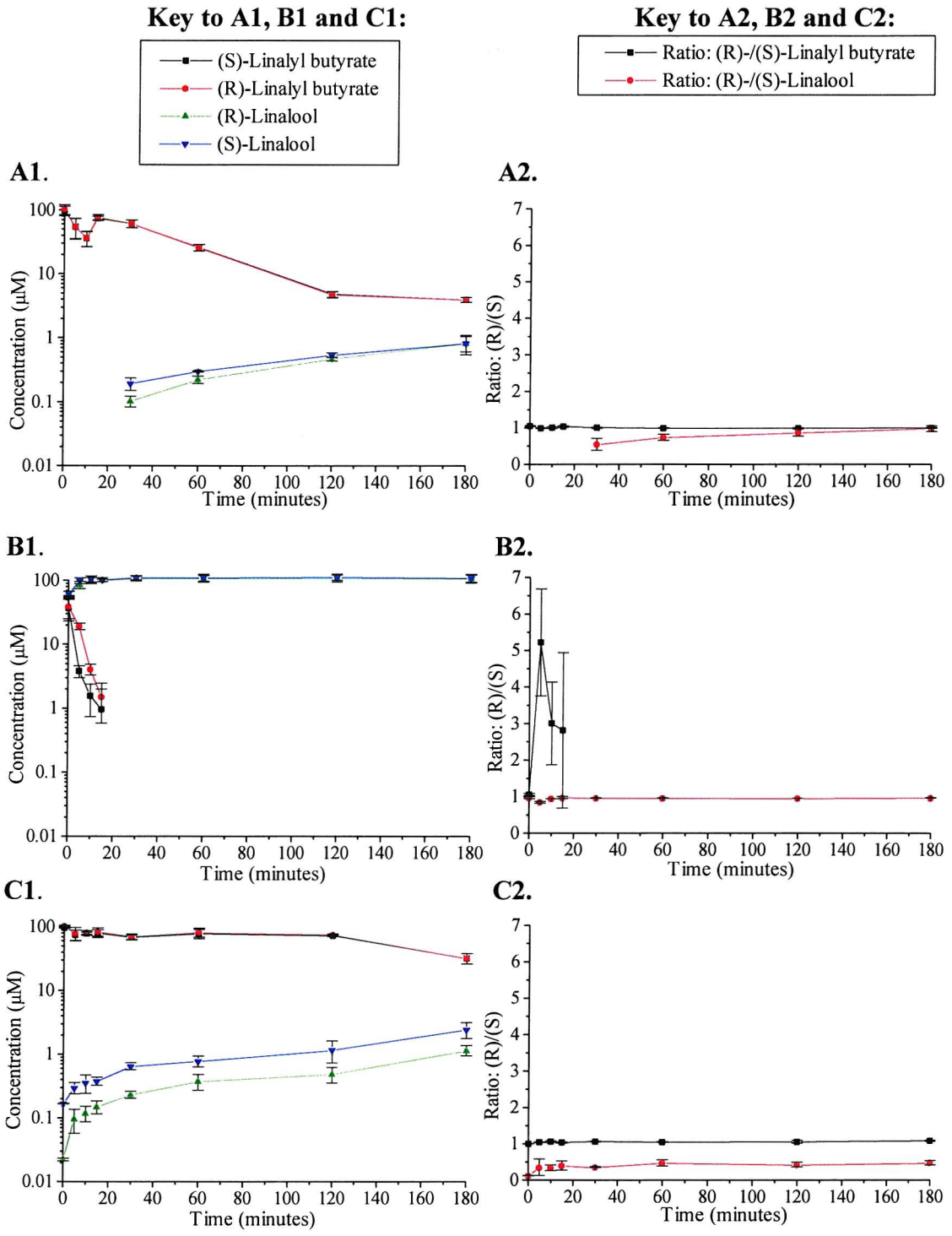


Figure 5.6: Stereoselective metabolism of 200 μM linalyl butyrate incubated in 5 % rat liver homogenate [mean ± SD (tissue buffer containing 0.1 mM $CaCl_2$)]. **A.** Incubations not containing liver homogenate (**A1** concentration versus time, **A2** ratio R/S versus time) **B.** Incubations containing liver homogenate (**B1** concentration versus time, **B2** ratio R/S versus time) **C.** Incubations containing liver homogenate and 100 μM paraoxon (**C1** concentration versus time, **C2** ratio R/S versus time). Initial ratio (R)/(S) –linalyl butyrate = 1:1.

Investigation and Compound	Control (no liver, no paraoxon)	Test (with 5 % liver, no paraoxon)	Test + Inhibitor (with 5 % liver, with paraoxon)
No Ca^{2+}			
(R)-Linalyl butyrate	39 ± 0	2.9 ± 0.2	140 ± 10
(S)-Linalyl butyrate	39 ± 0	1.2 ± 0.2	170 ± 30
(R)-Linalool	0.013 ± 0.001	0.31 ± 0.04	0.015 ± 0.004
(S)-Linalool	0.0095 ± 0.0021	0.42 ± 0.01	0.012 ± 0.002
0.1 mM Ca^{2+}			
(R)-Linalyl butyrate	29 ± 1	1.9 ± 0.3	304.5 ± 93.6
(S)-Linalyl butyrate	29 ± 1	1.0 ± 0.2	300 ± 80
(R)-Linalool	-	0.41 ± 0.02	0.020 ± 0.011
(S)-Linalool	-	0.43 ± 0.02	0.014 ± 0.005

Table 5.14: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD) rate of formation of alcohol = k ($minutes^{-1}$ ± SD)] of 200 µM linalyl butyrate incubated in 5 % liver homogenate with and without 100 µM paraoxon. Incubations performed at 37°C for 180 minutes. Investigations without Ca^{2+} utilised rat liver homogenate with a protein concentration of 17.31 ± 0.73 mg/ml, investigations with 0.1 mM Ca^{2+} present in the tissue buffer utilised rat liver with a protein concentration of 16.01 ± 1.10 mg/ml. All rates calculated from initial linear portions of log^{10} concentration versus time graphs.

As it was demonstrated that A-esterases do not have a major role in the hydrolysis of linalyl butyrate, and subsequent to limited data that known serine protease inhibitors may differentially inhibit B-carboxylesterase isoenzymes, further investigations were undertaken to study bi-phasic and stereoselective hydrolysis. These investigations used 200 µM linalyl butyrate incubated in 1 % rat liver homogenate, with and without the presence of 100 µM PMSF. This series of investigations utilised 1 % liver homogenate, as opposed to 5 %, in order to slow the rate of hydrolysis to allow a better elucidation of metabolic characteristics.

Figure 5.7 and table 5.15 illustrate the presence of at least two B-esterase isoenzymes in rat liver which hydrolyse linalyl butyrate. Under the conditions employed, one enzyme system was most active above 10 µM substrate concentration (low affinity / high capacity) and was highly stereoselective for (S)-linalyl butyrate. The second enzyme appeared to be saturated above 10 µM substrate concentration, but at lower concentrations was the most active system (high affinity / low capacity). This enzyme

demonstrated no stereoselectivity. The low affinity stereoselective enzyme was considerably more sensitive to inhibition by PMSF than the high affinity enzyme.

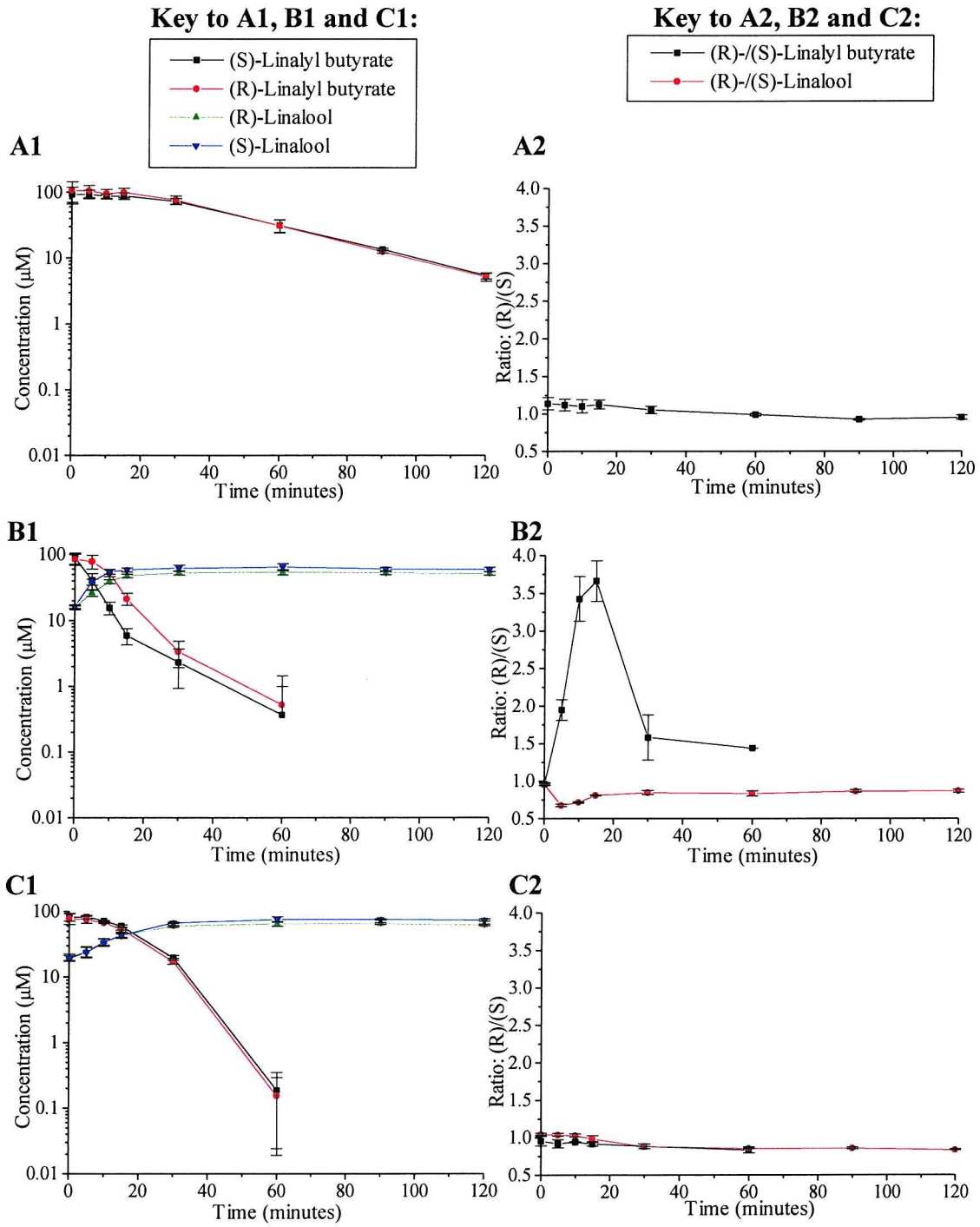


Figure 5.7: Stereoselective metabolism of 200 µM linalyl butyrate incubated in 1 % rat liver homogenate at 37°C over 120 minutes (mean ± SD). **A.** Incubations not containing liver homogenate (**A1** concentration versus time, **A2** ratio R/S versus time) **B.** Incubations containing liver homogenate (**B1** concentration versus time, **B2** ratio R/S versus time) **C.** Incubations containing liver homogenate and 100 µM PMSF (**C1** concentration versus time, **C2** ratio R/S versus time). Initial ratio (R)/(S) –linalyl butyrate = 1:1.

Time-Frame and Compound	Control (no liver, no PMSF)	Test (with 1 % liver, no PMSF)	Test + Inhibitor (with 1 % liver, with PMSF)
0.1 – 120 minutes			
(R)-linalyl butyrate	27 ± 2	-	-
(S)-linalyl butyrate	29 ± 2	-	-
0.1 – 15 minutes			
(R)-linalyl butyrate	-	7.7 ± 0.8	25 ± 5
(S)-linalyl butyrate	-	3.8 ± 0.2	26 ± 5
15 – 60 minutes			
(R)-linalyl butyrate	-	7.4 ± 3.8	6.6 ± 1.7
(S)-linalyl butyrate	-	11 ± 5	6.7 ± 1.7

Table 5.15: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD)] of 200 μM linalyl butyrate incubated in 1 % liver homogenate with and without 100 μM PMSF. Incubations performed at 37°C over 120 minutes. Rat liver homogenate protein concentration = 2.92 ± 0.53 mg/ml. Reaction rates are expressed based upon biphasic kinetics for incubations in liver homogenates. Rates of formation of linalool product are not illustrated.

Historically, A-esterases have been classified as aryl esterases. To further investigate and characterise possible differences in flavouring ester substrate specificity between A- and B-esterases in rat liver, linalyl cinnamate (200 μM), which contains an aromatic acyl moiety, and cinnamyl cinnamate (50 μM), which contains both aromatic aryl and acyl moieties, were employed as substrates. A substrate concentration of 50 μM cinnamyl cinnamate was used as this investigation additionally formed a part of previous work (see 5.3.2.5).

Figure 5.8 and table 5.16 illustrate that no appreciable A-esterase linalyl cinnamate hydrolytic activity could be detected in the presence of paraoxon, and that the extensive metabolism of this substrate by liver homogenate (as demonstrated by the production of linalool) was due to B-esterase activity. At two time points the ratio (R)/(S)-linalool were determined; the 30 minute time point and the final time point. The 30 minute point was selected as at this time approximately 50 % of substrate was hydrolysed by rat liver homogenate. This corresponded to the substrate concentration at which the greatest esterase stereoselective differences had been observed in investigations with linalyl butyrate (see figure 5.6).

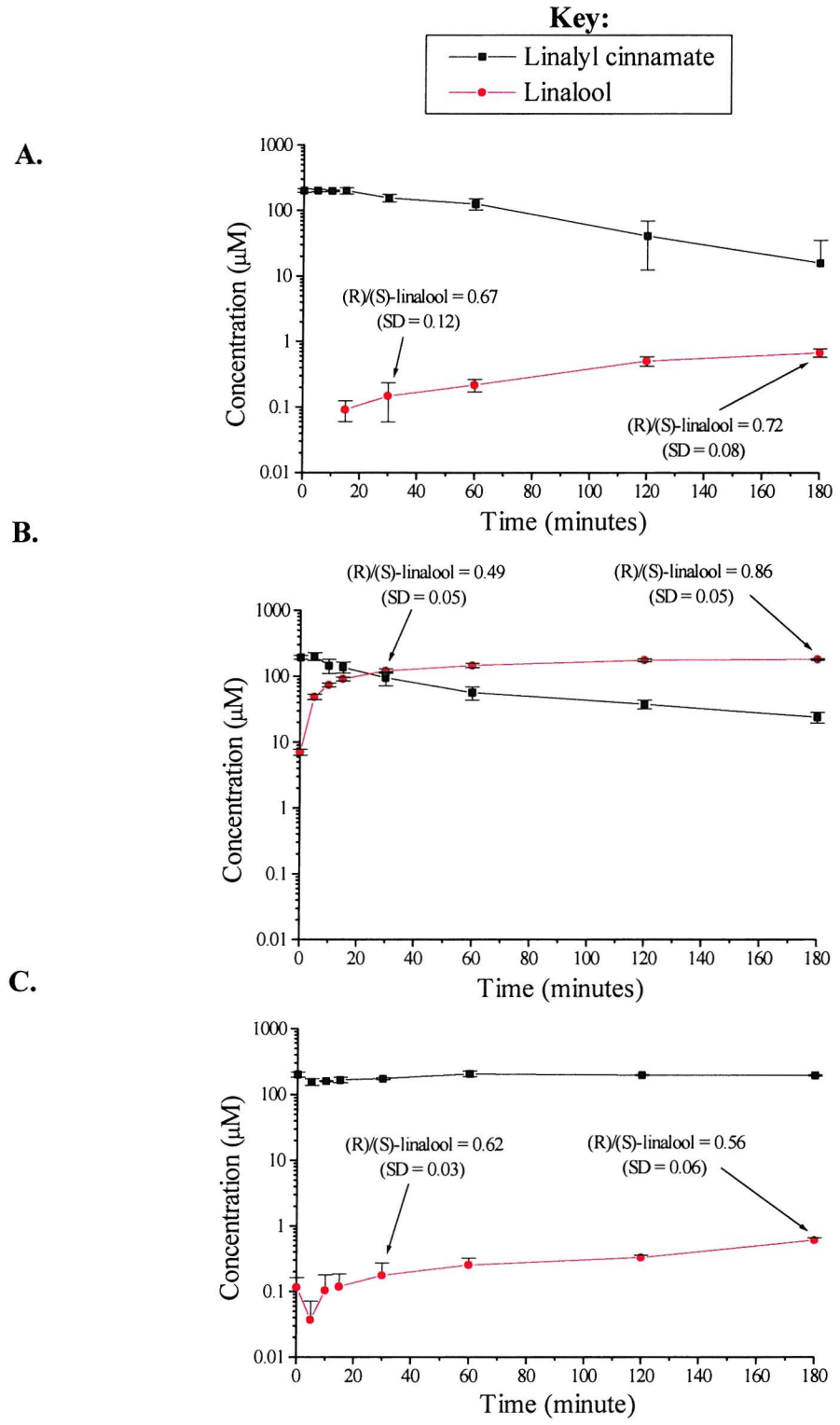


Figure 5.8: Metabolism of 200 μM linalyl cinnamate incubated in 5 % rat liver homogenate at 37°C over 180 minutes (mean ± SD). **A.** Incubations not containing liver homogenate **B.** Incubations containing liver homogenate **C.** Incubations containing liver homogenate and 100 μM paraoxon. Enantiomer ratios of (R)/(S) – linalool are indicated at 30 and 180 minutes incubation. Initial ratio (R)/(S) –linalyl cinnamate = 1:1.

The initial ratio (R)/(S) –linalyl cinnamate was 1:1, and as predicted from previous investigations, (S)-linalyl cinnamate was more unstable in an aqueous environment than the R-isomer, and rat liver homogenate displayed a distinct stereoselectivity for (S)-linalyl cinnamate. Figure 5.8B illustrates that even following 180 minutes incubation in rat liver homogenate, at a time when only approximately 10 % of the original substrate remained unhydrolysed, (S)-linalool was the major product.

Investigation and Compound	**Control (no liver, no paraoxon)**	**Test (with 5 % liver, no paraoxon)**	**Test + Inhibitor (with 5 % liver, with paraoxon)**
200 μM linalyl cinnamate			
Linalyl cinnamate	47 ± 24	60 ± 2	∞
Linalool	0.012 ± 0.003	0.21 ± 0.03	0.012 ± 0.003
50 μM cinnamyl cinnamate			
Cinnamyl cinnamate	52 ± 11	0.04 ± 0.00	240 ± 8
Cinnamyl alcohol	-	(> 70)	-

Table 5.16: Reaction rates [disappearance of ester = $t_{½}$ (minutes ± SD) and rate of formation of alcohol = k ($minutes^{-1}$ ± SD)] of 200 μM linalyl cinnamate, and 50 μM cinnamyl cinnamate incubated in 5 % rat liver homogenate with and without 100 μM paraoxon. Incubations performed at 37°C for 180 minutes. Investigations with linalyl cinnamate utilised rat liver homogenate with a protein concentration of 16.94 ± 0.82 mg/ml, investigations with cinnamyl cinnamate utilised homogenate with a protein concentration of 12.31 ± 0.22 mg/ml. All rates calculated from initial linear portions of log^{10} concentration versus time graphs.

Table 5.16 demonstrates that no rat liver A-esterase cinnamyl cinnamate activity was detected in the presence of paraoxon, and that the extensive metabolism of this substrate by liver homogenate was due to B-esterase activity. As such, no investigations had identified any appreciable hydrolysis of linalyl or cinnamyl flavouring esters by rat liver homogenate A-esterases.

5.4 Discussion

5.4.1 Linalyl Acetate

Linalyl acetate is an important and commonly used food flavouring compound, and some toxicological and metabolic data is available (discussed below). As such, linalyl acetate was utilised as the substrate in initial investigations with rat gastrointestinal tract and tissue homogenates.

Linalyl acetate was found to undergo hydrolysis in rat tissue and gastrointestinal tract homogenates by either chemical or enzymic means, the former resulting in the formation of linalool and α-terpineol, the latter resulting in the formation of linalool only. In cases where the sequestration of linalyl acetate (which is a poor esterase substrate) by organic material results in a longer half-life than control incubations, the product ratio linalool / α-terpineol is valuable in assessing the hydrolytic mechanism. As such, enzymic hydrolytic activity was seen to decrease between rat tissue and intestinal homogenates in the order: liver > intestinal mucosa > blood > intestinal contents. No statistically significant difference was seen between the product ratios of gastric contents homogenate and control incubations. However, the hydrolytic rate of linalyl acetate in gastric contents homogenate was significantly slower than control incubations ($P<0.1$). The sequestration of hydrophobic flavouring compounds within food material presumably has notable relevance to the risks associated with exposure.

The hydrolysis of linalyl acetate in rat intestinal homogenates and artificial pancreatic fluid (see table 3.1), both demonstrate no statistically significant increase in reaction rate in comparison to controls, but statistically significant differences ($P<0.05$) between the product ratios in test and control incubations. This suggests that artificial pancreatic fluid may be a useful model of duodenal hydrolysis.

In a FEMA 1979 report[238] the hydrolysis of linalyl acetate was found to have a half-life of 5.5 minutes in artificial gastric fluid and 53 minutes in artificial pancreatic fluid. These results are comparable to the findings presented in this report (see table 3.1), where hydrolysis in artificial gastric fluid produced a half-life of 0.31 ± 0.13

minutes, and in pancreatic fluid of 83 ± 3 minutes. Perhaps unreported differences in the experimental protocol of the FEMA report and the pancreatin utilised could help explain the moderate discrepancies. The pancreatin used in the investigations of this report was tested and certified to be United States Pharmacopoeia grade. It is also interesting to speculate that the difference in results may be associated with the stereochemistry of the linalyl acetate employed. It is possible that the substrate used in the 1979 investigations (source not specified) was plant-derived and as such may have been enantiometrically pure, where as the substrate used in the investigations of this report is synthetic and a racemic mixture.

Previously published toxicological data which specifically concerns linalyl acetate includes: an LD_{50} value in the mouse of 13,360 mg / kg body weight and in the rat of 14,550 mg / kg body weight (by gavage); an 84 day feeding study in the rat which showed no adverse effects at a 24 mg / kg body weight / day dose level (although this was a combined dose study with linalyl isobutyrate and geranyl acetate); and negative results in Ames, Unscheduled DNA Synthesis and Rec mutagenicity / genotoxicity assays[250]. Therefore from current data it would appear that linalyl acetate does not present a toxicological concern due to its use as a food flavouring additive.

From the available data it could be predicted that following ingestion, dependent upon sequestration in food material and stomach acidity, linalyl acetate will be hydrolysed to linalool (which will undergo structural isomerisation to form α-terpineol) and ethanoic acid. It is likely that monoterpene alcohol products and any remaining intact ester in contact with the stomach wall will be readily absorbed. It is possible that some linalyl acetate will also penetrate deeper into the gastrointestinal tract due to sequestration within food material. Intestinal digestive fluids and mucosal surfaces show hydrolytic activity against linalyl acetate, however any substrate absorbed intact may be hydrolysed by the blood, or almost certainly by the liver where the product linalool (which has low reported toxicity, see 4.1.2) is readily conjugated and/or oxidised (as demonstrated in Chapter 4) prior to excretion.

5.4.2 *Hydrolysis of Linalyl, Cinnamyl and Furfuryl Esters in Homogenates of Rat Gastric and Intestinal Contents*

Further to investigations into the behaviour of linalyl acetate in homogenates of rat gastric and intestinal contents, the behaviour of selected linalyl, cinnamyl and furfuryl esters in 5 % homogenates was studied. This was to enable a comparison with artificial human gastric and pancreatic fluids towards the construction of a food flavouring ester hydrolytic model.

Acid (pH 2.5) or enzymes present in homogenates of rat gastric contents had no measurable hydrolytic effect on linalyl propionate, linalyl cinnamate, cinnamyl cinnamate or furfuryl propionate. Linalyl propionate, linalyl cinnamate and furfuryl propionate demonstrated increased half-lives in test incubations in comparison to controls, and this was presumably due to sequestration of the esters, and inhibition of evaporation, by the organic material present. As discussed in relation to linalyl acetate, sequestration into food material may have important implications in respect to exposure following ingestion. In comparing artificial human gastric fluid and 5 % rat gastric contents homogenates, the esters investigated were more stable at pH 2.5 than they were at pH 1.2, and as such it would be anticipated that a greater degree of pre-absorption hydrolysis would occur in humans following ingestion than it would in rats.

Hydrolysis of the substrates by homogenates of rat intestinal contents and artificial human pancreatic fluid were in accord; furfuryl and cinnamyl esters were metabolised more rapidly than esters of linalool. Furthermore, in both systems furfuryl propionate was metabolised more rapidly than cinnamyl cinnamate. Direct comparisons of the mole rates of hydrolysis / minute / mg protein of substrates incubated in both rat intestinal contents homogenates and artificial pancreatic fluid are not meaningful due to the large amount of food protein material which was present in intestinal contents homogenates.

5.4.3 *Hydrolysis of Selected Linalyl, Cinnamyl and Furfuryl Esters by Preparations of Rat and Human Tissues*

Flavouring ester substrates were selected for study in homogenates of rat intestinal mucosa, rat and human blood, and rat and human liver subsequent to data relating to the hydrolysis 'screen' in artificial gastrointestinal fluids described in Chapter 3 (selection criteria is given in 5.1.2). In all cases 5 % tissue homogenates were used as the objective was to examine the effects of set amounts of different tissues upon set concentrations of substrate. Figure 5.9 illustrates a comparison of the initial reaction rates of linalyl propionate, linalyl cinnamate, cinnamyl cinnamate and furfuryl propionate in tissue homogenates. Rat tissue was found to have greater hydrolytic activity towards the substrates investigated than human tissue, and on a weight-for-weight basis rat liver homogenates illustrated the greatest overall esterolytic activity.

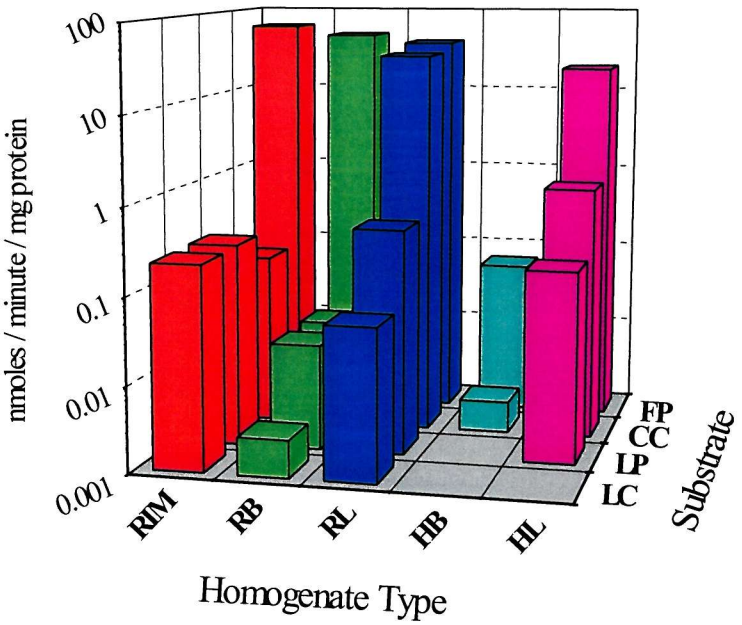


Figure 5.9: Consolidated data from 5.3.2.3 – 5.3.2.5, illustrating the initial rates of metabolism of flavouring esters in 5 % tissue homogenates. In all instances identical tissue samples (derived from the same 3 animals) were used in investigations with all substrates. Errors bars not shown as standard deviations too small for clarity. RIM = rat intestinal mucosa, RB = rat blood, RL = rat liver, HB = human blood, HL = human liver, LP = linalyl propionate, LC = linalyl cinnamate, CC = cinnamyl cinnamate, FP = furfuryl propionate. The rate of furfuryl propionate hydrolysis in rat and human liver homogenates exceeded 40 and 20 nmoles/min/mg protein respectively.

Rat intestinal mucosa was seen to possess the widest substrate specificity of all the tissue preparations studied, however distinct biphasic metabolism was observed. It is interesting to note that in the early work of Longland *et al* with a limited range of esters, no correlation was observed in the substrate specificities of intestinal and liver preparations[248]. Biphasic metabolism was possibly due to the progressive loss of esterase activity due to the action of intestinal proteases. The substrate concentration at which the cessation of metabolism occurred was higher the larger the ester under study (cinnamyl cinnamate was rapidly metabolised over the first 0.1 minutes of incubation following which approximately 80 % of substrate remained intact with virtually no further metabolism for the following 179.9 minutes. This effect was progressively less pronounced with linalyl cinnamate and linalyl propionate).

No clear stereoselectivity in the hydrolysis of linalyl propionate by homogenates of rat intestinal mucosa was observed, whilst the other tissue esterases which demonstrated rapid initial metabolism were stereoselective for (S)-linalyl butyrate (in the cases of pancreatin and rat liver). This possibly indicates that the observed biphasic reaction kinetics were not due to the action of a multi-enzyme system, and adds weight to the supposition that esterase inactivation (by proteases) occurred. It is however, interesting to further speculate on the results. Esterases with broad substrate specificity which effect rapid hydrolytic rates may not be able to undergo many enzymic turnover cycles before they become inactivated. It is interesting to postulate that the structure of a catalytic site which possesses wide substrate specificity is more prone to enduring acylation, this would correlate well to the observation of more pronounced cessation of hydrolysis of esters with larger acyl moieties (which may stabilise the ester link between enzyme and acyl moiety and inhibit de-acylation which normally occurs via nucleophilic attack).

As described in 1.2.4.2, limited published data has indicated that rat blood possesses substantially more hydrolytic activity than human blood, and this was borne out in the investigations presented in this report. Human blood homogenate was particularly poor at hydrolysing linalyl esters, whilst cinnamyl cinnamate was hydrolysed 7.5 times more slowly than it was in rat blood homogenate, and furfuryl propionate was hydrolysed 770 times more slowly than in rat blood homogenate. Linalyl cinnamate was hydrolysed approximately 6.2 times more slowly in rat blood homogenate than

linalyl propionate. No stereoselective preference was observed in the hydrolysis of linalyl propionate by rat blood.

It is interesting to note that human plasma has been found to contain more cholinesterase and more A-esterase (aryl esterase) activity than rat blood[69; 112; 128], and this may be related to the observed smaller difference between the rates of hydrolysis of furfuryl propionate and cinnamyl cinnamate in human blood when comparison is made to rat blood. As discussed in 1.2.4.1 a variety of data is available which suggests that plasma esterases may be secretory forms of hepatic esterases. As no stereoselectivity was observed in the hydrolysis of linalyl propionate, it is possible that the rat blood esterase(s) could be associated with the high affinity enzyme system identified in the rat liver isoenzyme stereoselectivity investigations presented in 5.3.3 (and discussed below).

Human liver was found to have substantially less hydrolytic activity towards the substrates studied than rat liver, this is in accord with the limited available data discussed in 1.2.4.2. Cinnamyl propionate and furfuryl propionate were metabolised too fast to enable the quantification of results in both rat and human liver homogenates. However, cinnamyl cinnamate and linalyl propionate were respectively hydrolysed approximately 35 and 3 times more rapidly by rat tissue than by human tissue. Linalyl butyrate, an additional substrate studied in rat liver homogenates, was the most rapidly hydrolysed linalyl ester. No metabolism of linalyl cinnamate by human liver homogenates was detected.

The hydrolysis of flavouring esters by rat and human liver illustrated biphasic kinetics similar to that of rat intestinal mucosa. Further investigations were undertaken using rat liver homogenates towards characterising the hepatic esterases involved in this metabolism.

As predicted from investigations with artificial pancreatic fluid, the hydrolysis of linalyl butyrate and linalyl cinnamate by rat hepatic preparations showed distinct preference for the (S)-linalyl ester. Also similar to pancreatic fluid investigations was the finding that this stereoselectivity was observed only in the initial few minutes of incubations, possibly indicative of the action of a multi-enzyme system, and

significant from the perspective of the observed biphasic reaction kinetics. Investigations into the hydrolysis of linalyl butyrate, linalyl cinnamate and cinnamyl cinnamate illustrated that A-esterases do not have an important role in the metabolism of these substrates.

Further investigations attempted to characterise B-esterase activity in respect of the observed biphasic metabolism. As discussed in 1.2.1.1, in 1975 Junge *et al*[26] identified two groups of rat hepatic B-esterases which showed different substrate specificities and inhibition profiles, and in 1994 Morgan *et al*[38; 39] identified two carboxylesterase isoenzymes which accounted for all the rat hepatic *p*-nitrophenylacetate hydrolytic activity. These studies indicated that known inhibitors of serine-protease activity may differentially inhibit B-esterase isoenzymes. As such investigations were conducted using the serine-protease inhibitor phenylmethylsulphonyl fluoride.

Rat hepatic linalyl butyrate hydrolytic activity was found to comprise at-least two B-esterase isoenzymes. One isoenzyme has low affinity / high capacity, is highly stereoselective for (S)-linalyl butyrate and is considerably more sensitive to inhibition by PMSF than a high affinity / low capacity isoenzyme which has no stereoselectivity for linalyl esters. The low affinity isoenzyme was responsible for the rapid initial metabolism of (S)-linalyl butyrate. In the studies of Morgan *et al*, the isoenzyme which was most readily inhibited by PMSF had high affinity for *p*-nitrophenylacetate[39]. As such, it is interesting to speculate that the enzyme which is highly sensitive to PMSF has greatest specificity for esters which have an aromatic alkyl moiety. The observation of such differences is important in better understanding the poorly characterised carboxylesterases and in predicting the fate of xenobiotic esters.

In all the tissues studied a general trend in the rates of hydrolysis of substrates was observed:

Furfuryl propionate > Cinnamyl cinnamate >Linalyl propionate > Linalyl cinnamate

Linalyl propionate was metabolised more rapidly than linalyl cinnamate, except in the case of rat intestinal mucosa which exhibited a more broad substrate specificity and human blood which did not metabolise linalyl esters. Cinnamyl cinnamate was metabolised more rapidly than linalyl cinnamate, except in the case of rat intestinal mucosa (due to the reasons outlined above). Cinnamyl cinnamate was metabolised rapidly in liver homogenates, although not as rapidly as the additional substrate cinnamyl propionate. Furfuryl propionate was the most rapidly metabolised substrate in all tissues investigated, except in liver investigations were it was hydrolysed at least as rapidly as the additional substrate cinnamyl propionate.

Table 5.17 illustrates a comparison of overall reaction rates between different alkyl groups (by comparing the rates of hydrolysis of different esters of specific carboxylic acids), and different acyl groups (by comparing the hydrolysis rates of different esters of linalyl or cinnamyl alcohol).

Differences between compounds in the rates of hydrolysis

Compounds Compared	Rat Intestinal Mucosa	Rat Blood	Rat Liver	Human Blood	Human Liver
Alkyl Comparisons					
CP / LP	-	-	>100	-	>130
FP / LP	270	2900	>100	*	>130
CP / FP	-	-	~ 1	-	~ 1
CC / LC	0.45	6.4	590	*	*
Acyl Comparisons					
LP / LB	-	-	0.19	-	-
LP / LC	1.0	6.2	7.4	*	*
LB / LC	-	-	39	-	-
CP / CC	-	-	>1.3	-	>20

Table 5.17: Differences in metabolism between ester compounds (calculated by division of mean reaction rates) illustrating alkyl and acyl moiety esterase substrate specificity between different 5 % tissue homogenates. Rates of metabolism of substrates in rat intestinal mucosa and liver incubations are based upon initial metabolism only. * - there was no detectable metabolism of linalyl esters, as such the comparison of reaction rates between compounds was not possible. LP = linalyl propionate, LB = linalyl butyrate, LC = linalyl cinnamate, CP = cinnamyl propionate, CC = cinnamyl cinnamate, FP = furfuryl propionate.

In all tissues investigated, the hydrolysis rates of flavouring esters due to substrate specificity associated with the alkyl moiety, decreased in the order:

$$\text{Furfuryl} \approx \text{Cinnamyl} > \text{Linalyl}$$

However, linalyl esters were moderately more favoured as hydrolysis substrates than cinnamyl esters by rat intestinal mucosa. In all the tissues investigated, esters of propionic acid were favoured substrates in comparison to esters of cinnamic acid. In the case of investigations with rat liver preparations, which included the use of a greater number of substrates, esters of butyric acid were the most favoured substrate. These sequences of substrate specificity are in agreement with the data available from the comprehensive study of hydrolysis rates in artificial pancreatic fluid (see Chapter 3), and thus demonstrate that the substrate specificity of porcine pancreatin is a good indicator to the substrate specificity of the rat and human tissues studied.

CHAPTER 6

INVESTIGATIONS INTO THE *IN VIVO* HYDROLYTIC METABOLISM OF SELECTED FOOD FLAVOURING ESTERS

6.0 Investigations into the *In Vivo* Hydrolytic Metabolism of Selected Food Flavouring Esters

6.1 Introduction: Background & Study Objectives

Few data are currently available concerning the *in vivo* hydrolytic metabolism of food flavouring esters (as discussed in Chapter 1). To further investigate and validate the trends in hydrolytic metabolism identified in Chapter 3 (comprehensive screen of the hydrolysis of monoterpenoid, cinnamyl, cinnamate and furfuryl esters in artificial gastrointestinal fluids) and Chapter 5 (hydrolysis of esters selected from Chapter 3 in homogenates of rat and human tissues), studies were undertaken into the *in vivo* metabolism of selected esters.

This series of investigations was to be conducted using the ester substrates selected for study in Chapter 5. The methodology was to administer substrates by gavage to rats and sample blood at set time-points from the tail tip. The principal substrate selected to aid in method development was terpinyl isobutyrate due to its very low hydrolysis rates identified in artificial pancreatic fluid. Linalyl cinnamate and citronellyl isobutyrate were also to be used. Linalyl cinnamate was selected as it was poorly hydrolysed by artificial pancreatic fluid but is more water soluble than terpinyl isobutyrate, and citronellyl isobutyrate was selected as it shares the same acyl group as terpinyl isobutyrate but was rapidly hydrolysed by artificial pancreatic fluid.

The Project License was specific in that the study was to develop and use the single dose gavage administration of substrates to rats followed by tail tip blood sampling as a model to investigate flavouring ester hydrolysis. Permission was additionally given for animals to be kept in metabolic cages and for the administration of substrates by intraperitoneal injection.

6.2 Methods

The methods and materials which were employed are as given in Chapter 2 with the exceptions below.

6.2.1 Compound Administration

6.2.1.1 Gavage Administration

Emulsified (20 mg substrate / ml distilled water containing 0.01 % w/v oleic acid) or pure substrate were administered via gavage at dose levels of 40 and 1000 mg / kg body weight respectively, to sets of 3 male Wistar albino rats (250 – 300 g unless otherwise stated) using graduated glass 1000 µl syringes (1001 gastight model, accuracy ± 2.5 µl at 20°C, Hamilton Ltd.) with 75 mm gavage needles. Animals were fasted overnight (16 hours) prior to administration of substrates unless otherwise stated.

6.2.1.2 Intraperitoneal Administration

1000 mg / kg body weight pure substrates were administered to sets of 3 male Wistar albino rats (245 – 250 g) via intraperitoneal injection using graduated glass syringes and 16 mm long 25 gauge needles.

6.2.2 Blood Sampling

For the collection of blood at the first time-point (5 or 10 minutes; time-courses are given in experimental results below) following gavage and intraperitoneal dosage, animals were placed in a restraining cage (Model 82, Linton & Co. Ltd.) and the terminal 1 – 2 mm of the rats' tails were removed with scissors.

At each time-point approximately 120 µl blood was collected using microvette capillary tubes coated with lithium heparin (Microvette CB300LH, Sarstedt Aktiengesellschaft & Co.). From these 100 µl blood was immediately placed in 0.5 ml eppendorf tubes containing 100 µl CHX/THN, followed by vigorous mixing. Samples

were stored frozen (-80°C) prior to gas chromatographic analysis. Between time-points the animals were removed from the restraining cage and were given water *ad libitum*, no food was given. At time-points subsequent to the first (to a maximum of 10 samples per animal over a period of 420 minutes), animals' tails were placed in water at approximately 55°C for 1 minute following which the tail vein was re-opened by gentle abrasion with paper tissue.

Following the completion of the investigations animals were killed by cervical dislocation.

6.2.3 Tissue Sampling

Blood, liver, and stomach contents were removed from male Wistar albino rats (330 – 350 g) according to the methods given in 2.2.6. Sacrifice of the animals was 2 hours following gavage dosing of sets of 3 animals, with no food or water given between dosing and sacrifice. Mesenteric fat samples, and both kidneys were additionally removed and processed using the same methods. The gastrointestinal tract was dissected last. Stomachs were taken following the removal of the stomach contents, and were washed in tissue buffer before being placed in pre-weighed vessels. Four consecutive 5 cm sections of the intestines were taken from each animal, the first section being measured from the base of the stomach. Each intestinal sample was placed in a pre-weighed vessel after being washed through with approximately 5 ml tissue buffer using a syringe, the effluent from each intestinal section wash was collected in a pre-weighed vessel. 50 % (w/v) tissue homogenates in tissue buffer were prepared and samples extracted with 0.5 volume CHX/THN. Samples of the intestinal washes were also extracted with 0.5 volume CHX/THN prior to gas chromatographic analysis.

6.2.4 Investigations using Metabolic Chambers

Metabolic chambers (rat model, Techniplast Ltd.) were used for the collection of urine and faeces, and for investigations which required fasting for 24 hours. At all times, animals in metabolic chambers were given water *ad libitum*.

To collect urine and faeces, following the administration of substrates by gavage to 3 male Wistar albino rats (265-275 g), animals were placed in individual metabolic chambers. Laboratory rat food (RM1 formulation, Special Diets Services Ltd.) was given *ad libitum* 8 hours following dosage (such that the total fast period did not exceed 24 hours). Urine was collected at time intervals of 2, 6 and 24 hours following dosage, the volumes were measured and samples were stored frozen (-80°C). Faecal matter was collected at 24 hours, weighed and stored frozen (-80°C).

Following the completion of the investigations animals were killed by cervical dislocation.

6.2.4.1 Analysis of Urine and Faeces

Samples of collected urine were extracted with 0.5 volume of CHX/THN prior to gas chromatographic analysis. 20 % (w/v) homogenates of the collected faecal material in distilled water were prepared and samples extracted with 0.1 volume of CHX/THN prior to gas chromatographic analysis.

6.2.5 Determination of Glucuronic Acid and Sulphate Conjugates in Samples of Urine, Faeces, Blood and Tissues

To identify and quantify glucuronic acid and sulphate conjugates, 100 µl aliquots were taken of urine, intestinal washes, 20 % (w/v) faecal homogenates, 50 % (w/v) homogenates of rat tissues, in addition to 50 µl of the aqueous layer of blood samples (which had previously been mixed with CHX/THN, see 6.2.2). Samples were diluted and mixed with 0.5 volume of 0.5 M sodium acetate buffer (pH 5.0), containing either 5000 units/ml β-glucuronidase, or 250 units/ml sulphatase and 17 mM D-saccharic acid 1,4-lactone (present in order to inhibit β-glucuronidase activity of the sulphatase). After incubation for 16 hours at 37°C, solutions were extracted with 0.5 volume CHX/THN prior to gas chromatographic analysis.

6.3 Results

6.3.1 Investigations into In Vivo Metabolism Following Gavage Administration

6.3.1.1 Gavage Administration of Terpinyl Isobutyrate and Linalyl Cinnamate

Terpinyl isobutyrate was administered by gavage to sets of rats at dose levels of 40 and 1000 mg/kg body weight. Linalyl cinnamate was administered by gavage to sets of animals at a dose level of 1000 mg/kg body weight. Blood was collected from the tail tip of each animal at 10, 20, 30, 60, 90, 120, 180, 240, 300 and 360 minutes following dosing. During the course of the investigations, all animals exhibited normal behaviour. At no time point were any substrates or products detected in the blood samples. Following the incubation of blood samples from the 1000 mg/kg body weight investigations in the presence of β-glucuronidase and sulphatase enzymes, no substrates or products could be detected in any samples.

6.3.1.2 Collection and Analysis of Urine and Faeces Following Gavage Administration of Terpinyl Isobutyrate

Due to the lack of identification of substrates or possible reaction products in blood following the gavage administration of 1000 mg/kg body weight terpinyl isobutyrate and linalyl cinnamate, collection and analysis of urine and faeces for 24 hours following dosing with 1000 mg/kg body weight terpinyl isobutyrate was undertaken.

No free terpinyl isobutyrate or α-terpineol were detected in the urine samples. Figures 6.1 and 6.2 illustrate respectively that low concentrations of glucuronic acid and sulphate conjugates of α-terpineol were constantly excreted over 24 hours. Approximately 9 times more α-terpineol glucuronic acid conjugate was excreted than sulphate conjugate. Following the analysis of faecal material, terpinyl isobutyrate, and α-terpineol along with the glucuronic acid conjugate of α-terpineol were detected as illustrated in table 6.1.

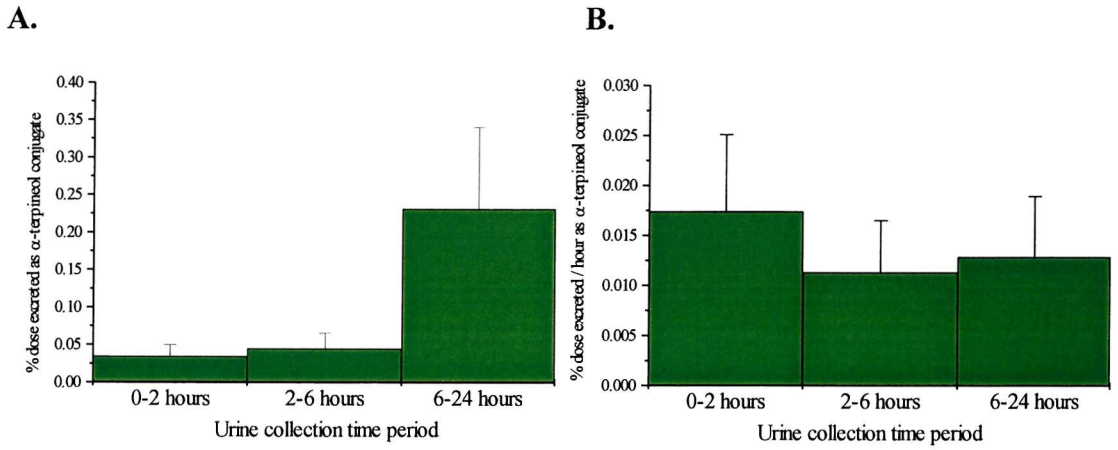


Figure 6.1: Excretion of α-terpineol as a glucuronic acid conjugate in urine, following gavage dosage with 1000 mg/kg body weight terpinyl isobutyrate (mean ± SD). **A.** % dose excreted per urine collection period **B.** % dose excreted per hour.

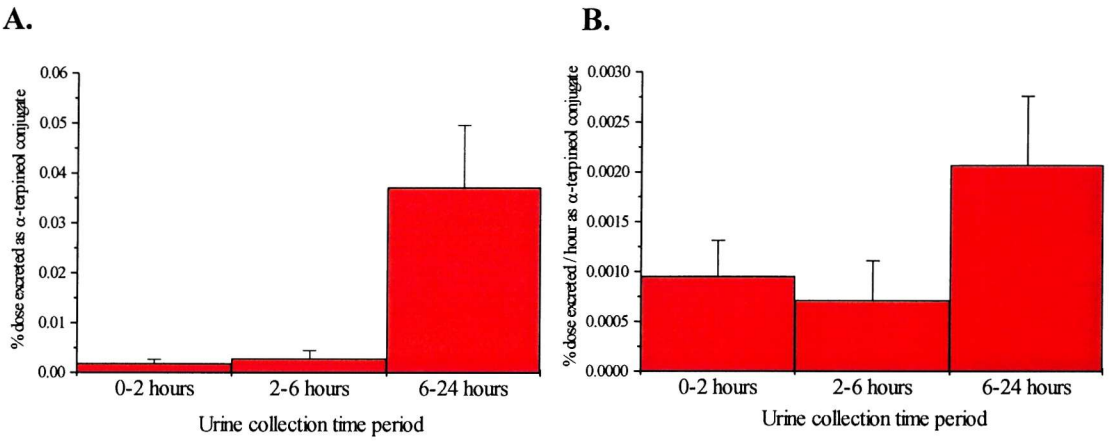


Figure 6.2: Excretion of α-terpineol as a sulphate conjugate in urine, following gavage dosage with 1000 mg/kg body weight terpinyl isobutyrate (mean ± SD). **A.** % dose excreted per urine collection period **B.** % dose excreted per hour.

Terpinyl isobutyrate	α-Terpineol	Glucuronic acid conjugate of α-terpineol	Sulphate conjugate of α-terpineol
16 ± 6	0.12 ± 0.03	0.090 ± 0.037	None identified

Table 6.1: Excretion of terpinyl isobutyrate and reaction products (% dose ± SD) in rat faeces over 24 hours following gavage dosing with 1000 mg/kg body weight terpinyl isobutyrate.

Approximately 16 % of the dose was identified as excreted unchanged in the faeces, along with low concentrations of the alkyl hydrolysis product and the glucuronic acid conjugate (approximately 0.2 % of the dose). However, it is possible that evaporative loss of substrate / product(s) from the faecal material occurred over the period of the study from the faeces collection containers. Approximately 0.3 % of the dose was identified in the urine as glucuronic acid and sulphate conjugates, therefore in total only approximately 0.4 % of the dose was excreted as conjugates following absorption from the gastrointestinal tract. These results indicated that either the majority of the dose remained in the gastrointestinal tract for a period longer than 24 hours (assuming that evaporative loss from the faeces containers was minimal), or that the substrate was absorbed and sequestered in animal tissue(s).

6.3.1.3 Analysis of Tissues Following Gavage Administration of Terpinyl Isobutyrate, Linalyl Cinnamate and Citronellyl Isobutyrate

In order to further investigate the fate of 1000 mg/kg body weight terpinyl isobutyrate, linalyl cinnamate and citronellyl isobutyrate following gavage administration to rats, animals were killed 2 hours subsequent to dosing and tissues were removed for analysis. Tables 6.2, 6.3 and 6.4 illustrate the results of these investigations.

The results indicate that in the case of all the substrates studied, very little absorption from the gastrointestinal tract occurred (no more than 3 % of the doses were recovered in the tissues investigated, almost all of which was recovered from stomach walls). Although a maximum of no more than 50 % of the doses were recovered in total, it is likely that the majority of the doses which were not recovered were lost during the procedures for opening, sectioning and washing the gastric and intestinal tissues. It is therefore clear that the substrates were not well absorbed due to sequestration in ingested organic material (which included laboratory food, bedding material and faeces). However, it is additionally possible that poorly absorbed lipophilic droplets formed following gavage administration.

Homogenate type	Free terpinyl isobutyrate	Free α-terpineol	Glucuronic acid conjugated α-terpineol	Sulphate conjugated α-terpineol
Stomach contents	43 ± 20	0.19 ± 0.04	-	-
Stomach	2.1 ± 1.1	0.019 ± 0.002	-	-
0-5cm intestinal contents	0.84 ± 0.17	0.0021 ± 0.0003	nd	nd
5-10cm intestinal contents	0.61 ± 0.26	0.0019 ± 0.0002	nd	nd
10-15cm intestinal contents	0.55 ± 0.32	0.0020 ± 0.0001	nd	nd
15-20cm intestinal contents	0.55 ± 0.14	0.0021 ± 0.0003	nd	nd
0-5cm intestine	0.013 ± 0.004	0.0014 ± 0.0006	nd	nd
5-10cm intestine	0.012 ± 0.006	0.0012 ± 0.0006	nd	nd
10-15cm intestine	0.010 ± 0.005	0.0012 ± 0.0010	nd	nd
15-20cm intestine	0.011 ± 0.005	0.0014 ± 0.0012	nd	nd
Blood	nd	nd	nd	nd
Liver	0.0046 ± 0.0020	0.0014 ± 0.0007	0.0070 ± 0.0023	nd
Kidneys	nd	nd	nd	nd
Mesenteric fat	0.010 ± 0.001	nd	nd	nd

Table 6.2: Localisation of substrate and reaction products (% of dose ± SD except in the case of mesenteric fat which was measured as mg/g tissue ± SD, and blood which was measured as mg/ml ± SD) 2 hours following gavage administration of 1000 mg/kg body weight terpinyl isobutyrate to rats (340-350g). nd = none detected.

Homogenate type	Free linalyl cinnamate	Free linalool	Glucuronic acid conjugated linalool	Sulphate conjugated linalool
Stomach contents	27 ± 12	0.33 ± 0.15	-	-
Stomach	0.87 ± 0.09	0.036 ± 0.003	-	-
0-5cm intestinal contents	0.60 ± 0.09	0.0027 ± 0.0007	nd	nd
5-10cm intestinal contents	0.60 ± 0.18	0.0030 ± 0.0006	nd	nd
10-15cm intestinal contents	0.48 ± 0.30	0.026 ± 0.010	nd	nd
15-20cm intestinal contents	0.45 ± 0.27	0.0042 ± 0.0018	nd	nd
0-5cm intestine	0.012 ± 0.009	0.014 ± 0.003	nd	nd
5-10cm intestine	0.013 ± 0.008	0.0090 ± 0.0036	nd	nd
10-15cm intestine	0.011 ± 0.013	0.0096 ± 0.0063	nd	nd
15-20cm intestine	0.011 ± 0.003	0.0093 ± 0.0051	nd	nd
Blood	nd	nd	nd	nd
Liver	nd	0.0003 ± 0.0001	0.012 ± 0.001	nd
Kidneys	nd	nd	nd	nd
Mesenteric fat	0.0048 ± 0.0028	nd	nd	nd

Table 6.3: Localisation of substrate and reaction products (% dose ± SD except in the case of mesenteric fat which was measured as mg/g tissue ± SD, and blood which was measured as mg/ml ± SD) 2 hours following gavage administration of 1000 mg/kg body weight linalyl cinnamate to rats (330-340g). nd = none detected.

Homogenate type	Free citronellyl isobutyrate	Free citronellol	Glucuronic acid conjugated citronellol	Sulphate conjugated citronellol
Stomach contents	30 ± 9	13 ± 5	-	-
Stomach	0.33 ± 0.04	0.39 ± 0.06	-	-
0-5cm intestinal contents	0.25 ± 0.04	0.24 ± 0.10	nd	nd
5-10cm intestinal contents	0.063 ± 0.057	0.60 ± 0.09	nd	nd
10-15cm intestinal contents	0.096 ± 0.002	0.78 ± 0.21	nd	nd
15-20cm intestinal contents	0.060 ± 0.030	0.81 ± 0.03	nd	nd
0-5cm intestine	None detected	0.0028 ± 0.0015	nd	nd
5-10cm intestine	None detected	0.0019 ± 0.0012	nd	nd
10-15cm intestine	None detected	0.0014 ± 0.0011	nd	nd
15-20cm intestine	None detected	0.0018 ± 0.0006	nd	nd
Blood	nd	nd	nd	nd
Liver	nd	0.0003 ± 0.0001	0.0011 ± 0.0003	nd
Kidneys	nd	nd	nd	nd
Mesenteric fat	0.0043 ± 0.0013	nd	nd	nd

Table 6.4: Localisation of substrate and reaction products (% dose ± SD except in the case of mesenteric fat which was measured as mg/g tissue ± SD, and blood which was measured as mg/ml ± SD) 2 hours following gavage administration of 1000 mg/kg body weight citronellyl isobutyrate to rats (330-340g). nd = none detected.

Table 6.5 illustrates the ratios of substrate / alcohol hydrolysis product(s) in the samples analysed 2 hours following the gavage administration of ester compounds. In all the organs, tissues, and gastrointestinal contents studied the degree of hydrolysis was found to be substrate specific decreasing in the order:

Citronellyl isobutyrate > Linalyl cinnamate > Terpinyl isobutyrate

Homogenate type	Terpinyl isobutyrate	Linalyl cinnamate	Citronellyl isobutyrate
Stomach contents	230	83	2.3
Stomach	110	24	0.85
0-5cm intestinal contents	400	220	1.1
5-10cm intestinal contents	330	200	0.11
10-15cm intestinal contents	280	18	0.12
15-20cm intestinal contents	260	110	0.074
0-5cm intestine	9.2	0.85	†
5-10cm intestine	10	1.4	†
10-15cm intestine	8.5	1.2	†
15-20cm intestine	7.7	1.2	†
Blood	*	*	*
Liver	0.56	†	†
Kidneys	*	*	*
Mesenteric fat	◊	◊	◊

Table 6.5: Ratios of mean substrate (% dose) / mean total alkyl product(s) (% dose) in rat tissues 2 hours following dosage with 1000 mg/kg body weight terpinyl isobutyrate, linalyl cinnamate or citronellyl isobutyrate. Mean alkyl hydrolysis product in the case of the liver investigations includes conjugated product. * = no substrate or product detected, ◊ = no product detected, † = no substrate detected.

No conjugates of alkyl hydrolysis products were identified except in livers where glucuronic acid conjugates were detected in the case of all the substrates investigated. In all cases a greater concentration of conjugated alcohol was found in livers than free alcohol (this ratio was approximately 5, 45 and 3 in respect of animals dosed with terpinyl isobutyrate, linalyl cinnamate and citronellyl isobutyrate respectively). However, only following dosage with terpinyl isobutyrate was free ester detected in livers.

6.3.1.4 Gavage Administration of Terpinyl Isobutyrate Following 24 Hour Fast in Metabolic Chambers

The previous studies (6.3.1.3) demonstrated that the absorption of substrates administered by gavage following 16 hours fast was extremely limited due possibly to the presence of ingested organic material in the gastrointestinal tract (which included laboratory food, bedding material and faeces). As such, in an attempt to develop the methodology of gavage dosing and tail tip blood sampling as a model to investigate the hydrolysis of food flavouring esters, a trial study was undertaken in which rats (230-235g) were fasted for 24 hours in metabolic cages prior to the gavage administration of 1000 mg/kg body weight terpinyl isobutyrate.

Blood samples were taken from the tail tip of each animal at 5, 10, 20, 60, 90, 120, 180, 240, 360 and 420 minutes following dosing. No terpinyl isobutyrate, α-terpineol or conjugates of α-terpineol were detected in any blood samples. Following the final time-point the stomach contents and the contents of the intestines (which were found to contain faecal material and laboratory food) were removed. 63 ± 28 % of the dose was recovered from the gastrointestinal tract, 0.20 ± 0.05 % of which had been hydrolysed to form α-terpineol.

Gavage dosing was shown to be an unsuitable method of administration, in respect of the blood sampling model of *in vivo* hydrolysis, due to poor absorption possibly due to sequestration of substrates in ingested food material present in the gastrointestinal tract. A fasting period of longer than 24 hours was not possible due to the limitations imposed by the Project License.

6.3.2 Investigations into In Vivo Metabolism Following Intraperitoneal Administration

6.3.2.1 Intraperitoneal Administration of Terpinyl Isobutyrate, Linalyl Cinnamate and Citronellyl Isobutyrate

As no substrates or products could be detected in blood samples following the gavage dosing of selected food flavouring esters, substrates were administered by

intraperitoneal injection. Following the administration of 1000 mg/kg body weight terpinyl isobutyrate, linalyl cinnamate and citronellyl isobutyrate to sets of rats, blood samples were taken from the tail tip at the time-points 10, 20, 40, 60, 90, 120, 180, 240, and 300 minutes. Subsequent to the final time-points samples of mesenteric fat were taken from the animals.

Animals dosed with linalyl cinnamate and citronellyl isobutyrate exhibited normal behaviour over the entire course of the studies. Animals dosed with terpinyl isobutyrate exhibited severe cyanosis, muscle flaccidity and respiratory difficulty from 3 minutes to 90 minutes following dosing, after which time effects were progressively reduced such that animals returned to normal behaviour over the subsequent 150 minutes. Under the terms of the Project License no further studies were undertaken.

No substrates, alkyl hydrolysis products or conjugated alkyl hydrolysis products (either conjugated with glucuronic acid or sulphate) were detected in blood following the administration of terpinyl isobutyrate or linalyl cinnamate. However, as illustrated in figure 6.3 low concentrations of citronellol and the glucuronic acid conjugate of citronellol were detected in blood samples following the administration of citronellyl isobutyrate. Following the final time-points samples of mesenteric fat were removed from the animals, as illustrated in table 6.6 these were found to contain high concentrations of the ester substrates.

The concentrations of alcohol products in comparison to the concentrations of ester substrates in mesenteric fat decreased in the order; citronellyl isobutyrate > linalyl cinnamate > terpinyl isobutyrate. In addition, lower concentrations of ester were present in the tissue for substrates which demonstrated greater evidence of hydrolysis.

Figure 6.3 demonstrates that up to 90 minutes following citronellyl isobutyrate administration an increasing concentration of free citronellol was present in the blood, following which the concentration in blood appeared to be in equilibrium with site(s) of storage / hydrolysis. From the time of reaching this equilibrium, an increasing concentration of glucuronic acid conjugated citronellol was detected in blood.

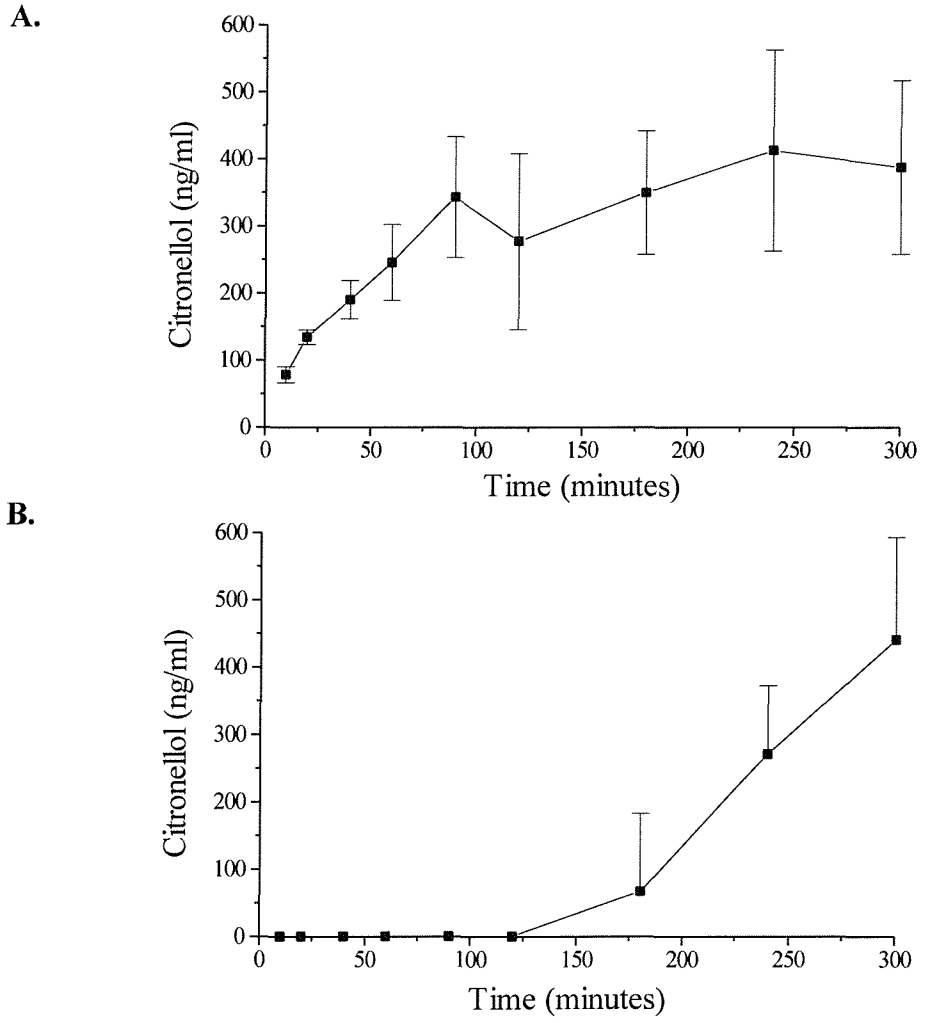


Figure 6.3: Concentrations of alkyl hydrolysis product identified in rat blood samples following the intraperitoneal injection of 1000 mg/kg body weight (245-250 mg) citronellyl isobutyrate (mean ± SD). **A.** Free citronellol **B.** Citronellol conjugated with glucuronic acid.

Compound identified	Terpinyl isobutyrate	Linalyl cinnamate	Citronellyl isobutyrate
Substrate	4.2 ± 1.8	3.3 ± 0.2	2.1 ± 0.2
Alkyl hydrolysis product	0.0017 ± 0.0005	0.19 ± 0.01	0.64 ± 0.02

Table 6.6: Ester substrates and alkyl hydrolysis products (mg/g ± SD) in mesenteric fat following the intraperitoneal administration to rats of 1000 mg/kg body weight (245-250 mg) terpinyl isobutyrate, linalyl cinnamate and citronellyl isobutyrate.

It is likely that the observed psuedo-steady-state concentration of free citronellol in blood was as a result of both the distribution of intact ester from sites of administration and sequestration to the blood where subsequent hydrolysis occurred, and the movement of the hydrolysis product (citronellol) from the tissues (sites of ester sequestration and hydrolysis) to the blood.

Intraperitoneal injection and blood sampling was found to be a poor model in respect of studying the hydrolytic fate of flavouring esters, as the administration of only the most readily hydrolysed substrate resulted in measurable concentrations of alcohol product in the blood. However these concentrations were low, possibly subject to equilibrium with sites of sequestration, and presumably only detectable following extensive hydrolysis in the tissues.

6.4 Discussion

To investigate the *in vivo* hydrolysis of selected food flavouring esters a methodology was to be developed which involved the gavage administration of substrates to rats followed by blood sampling from the tail tip. This technique was unsuccessful due to the limited absorption of the lipophilic substrates from the gastrointestinal tract. This was particularly illustrated by the identification of the majority of the doses in the contents of the gastrointestinal tract of rats up to 7 hours following dosage, and the large differences in substrate concentration identified in mesenteric fat following gavage and intraperitoneal administration (in the case of all the compounds investigated the concentration of substrates identified in mesenteric fat following intraperitoneal administration was >400 fold that identified following gavage administration). Lack of absorption from the gastrointestinal tract was as a consequence of sequestration in previously ingested organic food materials (present even following a fast period of 24 hours), and/or because of the formation of lipophilic substrate droplets following gavage administration. The *in vitro* investigations described in Chapters 3 and 5 illustrated the sequestration of esters in organic materials present in incubations, as such it is likely that sequestration in food materials was the primary cause of low bioavailability.

As discussed in 1.3.3, a number of studies have indicated that following oral administration the rapid absorption of the flavouring esters methyl cinnamate[259] (rats and rabbits), cinnamyl anthranilate[136] (rats and mice) and propyl anthranilate[263] (rats) occurs. However, these esters are less lipophilic than the monoterpenoid esters studied and may not have been as readily sequestered in ingested food materials. Furthermore, the rapid absorption of iso-amyl-3-[2'-furyl] propionate, allyl phenylacetate, methyl-*N*-methyl anthranilate and dimethyl benzyl carbinyl acetate has been reported following the direct administration into guinea pig ileum[261]. These esters are also possibly less lipophilic than the monoterpenoid compounds studied, and presumably by direct administration into the ileum would not encounter sizeable quantities of organic food material before absorption into the gastrointestinal wall could occur. Parke *et al* have demonstrated the rapid absorption, ready conjugation and excretion of linalool following oral and intraperitoneal dosing[301], with only 3 % of the radioactivity from the radio-labelled dose remaining in tissues after 72 hours (and investigations presented in 4.3.3 have demonstrated the ready movement of linalool through preparations of rat intestinal wall). Monoterpenoid esters are more lipophilic than their parent alcohols and this clearly has a substantial influence on their absorption and subsequent distribution. It is interesting to note that the sequestration of ingested lipophilic esters may afford some degree of protection form exposure. In such cases, for esters which are readily hydrolysed in the gut lumen, the majority of the absorbed dose may be in the form of the parent alcohol and carboxylic acid products.

The dose of terpinyl isobutyrate which was absorbed (following a gavage dose of 1000 mg/kg body weight) resulted in the continuous urinal and faecal excretion over a 24 hour period, of low concentrations of the glucuronic acid conjugate of α-terpineol (approximately 30 μg α-terpineol / hour in the urine, with approximately 10 μg α-terpineol / hour excreted in the faeces) with lower concentrations of the sulphate conjugate (approximately 3.5 μg α-terpineol / hour in the urine). The concentrations of these conjugates in the urine were associated with levels in the blood which were too low to detect in small blood samples. The presence of hydrolysis product(s) which had undergone further phase I metabolism (such as oxidation) was not determined.

The behaviour and hydrolysis of the small proportions of gavage administered doses which were absorbed, resulted in observations which were consistent with the hydrolytic trends identified between different substrates in Chapter 3 using artificial pancreatic fluid. In the case of all substrates investigated, intact ester was detected in the stomach wall and mesenteric fat. Furthermore, intact terpinyl isobutyrate was detected in the intestinal wall and liver, intact linalyl cinnamate was detected in the intestinal wall but not the liver, and citronellyl isobutyrate was not detected in either the intestinal wall or the liver. In addition, in all cases a greater proportion of the dose of citronellyl isobutyrate was identified in rat tissues as the alkyl hydrolysis product, and this proportion was progressively less for linalyl cinnamate and terpinyl isobutyrate respectively. This trend was also true in the case of the stomach and intestinal contents analysed. Additionally, a significant proportion of the dose of terpinyl isobutyrate was recovered intact in rat faeces over a period of 24 hours following gavage administration, thus demonstrating the lack of hydrolysis of this ester in the gastrointestinal tract.

Further to the gavage administration of substrates, administration by intraperitoneal injection also resulted in limited success in respect of the detection of esters / products in blood. Considering the large doses administered, it is unlikely that ester substrates were rapidly and completely transformed *in vivo* to substances which were not detected. The results therefore indicate that esters were sequestered and hydrolysed in tissues (such as mesenteric fat) and thus were not detected in blood samples. The presence of low concentrations of hydrolysis products in the blood following the intraperitoneal administration of citronellyl isobutyrate (which had been shown in chapter 3 to be readily hydrolysed), indicated that the equilibrium of concentration in the tissues verses the blood was strongly in favour of the tissues. Esters were sequestered to a greater extent than their alcohol hydrolysis products, although this observation may be partly related to the possibly greater hydrolytic activity of blood in comparison to a number of other tissues. The glucuronic acid conjugation and subsequent removal of citronellol from the blood may have resulted in the continuous movement of citronellol from the tissues to the blood. It would have been interesting to continue these studies for longer time periods to ascertain the maximum concentration of conjugated citronellol in the blood and the rate of excretion. For

esters which are resistant to hydrolysis, their preferential distribution in fatty tissues and their potential bioaccumulation should be studied further.

Following the intraperitoneal administration of terpinyl isobutyrate, but no other substrates, animals demonstrated symptoms indicative of central nervous system (CNS) depression. Assuming that this effect was not due to a unique and unidentified toxic characteristic of the ester or metabolite(s) (or the presence of low concentrations of an unknown and undetected contaminant), the concentration of intact ester which entered the systemic circulation and reached the fatty tissue of the CNS was greatest following dosage with terpinyl isobutyrate than the other esters investigated. Many organic xenobiotics which become absorbed into the central nervous system produce anaesthetic-like states by altering the properties of neuronal axons thus inhibiting the spread of action potentials. The slow recovery of the animals dosed with terpinyl isobutyrate from apparent anaesthesia to normal behaviour would possibly indicate the limited rate of hydrolysis of this ester in the CNS (although the continuous delivery to the CNS may also be an important factor). Further studies, which could have better characterised the disposition and effects of terpinyl isobutyrate and similar esters, were not permitted under the terms of the Project License which specifically concerned the administration of doses of flavouring additives such that no toxicity resulted, followed by tail tip blood sampling.

The results obtained from *in vivo* investigations support the findings of Chapters 3 and 5 which concerned hydrolysis in artificial pancreatic fluid and tissue homogenates respectively. Steric hindrance around the ester bond was observed to be the major determinant in hydrolytic preference between substrates, with the structure of the acyl moiety being of secondary importance. The susceptibility of esters to hydrolysis decreased in the order:

Citronellyl esters > Linalyl esters > Terpinyl esters

Citronellol isobutyrate (which has no steric hindrance due to side chains attached to the functional alkyl carbon atom) was hydrolysed more readily than linalyl cinnamate (which has methyl and allyl chains attached to the functional alkyl carbon atom, and also has a rigid and bulky aromatic acyl moiety), which in turn was hydrolysed more

readily than terpinyl isobutyrate (which has two methyl chains attached to the functional alkyl carbon atom, and has the same acyl moiety as the ester of citronellol investigated). Chapter 4 demonstrated the ready conjugation of linalool with glucuronic acid by *in vitro* preparations of rat liver, *in vivo* investigations supported these observations and illustrated that all the monoterpenoid alcohols studied underwent glucuronic acid conjugation in the liver.

The presence of intact terpinyl isobutyrate in the livers of gavage-dosed rats despite poor absorption across the gastrointestinal tract, and evidence of its incomplete first-pass metabolism and subsequent disposition in the CNS following intraperitoneal dosing, is of possible importance in respect of safety evaluations which have been based upon the premise of rapid and complete hydrolysis. Furthermore, intact esters were detected to varying degrees in the stomach wall, intestinal wall and mesenteric fat following gavage dosing in the case of all the substrates investigated (although citronellyl isobutyrate was not detected in intestinal walls). It is clear that tissues, particularly those which are rich in lipid, may sequester ingested and absorbed lipophilic esters, and in the case of compounds which are resistant to hydrolysis their potential bioaccumulation should be considered. This is particularly salient when consideration is given to the observations of more rapid hydrolysis of esters by the blood and liver of rats in comparison to human blood and liver (see Chapter 5). At the least, for esters with structural features which have been demonstrated to result in low hydrolysis rates, it would be considered wise to base safety evaluations upon some toxicological knowledge of the ester in addition to the parent alcohol and carboxylic acid moieties.

CHAPTER 7

CONCLUSIONS

7.0 Conclusions

There are currently approximately 1800 food flavouring additives recognised by the Flavor and Extract Manufacturers' Association as being in the consumer market. Up to 60 % of the compounds are esters[342] which may be hydrolysed following ingestion by gastrointestinal digestive fluids, gastrointestinal mucosa, the blood or the liver. However, the identity and activity of tissue esterases remains poorly characterised (see 1.2). As discussed in Appendix I, the metabolism and toxicity of the majority of flavours have not been investigated, and their presence in the food supply is accepted by regulatory bodies due to criteria concerning their histories of usage, exposure and consumption patterns, structure and predicted metabolism. For esters that are predicted to be rapidly and completely hydrolysed, safety assessment may be based upon their parent alcohol and carboxylic acid moieties, the further metabolism and safety of which are generally well established.

The flavour manufacturing industry is located predominantly in the USA, where GRAS status is accepted by FEMA based upon the concept of *lack of evidence of hazard.* This is not the case internationally, and other advisory bodies have classically evaluated flavours for their *evidence of safety.* This process is hindered by the lack of data available concerning flavouring additives and the large number currently present in the food supply. However, exposure to flavours is small in comparison to most other classes of food additive, and due to their organoleptic properties, their usage in food is self-limiting. As such, to advance the safety evaluation of flavouring additives, JECFA have recently implemented a 'decision tree' protocol, which does not rely upon the classical threshold ADI paradigm[341]. In accordance with the new protocol, in order to establish the safety of a flavouring compound in the absence of toxicological data, the pathways and consequences of metabolism must be known or predictable. For groups of related compounds, the metabolic fate for the entire group is often interpreted from information available upon an individual member. This has created concern about the validity of extrapolating toxicity data from one member of a structural class of flavouring compounds to another without adequate evidence of common underlying metabolic fate, particularly hydrolysis in the case of esters.

To facilitate the safety assessment process, this project was undertaken towards building a predictive model of flavouring ester hydrolysis. This has been achieved via the research presented above (Chapters 3, 5 and 6) which includes supporting studies towards characterising the esterases hydrolysing the flavouring compounds studied. Research work additionally included an investigation of the unusual behaviour of monoterpene alcohols in solution, identified during the construction of the hydrolytic model, and further studies into the absorption and metabolism of this important group of food additives (Chapter 4).

7.1 Hydrolysis of Esters in the Gastric Environment, and Metabolism of Monoterpene Alcohols

Monoterpenoid esters, and the propionic acid esters of cinnamyl and furfuryl alcohols underwent hydrolysis in artificial gastric fluid due to the pH of the solution. Hydrolysis of esters in artificial gastric fluid at pH 1.2 was considerably more rapid than hydrolysis in homogenates of rat gastric contents at pH 2.5 (in which hydrolysis of the selected esters was not detected). The rate of acid-catalysed hydrolysis was determined by the structures of both the alkyl and acyl ester moieties and was probably a function of the stability of charged reaction intermediates. In the case of monoterpenoid esters which have a double bond one carbon atom distant from the functional alkyl carbon atom (esters of linalool, geraniol and nerol), the alcohol products formed in acidic and neutral environments were found to be indicative of distinct reaction mechanisms. It is also interesting to note that enzymic hydrolysis of these esters by artificial pancreatic fluid and tissue homogenates could be differentiated from non-enzymic hydrolysis, because enzymic hydrolysis resulted in the production of the parent alcohol only, whereas non-enzymic hydrolysis resulted in the formation of multiple characteristic products. The alcohol products produced in an acidic environment were found to undergo further reactions in the form of structural isomerisations.

The unusual structural isomerisations of monoterpenoid alcohols in acidic artificial gastric fluids were investigated, and α-terpineol was found to be the final product of the acid-catalysed rearrangement of linalool, geraniol and nerol. However, the rate of

linalool rearrangement was found to be at least 20 times slower in rat artificial gastric fluid (pH 2.5) and homogenates of rat gastric contents, than it was in human artificial gastric fluid (pH 1.2). Caution should as such be applied in interpreting animal studies for assessing the human health risks associated with potentially acid labile esters and their hydrolysis products.

These data suggest that following the consumption of food / beverage items containing esters of monoterpene alcohols, the absorbed compounds will be a mixture of the intact ester, it's hydrolysis products, and isomerisation product(s) of the parent alcohol (in the case of monoterpene alcohols which may form stable carbocations following the loss of the hydroxy moiety). The amounts of the different compounds absorbed from the stomach will depend upon the extent of sequestration and retention of the lipophilic compounds in food material present in the gastrointestinal tract, and the degree of stability of the ester bond to acid-catalysed hydrolysis.

Linalool and citronellol were found not to be metabolised by artificial pancreatic fluid, and linalool was found to be rapidly absorbed across preparations of rat intestine. Linalool was not metabolised by simple homogenates of rat intestinal contents, intestinal mucosa, blood and liver. However, rat hepatic preparations containing activation systems for cytochrome P-450 oxidation and glucuronic acid conjugation both gave rapid metabolism of linalool, with a stereoselective preference for (R)-linalool in the case of oxidation (no metabolism was detected with rat liver homogenates containing sulphate conjugation activation system, or with rat intestinal preparations containing activation systems for cytochrome P-450 oxidation, sulphate or glucuronic acid conjugation).

Linalool and simple esters of linalool are economically important food flavouring compounds which represent the majority of monoterpene additives consumed[274]. As linalool was shown to be a poor substrate for metabolism by rat intestinal mucosa and is known to be a poor substrate for alcohol and aldehyde dehydrogenase (see 4.1.3), metabolism is likely to occur in the liver by both cytochrome P-450-mediated oxidation and conjugation with glucuronic acid. This is in contrast to the currently accepted view, based mainly upon high dose animal investigations (500-800 mg / kg body weight / day[301; 306]) discussed in 4.1.3, that oxidation only occurs following

repeated dosing and that elimination is almost entirely via glucuronic acid conjugation. It is possible that cytochrome P-450-mediated oxidative metabolism is saturated following high dosage, which consequently will not effectively represent metabolism following low dose human exposure. Monoterpenoid alcohols and aldehydes have been shown to be hydroxylated at a common site[305; 306; 308-310], and of the limited data available (discussed in 4.4.3), the ingestion of a solution containing α-terpineol by a human has been shown to lead to metabolites indicative of epoxide formation[300]. As such, it would appear necessary to further evaluate the metabolism of monoterpenoid alcohols at low doses.

7.2 Characterisation of Esterase Enzymes

The available data indicate that the esterases present in the exocrine digestive secretions of the pancreas belong to the B-esterase family[53] (see 1.2.1.1.2). Investigations with the serine esterase inhibitor paraoxon demonstrated that the hydrolysis of linalyl butyrate by porcine pancreatin was entirely due to the activity of B-esterases. It has been estimated that approximately 3 % of total rat liver microsomal protein is comprised of esterases[13; 38] and approximately 5 % of rat liver homogenate esterase activity has been found to be due to A-esterases[26]. No appreciable hydrolysis of linalyl butyrate, linalyl cinnamate or cinnamyl cinnamate was detected in homogenates of rat liver following inhibition with paraoxon. Therefore, A-esterase enzymes were demonstrated to have no importance in the hydrolysis of the selected flavouring compounds by artificial pancreatic fluid or by rat liver.

As discussed in 1.2.1.1.1, Junge *et al* and Hosokawa *et al*, have identified two groups of rat liver serine-esterases[26; 36], and more recently Morgan *et al* have utilised the serine-protease inhibitor PMSF to demonstrate the presence of two serine carboxylesterase isoenzymes which constitute the entire hydrolytic activity in the rat liver towards *p*-nitrophenylacetate[39]. To investigate the B-esterase isoenzymes metabolising food flavouring additives, the stereoselective hydrolysis of linalyl esters (the carbon atom to which the functional group of linalool is bonded is a chiral centre) was studied, as a potential marker to differentiate isoenzyme activity, in conjunction with PMSF.

Immediately following the addition of linalyl butyrate to incubations of artificial pancreatic fluid, stereoselectivity was observed for the S-stereoisomer, no stereoselectivity was observed at subsequent time-points. Stereoselective and non-stereoselective hydrolysis could not be separated when the incubations contained PMSF. As such, it was not possible to identify the presence of multiple B-esterases. The R-stereoisomer of linalyl butyrate has possibly greater steric hindrance around the ester bond to nucleophilic attack than the S-stereoisomer (see figure 3.20). Therefore, the stereoselectivity of porcine pancreatin which was observed during the first 0 to 6 seconds of incubations was probably due to differences in enzyme affinity.

No conclusive stereoselectivity was observed in the hydrolysis of linalyl propionate by homogenates of rat blood or rat intestinal mucosa. Hydrolysis of substrates by rat intestinal mucosa demonstrated distinct biphasic kinetics (an initial period of rapid metabolism followed by a period of negligible metabolism) which was probably due to the action of intestinal proteases. Hydrolysis of substrates by homogenates of rat liver demonstrated less distinct biphasic kinetics, which were the result of the activity of multiple B-esterase isoenzymes. One isoenzyme illustrated low affinity / high capacity was highly stereoselective for (S)-linalyl esters and was highly sensitive to inhibition by PMSF, whereas the second isoenzyme illustrated high affinity / low capacity but was not stereoselective or sensitive to inhibition by PMSF. Serine esterase isoenzymes are currently poorly characterised due particularly to their wide substrate specificities, this is the first time that hydrolytic stereoselectivity has been successfully used as a tool for investigating serine esterase isoenzymes, and is therefore a potentially valuable advance in knowledge.

7.3 Construction of a Model of the Enzymic Hydrolysis of Food Flavouring Esters

To construct a predictive model of the enzyme-mediated hydrolysis of food flavouring esters, groups of compounds were selected for study based upon an evaluation of the effects upon hydrolytic rates of defined structural criteria (which are discussed in 1.4 and below). This was achieved by investigating and comparing the hydrolysis of 44

esters of monoterpene alcohols (esters of citronellol, geraniol, nerol, linalool, and α-terpineol), cinnamyl alcohol, cinnamic acid and furfuryl alcohol. The first stage of the work was an examination of the hydrolysis rates of all the esters in artificial pancreatic fluid, from which compounds were selected for study in the second and third stages which were respectively the investigation of hydrolysis in 5 % tissue homogenates, and *in vivo* following oral and intraperitoneal administration to rats. Table 7.1 illustrates a comparative ranking of the fastest to the slowest hydrolysed esters in the second and third stages of the investigations in comparison to artificial pancreatic fluid.

		5 % Intestinal Contents / Tissue Homogenates						
Rate	**Artificial Pancreatic Fluid**	**Rat Intestinal Contents**	**Rat Intestinal Mucosa**	**Rat Blood**	**Rat Liver**	**Human Blood**	**Human Liver**	**Rat *in vivo***
	FP=CP	FP	FP	FP	FP=CP	FP	FP=CP	
	CC	CC	LP=LC	CC=LP	CC	CC	CC	
	CitI		CC					CitI
	LB				LB			
	LP	LP			LP	LP	LP	
	LC	LC		LC	LC	LC	LC	LC
	TI							TI

Table 7.1: Ranking of the rates of hydrolysis of esters selected for study in 5 % tissue homogenates (see Chapter 5) and *in vivo* in rats (see Chapter 6) compared to hydrolysis of the selected esters in artificial pancreatic fluid (see Chapter 3). FP = furfuryl propionate, CP = cinnamyl propionate, CC = cinnamyl cinnamate, CitI = citronellyl isobutyrate, LB = linalyl butyrate, LP = linalyl propionate, LC = linalyl cinnamate, TI = terpinyl isobutyrate. Blue figures are esters for which no measurable hydrolysis occurred in the system investigated. Green figures are esters for which hydrolysis was too rapid in the system investigated to enable the quantification of rates. Red figures are esters which are not in the same ranking sequence of hydrolysis rates identified with artificial pancreatic fluid.

Trends in the ranking of substrate specificity identified in all the systems studied were identical, except for studies with rat intestinal mucosa homogenates which demonstrated a broader substrate specificity than the other systems investigated. This is in agreement with Longland *et al* who have previously demonstrated dissimilar

substrate specificities of rat intestinal mucosa and liver[248]. The rates of hydrolysis of cinnamyl cinnamate and linalyl propionate in homogenates of rat blood were found to be equal when calculated as nmoles of substrate hydrolysed / minute / mg protein, however, the rate of formation (k, $minute^{-1}$) of the alcohol hydrolysis product was more rapid in the case of cinnamyl cinnamate than it was with linalyl propionate. As such, the substrate specificity of artificial pancreatic fluid (discussed in 3.3.2 and below) has been shown to be valid for predicting the trends in substrate specificity of tissue esterases and rat intestinal digestive enzymes. Furthermore, these results are consistent with the limited amount of data available concerning the substrate specificity of hepatic esterases (discussed in 1.2.1.1.1), pancreatic esterases (discussed in 1.2.1.1.2), and from previous investigations with food flavouring esters (discussed in 1.3).

Although the trends in substrate specificity were consistent in the different systems studied, it was generally found that the greater the overall esterolytic activity possessed by the system investigated, the greater were the differences between substrates in their rates of hydrolysis. A comparison of all available data demonstrates that in the systems investigated esterolytic activity decreased in the order:

Rat liver > Human liver ≥ Rat intestinal mucosa > Rat blood > Human blood

The esterolytic activity of artificial pancreatic fluid was at least as active as rat blood. The hydrolysis of substrates by homogenates of rat blood were up to 770 fold more rapid than hydrolysis by human blood, and hydrolysis by rat liver was up to 35 fold more rapid than that catalysed by human liver. These results, in conjunction with the available data discussed in 1.2.4.2, indicates that the rat is a poor model of human esterolytic activity.

It has previously been suggested that substrate lipophilicity is an important factor in determining esterase substrate specificity (the greater the lipophilicity the more rapid the hydrolysis)[16]. However, in the results given above substrate lipophilicity has not been found to influence substrate specificity. For example, although esters of aromatic carboxylic acids are poorly hydrolysed in comparison to aliphatic esters, esters of aromatic alcohols are metabolised rapidly, and esters of furfuryl alcohol are

hydrolysed more rapidly than compounds with greater lipophilicity such as monoterpenoid esters. Structural criteria were observed to be predominant in determining substrate specificity.

Substrate specificity was associated with the following structural criteria, each of which had some degree of negative influence on hydrolysis rates. Numbers representing the degrees of effects attributable to the structural criteria are derived from investigations with porcine pancreatin, and indicate the difference in hydrolytic rates between comparable esters which have and do not have the structural features investigated. Although the degrees of influence of the different criteria on hydrolytic rates may overlap, the criteria are ranked below in order of decreasing importance:

1. **Major Influence** - Steric hindrance around the ester bond

Steric hindrance of catalysis by esterases due to the presence of hydrocarbon groups bonded to carbon atoms directly adjacent to the ester bond, was found to have a major impact upon hydrolytic rates (up to over 17,000 fold). This was further demonstrated by the identification of stereoselectivity in the case of linalyl esters (which have a chiral centre adjacent to the ester bond).

2. **Medium to Major Influence** - Branched / aromatic groups

Carbon chain branching (distal to the ester bond) and the presence of aromatic or cyclic structures did not have an important effect upon hydrolytic rates when they were present as part of the alkyl moiety. However, such structures did have a substantial impact upon hydrolytic rates when they were present as part of the acyl moiety (esters with branched acyl carbon chains were hydrolysed at least 30 fold slower than linear analogues). Aromatic (such as cinnamic acid) and branched aromatic (such as anthranilic acid) structures had the greatest effect (reducing hydrolysis rates up to several thousand fold).

3. **Minor to Medium Influence** - Linear length of alkyl / acyl moieties and unsaturation

The linear length of acyl moiety carbon chains resulted in trends in esterase substrate specificity which were consistent irrespective of the structure of the alkyl moiety (acyl moiety linear carbon chain length had an effect on hydrolysis rates of up to 20 fold, esters of butyric acid were the favoured substrates). The linear length of the alkyl moiety was found to be of importance, in respect of having a negative impact on hydrolysis rates, only if the number of carbon atoms was less than 4 (in which case an effect of up to 350 fold was observed).

Double bonds proximal to the ester bond were found to have no measurable impact upon hydrolytic rates in the case of cinnamyl esters, but resulted in a possibly slight negative impact in the case of geranyl and neryl esters (having an effect of approximately 2 fold in the rate of hydrolysis).

7.4 Recommendations & Further Work

The results presented in Chapter 3 and 4 demonstrated distinct differences between models of human and rat gastric environments in the behaviour of esters and alcohols which undergo reactions in an acidic environment. As such, it is recommended that:

- Caution be applied in interpreting data available for acid-labile compounds from animal studies for human safety assessment purposes, and that acid-catalysed reaction products be identified.

It has been suggested in Chapter 4 that following the low dose administration of monoterpene alcohols, cytochrome P-450-mediated oxidation is a more important pathway of metabolism than has been assumed from the results of high dose *in vivo* studies. The available data indicates common sites of hydroxylation[305; 306; 308-310], and that if hydroxylation occurs proximal to the alcohol functional group[308] or allylic oxidation of cyclic monoterpenes occurs[334; 340], epoxides may form. Therefore it is recommended that:

- Studied be undertaken into the low dose metabolism of monoterpene alcohols such as α-terpineol.

The available data (discussed in 1.2.4.2) and the results of Chapter 5 indicate that the esterolytic activity of the rat is substantially greater than that of humans, and that uncertainty factors which account for inter-species variation may be exceeded. Although *in vivo* hydrolysis data are not available and have rarely been utilised for the safety assessment of food flavouring esters (esters of cinnamyl alcohol / cinnamic acid being the principal exceptions), this does have important ramifications in respect of the safety evaluation of other xenobiotic esters such as pharmaceuticals (where safety evaluations have relied in part in the interpretation of data from animal studies), therefore:

- Greater information is required to adequately account for species differences in esterolytic activity. This may be achieved by experimentation and the analysis of available data on the pharmacokinetics of ester drugs.

Not only does inter-species esterolytic activity require more precise quantification, but esterase isoenzymes remain almost entirely uncharacterised. The stereospecific hydrolysis of esters of linalool has been demonstrated to be a possibly valuable tool in aiding the characterisation of B-esterase isoenzymes, therefore:

- It is important to further this work and examine hydrolytic stereoselectivity of linalyl esters in conjunction with putative differential inhibitors of esterase isoenzymes.

The results of high dose gavage administration (1000 mg / kg body weight) studies with rats illustrated the poor bioavailability of lipophilic esters due to sequestration in food material and / or the formation of lipid droplets. Therefore, to enhance knowledge available for safety evaluation purposes, it is recommended that:

- Studies be undertaken to identify the reasons for poor bioavailability and further characterise the bioavailability of food flavouring esters administered with and without wet and dry diets.

The results of high dose intraperitoneal administration (1000 mg / kg body weight) studies with rats indicated the partitioning of ester substrates into tissues, such as adipose tissue. This has potential implications in respect of the bioaccumulation of esters which are resistant to hydrolysis. Therefore it is recommended that the behaviour of food flavouring esters *in vivo* be further evaluated by:

- Re-evaluation of the *in vivo* studies presented in Chapter 6 utilising more sensitive analytical equipment, and preferably the determination of the fate of a radio-labelled ester substrate which has been shown to be resistant to hydrolysis (such as terpinyl isobutyrate).

During the safety assessment of food flavouring esters assumptions have been made concerning hydrolytic metabolism. These are that hydrolysis of ingested food flavouring esters is rapid and complete, and that data available for one member of a structurally related group of ester compounds can be used to interpret the hydrolytic fate of all the members of the group. These assumptions are not accurate as wide differences between the rates of hydrolysis of flavouring esters have been demonstrated. These differences are based upon structural criteria which have been defined. A simple incubation system containing porcine pancreatin has been shown to accurately indicate the differences between the hydrolytic metabolism of substrates by tissue esterases. Therefore, as a part of the decision process to determine whether the safety assessment of an ester food flavouring additive may be based solely upon it's hydrolysis products, it is recommended that:

- Regard is taken to the structural criteria above (see 7.3) in respect of the level of negative influence on hydrolytic rates, and if necessary:
- An assessment be made of the rate of hydrolysis in artificial pancreatic fluid (in comparison to esters which have been shown to be rapidly, moderately and slowly hydrolysed in that medium).

Limited data are available concerning the toxicology and metabolism of food flavouring compounds. The safety assessment procedure recently adopted by JECFA (see Appendix I A.5.1.2) is a system which enables the best use of available data, including exposure information, within the context of established knowledge of toxicology and metabolism. In respect of the hydrolysis of food flavouring esters, both available data and established knowledge of metabolism are deficient, therefore predictions concerning hydrolysis may not be accurate. This Thesis Report was produced to enable greater confidence in making hydrolytic predictions during the safety assessment of ester food additives.

APPENDIX I

FLAVOURING ADDITIVES: LEGISLATION & ADMINISTRATION OF SAFETY ASSESSMENT & RISK MANAGEMENT

A.0 Flavouring Additives: Legislation & Administration of Safety Assessment & Risk Management

A.1 Introduction

A.1.1 Market History of Flavouring Additives

Food flavouring and aroma chemicals, in addition to fine fragrance substances, satisfy the two most primitive and possibly least understood senses, and have profound implications toward behaviour[343]. An insight into the commercial history of these substances represents an insight into the very earliest forms of human trade[344; 345]. A trade which has undergone three major revolutions since its inception, and is currently undergoing a fourth.

Continents have been explored and conquered, and the most bloody of regimes organised to satisfy the flavouring industry's commercial interests. The control and monopolisation of supply was the first major revolution. The largest and most notorious concern of this period was the Dutch East India Company, a chartered joint-stock organisation. At it's most prosperous (and brutal) in 1617-1624 the organisation held a complete monopoly upon pepper, cinnamon, cloves and nutmeg[344]. This international trade was testament to the economic power of Europe and the demand which existed for these new products of limited supply from far distant lands. Interestingly, for some time peppercorns could actually be utilised as a single international unit of currency[344].

The colonisation of newly conquered lands, and the development of the science of botany broke the spice monopoly, with associated significant price reductions. This was the second major revolution[344]. In the mid 1800's came the beginning of the third major revolution, a revolution which affected the fine fragrance industry as much as food additive producers and suppliers. The newly developed techniques of organic chemistry enabled synthetic, and sometimes completely new, compounds to be produced[1]. Until that time perfumery had relied upon the simple extraction of plant products (higher-order plants can synthesise combinations of related chemicals

belonging to 3 groups of compounds[346]). In 1868 the first synthetic chemical was introduced into a perfume[347], although it was not until 1921 with the introduction of Chanel No. 5 (which has since created sales of over $1 billion), that a perfume had a main note dominated by synthetics[347]. Natural ingredients remain vital in-order to give 'body' as opposed to the 'sparkle' of synthetics[1; 348].

The use of synthetics not only brought advantage in the variety of chemicals available, but also created great advantage in price, reliability of supply, and quality control. Today, there are 1815 substances recognised as safe food flavouring additives by the Flavor and Extract Manufacturers' Association of the US (FEMA), over 75% of these are produced by synthetic means[3; 349].

The fourth revolution which is at present occurring in the industry has developed particularly from the success of synthetic compounds. As history testifies, the industry has always been technology driven, the products of the application of technology have created substantial reward, thus itself stimulating the increasing use of new technologies. However, recent societal and cultural factors are generating increasing impetus for change. The earliest indications of this change came when household products, such as cleaning agents, stopped having generic scents irrespective of brand, and product differentiation through scent began[1].

Through the technology and skills of it's corporations, Europe and then the USA became the major exporters of flavour and fragrance compounds[1]. The flavour and fragrance industry now comprises 6 large multi-national corporations (which have developed over the past 170 years from a number of what were small manufacturing and marketing companies) and several hundred small to medium sized suppliers of specialist products. These organisations provide services to manufacturers of consumer goods. The relationship between producer and customer is extremely close, and chemical formulations are regarded as secret. However, international patent law does not allow for the patenting of formulas. This is reflected in labelling regulations throughout the world, such that the flavouring content of food need not be disclosed. Instead the word 'flavourings' is required to be stated[1] (a safeguard which is increasingly circumvented by the development of more capable analytical equipment).

The combination of societal change (in the form of globalisation, income trends, family structure and lifestyle changes) and technological changes are currently having major impacts on manufacturers and suppliers of consumer goods. As a result, the pace of development in the way in which existing flavouring compounds are utilised and the creation / discovery of new compounds is more rapid than at any other time in history.

A.1.2 Regulatory Background

The importance of food safety for health and development has been recognised by many international fora, and although the addition of flavouring compounds to foods is generally at low levels and is self-limiting, the compounds and their usage are under close regulatory scrutiny in many nations. A number of factors necessitate rigorous safety and surveillance procedures and appropriate legislative frameworks: the large number of flavouring substances in current use; the lack of toxicological information concerning commonly used food flavour compounds; the continuous development of new flavours; and new uses for existing flavours.

The major industrialised countries have developed food legislation independently over a considerable period of time (the British Industrial Biological Research Association issues Information Bulletins which contain regular sections on current developments in food legislation by country[350]). Through the harmonisation of trading practices, consolidation of the economic and social structure of Europe, and industrial representation by trade associations, this situation is changing somewhat. However, on the world stage, legislation dealing with flavouring substances still often remains clearly distinct between nations. In many, no specific legislative provisions are made for flavouring compounds, in others, lists of permitted or banned substances are maintained, and in several, all flavouring compounds require specific government authorisation before use[1].

The risk assessment process for food additives may involve either quantitative risk assessment (as is the case for genotoxic carcinogens), or more usually safety assurance where animal and *in vitro* studies are used to extrapolate risk to humans and determine acceptable limits of usage[351].

The fragrance industry is closely aligned with the flavour industry in respect of the products which it manufactures, however it is not subject to legislative approval processes despite a reported increasing incidence of adverse reactions to perfume ingredients, and a number of documented toxicity problems[352]. Instead, trade associations of the perfume industry are expected to co-ordinate the maintenance of best-practice toward eliminating the use of hazardous compounds. The International Fragrance Association (IFRA) maintains a Panel of Experts to assess information presented to it by the Research Institute for Fragrance Materials (RIFM)[353]. Recently, in an attempt to implement a more rigorous approach to the safety evaluation of fragrance compounds, an ingredient database has been developed to identify compounds for which review should be considered a high priority based upon quantities of use, consumer exposure and chemical structure[354].

Despite the various administrative structures in place to regulate the use of flavouring additives, formalised scientific risk and safety assessments are predominantly undertaken by two organisations: the Expert Committee of FEMA (FEXPAN), which can provide legally recognised judgement in the USA[355]; and the Joint FAO / WHO Expert Committee on Food Additives (JECFA), which undertakes an advisory role. However, a number of other governmental and intergovernmental bodies also evaluate flavouring additives for safety, and there is a current need for harmonisation. Recently, formalised JECFA procedures for the safety assessment of flavouring compounds have been consolidated and applied[250].

A.2 Organisations Of The Flavour Industry

In order to deal effectively with government agencies, and support the overall objectives of the flavour industry as a single body, in most countries the industry has formed a trade association (see table A.1). On an international level, the International Life Sciences Institute is funded by food manufacturing corporations and is a forum for debate and research concerning food safety issues.

Country	Trade Association
Australia	Flavour and Fragrance Association of Australia
Austria	Fachverband der Nahrungs-und Genussmittelhindustrie
Belgium	Groupement des Fabricants, Importeurs et Malangeurs d'Aromes, Essences, Extraits et Produit Aromatiques
Brazil	Associacao Brasileira das Industrias da Alimentoaco
Canada	Flavour Manufacturers Association of Canada
Colombia	Asociacion Naxional de Industriales
Denmark	Essens Fabrikant Foreningen
France	Syndicat National dea Industris Aromatiques Alimentaires
Germany	Verband der Deutschen Essenzenindustrie
India	Perfumes and Flavours Association of India
Italy	Federazione Nazionale dell' Industria Chimica (Grupppo Essence Naturali e Sintetiche)
Japan	Japan Flavor and Fragrance Manufacturers' Association
Mexico	Associacion Nacional de Fabricantes de Productos Aromaticos
Netherlands	Vereniging van Geuren Smaakstoffenfabrikanten
Norway	Norske Aromaprodusenters
South Africa	The South African Association of Industrial Flavour and Fragrance Manufacturers
Spain	Associacion Espagnola de Fadricantes de Aromas para Alimentacion
Sweden	Foreningen Svenska Aromtillverkare
Switzerland	Schweizerische Gesellschaft fur Chemische Industrie
United Kingdom	The British Essence Manufacturers' Association
United States of America	Flavor and Extract Manufacturers' Association
International	The International Life Sciences Institute

Table A.1: National / International trade associations of the flavour industry[1]

A.2.1 The Flavor and Extract Manufacturers' Association (FEMA)[1; 10; 349; 355-357]

Formed in 1909, the Flavor and Extract Manufacturers' Association of the United States aims primarily to: keep the industry informed of legislative issues; represent it's corporate members in meetings with government and other organisations; and co-ordinate work on problems which effect the industry as a whole.

In 1959 and further in 1968 FEMA conducted a survey to provide basic data for a program to determine which flavour ingredients were in use in the US, and to establish in what products and in what concentrations they were used. In addition,

much information on the safety of these chemicals was collected and collated. As such the first list of flavour ingredients was produced. FEMA also established a panel (initially consisting of six members) of experts in pharmacology, biochemistry, toxicology, metabolism and medicine to aid in the safety evaluation of flavouring agents (see A.4.3).

FEMA, in association with the International Organisation of the Flavour Industry (IOFI, see A.2.3), has conducted two surveys on the usage of flavours in foods, and more recently in co-operation with the National Academy of Sciences for the Food and Drug Administration. This information is extremely valuable for regulatory agencies and in the setting of standards for food additives.

A.2.2 The British Essence Manufacturers' Association (BEMA)[1]

Established in 1917, the British Essence Manufacturers' Association engage in similar activities for the British industry as FEMA does for its corporate members in the US. However, BEMA also covers the remit of food colours and other miscellaneous products used by the food, beverage, confectionery, perfumery, tobacco and similar trades.

BEMA is highly active in promoting the harmonisation of legislation which exists throughout Europe. Representatives of the Association are also members of IOFI (see A.2.3) and sit on the Food and Drinks Industries Council (FDIC), which represents all the food and beverage companies in the UK via their own associations. Representatives from FDIC are members of the Council of Europe, and as such the flavour industry of the UK, and its related industries, are represented at a high level within the structure of European administration.

A.2.3 The International Organisation of the Flavour Industry (IOFI)[1]

With regard for the need of a single body to represent the flavour industry's international legislative concerns, IOFI was established in 1969. IOFI maintains a detailed Code of Practice and close collaboration with IFRA, and is closely linked to the European Flavour and Fragrance Association (EFFA; which represents the

industry specifically within the European Union). Present membership numbers 22 countries, with the main activities of IOFI being in such areas as:

- Collection, analysis and harmonisation of regulations.
- Elaboration of Codes of Practice and industry guidelines.
- Collection, generation and evaluation of safety data.
- Inventories of raw materials and specifications.
- Methods of analysis.
- Environmental issues.

IOFI maintains a monthly information service and meets at sessions of the General Assembly in Geneva, which is administered by a Board of Directors on which each member country has a delegate. A Committee of Experts handles scientific affairs, and working groups exist to evaluate individual concerns.

IOFI has the status of permanent observer at the United Nations Joint Food and Agriculture Organisation / World Health Organisation Food Standards Commission, which is a committee reporting to the *Codex Alimentarius* Commission (an international organisation concerned with the harmonisation of trading standards). As such IOFI aims to facilitate co-operation and trade between member countries.

A.3 The United Kingdom & Europe

In the United Kingdom, further to The Food and Drugs Act 1955 and The Labelling of Food Regulations 1970[1], The Food Safety Act 1990 prohibits the addition to food of any substance which renders it injurious to health. It also provides the legal basis for specific regulations controlling particular classes of additives and the composition of particular foods[358].

However, in December 1988, European Directive 89/107/EEC, commonly referred to as the 'Food Additives Framework Directive' was adopted. This established a procedure for agreeing comprehensive Directives for virtually all classes of food

additives[358]. As such, the use of flavourings in the UK is controlled by the Flavourings in Food Regulations 1992 (Statutory Instrument 1992 No. 1971 as amended by Statutory Instrument 1994 No. 1486)[359]. These regulations implement certain provisions contained in European Council Directive 88/388/EEC on the approximation of the laws of the Member States relating to flavourings; the 'Flavourings Framework Directive'[360].

The Flavourings Framework Directive aims at the full harmonisation of Member States' national controls on flavourings, this is further to the principal of 'mutual recognition' where any product legally produced and sold in one member state can be marketed in any other member state. A European Parliament and Council Regulation establishing a Community Procedure for flavouring substances used or intended for use in or on foodstuffs came into force on 23 November 1996[359], and a Commission Regulation on 18 July 2000 established the measures necessary for the adoption of the Procedure[361]. This regulation will lead to the establishment of a positive register of permitted chemically-defined flavouring substances (which will update and replace the Council of Europe 'Blue Book'[362]), following a programme of evaluation administered by the European Union Scientific Committee for Food (SCF). Prior to evaluation, manufacturers are required to place a list of compounds at market, along with a variety of data relating to those compounds, on an internet-linked database [the Flavis Database (http://www.flavis.net/), in which compounds are grouped according to their chemical structure].

The SCF is pressing to have the positive register finalised by early in the new millennium, and as such is placing increased pressure upon expert committees such as JECFA and FEXPAN to scientifically evaluate and advise upon the safety of flavouring compounds (see A.5 on international safety assessment). This naturally necessitates some commonality in the safety assessment protocols employed by different committees, and some movement towards this has taken place recently with a new scheme adopted by JECFA (see A.5.1.2).

The provisions of The Flavouring Framework Directive, having been implemented in the UK in the Flavouring in Food Regulation 1992, came into force on 1 January 1996[358]. This legislation lists the additives which are permitted, and sets out technical

specifications for flavourings and their use, including the setting of maximum levels in food. The legislation also covers labelling and other marketing issues.

It is interesting to note that The Flavouring Framework Directive[360] contains two annexes of specific lists: Annex I entitled '*Maximum limits for certain undesirable substances in foodstuffs as consumed as a result of the use of flavourings*' (containing only one entry: 3,4 benzopyrene); and Annex II entitled '*Maximum limits for certain substances obtained from flavourings and other food ingredients with flavouring properties present in foodstuffs as consumed in which flavourings have been used*'.

To date, Member States have notified the *Codex Alimentarius* Commission of lists of flavouring substances used in their territories, and a draft list has been drawn and circulated amongst Member States for comment[363]. Initial problems over the drafting of the list included; the precise definition of a flavouring agent; the commercial sensitivity of the information and as such confidentiality, and the continuous process of safety evaluation which can re-assign the status of compounds (for example, the Scientific Committee for Food have recently advised that the maximum limit for coumarin, as advised in Annex II of the Flavouring Framework Directive, should be reduced). However, of significant concern is the requirement of Member States to monitor consumption and usage[364], how this costly and complex procedure is to be performed across Europe has not been made clear.

A.3.1 Food Standards Agencies

The UK Government has proposed a new agency for the protection of public health in relation to food safety[365]. This proposal has been drawn partly in the light of a report by Professor Philip James on behalf of the Government, detailing the state of food safety and public protection[366]. Particularly noted was the erosion of public and producer confidence in the current systems of food controls[367] (factors heavily influenced undoubtedly by the public perception and economic issues surrounding the Bovine Spongiform Encaphalopathy and *Escherichia coli* meat contamination issues, which are further to the already somewhat confusing and apparently contradictory advice offered by a variety of Government agencies upon diet). Particular key factors which the Government have stated require to be addressed include[366]:

- The potential for conflicts of interest within the Ministry of Agriculture Fisheries and Food arising from its dual responsibilities.
- Fragmentation and lack of co-ordination between various Government bodies.
- Uneven enforcement of food law.

The Governmental White Paper *The Food Standards Agency: A Force for Change* was published on 14 January 1998[365], this set out plans for the new public body which will manage food safety and standards issues. The Agency will have the power to monitor the safety and quality of food, provide advice, and enable current legislative provisions on food safety.

The Agency was recently launched on the 3rd of April 2000 with twelve independent members supported by an executive staff and headed by a chief executive (Professor Sir John Krebs)[367]. It is planned that it will eventually have a £100 million budget, with £25 million for research and £6 million for surveillance functions, with at least part of these monies being raised through a charge on industry (a vigorously debated issue at present)[368; 369]. It will posses its own Communications Unit[367] to help alleviate some of the problems currently associated with the poor communication of scientific information and associated negative public perception. All matters concerning food additives are to be managed by the Agency, including flavouring regulation and safety management[365], the risk assessment of which will continue to be conducted by international committee (see A.5).

The European Parliament has recently voted on the establishment by the European Commission of a European Food Standards Agency. It is anticipated that this institution will be established by 2002. Originally envisaged as a European counterpart to the US Food and Drug Administration, in respect of all matters concerning the safety and regulation of the food supply, it has recently become clear that regulatory powers will be limited and that ultimate decisions on the safety of food will rest with politicians and not scientists[370]. How the operations of a European Food Standards Agency are to be integrated with similar agencies in individual nation states remains to be determined.

A.4 The United States of America

The first federal legislation governing food and drugs was the Food and Drug Law 1906[371]. This was a relatively simple piece of legislation aimed at controlling misrepresentation, limiting the use of chemicals and establishing hygiene requirements for food processors. This Act was replaced by the Federal Drug and Cosmetic Act 1938[371]. This is still the basic legislation but new provisions to meet the requirements of better consumer protection have subsequently been incorporated.

Between 1950-1952, Congressman J.J. Delaney chaired a House select committee investigating the use of chemicals in foods and cosmetics. In June 1952, the Committee filed a report urging that the chemicals used in food be subject to essentially the same safety requirements as those for new drugs[372] (large numbers of animals are now utilised toward the safety assessment of food additives[373]). The existing 1938 Federal Food, Drug and Cosmetic Act was not viewed as providing an adequate mechanism for ensuring the safe use of additives in food as no pre-clearance procedures were required[371]. The responsibility was for the Food and Drug Administration (FDA) to prove that a chemical was injurious to health after it was placed on the consumer market[355]. Furthermore, the Act did not take account of the fact that chemicals added to food may be toxic at high doses but prove acceptably safe at normal usage levels[371]. As such both food safety and scientific innovation were hampered. Furthermore the famous 'Delaney Clause' which levelled a blanket prohibition on the use of substances found to induce cancer, has often given rise to possibly unsound restrictions upon food additives, discussion upon reform of this provision continue[374].

In 1958, the United States Congress enacted the Food Additives Amendment (FAA) to the Federal Food, Drug, and Cosmetic Act (FFDCA section 201(s))[375]. The centrepiece of the FAA is the definition of food additive and an exclusion specifically provided by Congress for substances 'generally recognised as safe'[349]:

'The term food additive means any substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food ..... if such substance is not generally recognised, among experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures (or, in the case of a substance used in food prior to January 1, 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of intended use ..... '

This legislation requires that the FDA approve all food additives before they are used in food. Information on the proposed additive's production, it's expected consumption in the food supply, and it's safety must be submitted in the form of a Food Additive Petition for the FDA's review[371].

However, in enacting the amendment, Congress also needed to consider the thousands of food ingredients which had already been in use for many years. If approval was required for all substances, the food supply would be disrupted and the food industry subject to a serious financial burden which was probably unnecessary[355].

As such, two groups of substances were exempted from the food additive regulation process[375]:

1. 'Prior sanctioned substances' applies to substances the FDA or the US Department of Agriculture had determined were safe for use in food prior 1958.
2. 'Generally Recognised as Safe' (GRAS) substances are assessed by an appropriately qualified panel of experts (whom represents an expert is given no further definition) on the basis of past history of use, and scientific safety assessment criteria, to be safe as a food additive in respect of intended use.

The 'prior sanctioned' category immediately ran into difficulties, as the FDA had no accurate or complete records of what decisions they had taken in the past. The FDA had to advise that if anyone knew or suspected a substance to have been previously sanctioned they should be informed[375].

If a substance was in use prior 1958, it may be determined GRAS (the concept being originally taken from existing drug approval legislation) based upon common use in food and on the available scientific literature. Flavour ingredients which are classified as GRAS are removed from consideration as food additives, and as such exempt from the process of pre-market approval[357]. GRAS substances should not however, be assumed to be safer than food additives[376]. The concept of GRAS (although being clearly ambiguous as a legal term[371; 372]) is unique, no other country has such a category for food ingredients. The Code of Federal Regulations covering GRAS is searchable from http://www.access.gpo.gov/nara/cfr/cfr-retrieve.html#page 1.

A.4.1 Determining GRAS Status

According to the 1958 Act, a manufacturer or user of an ingredient can determine itself, whether the ingredient satisfies the regulatory definition of a GRAS substance and so claim exemption from the lengthy approval process for food additives[376]. A rule was consolidated in a 1988 amendment in that 'persons have the right to make independent GRAS determinations'[372]. As such, ingredient manufacturers have a level of self regulation over food safety issues in the US, and the responsibility is for the FDA to challenge the GRAS status of substances. However, although not a legal requirement, companies may elect to also file a GRAS Affirmation Petition with the FDA in order to seek the agency's concurrence[377]. The FDA actively encourage the submission of GRAS Affirmation Petitions which involve gathering comment from interested parties concerning the substances in question[353]. This affirmation process is soon to be replaced by a simplified system were the FDA simply notifies the manufacturer of agreement or disagreement with the information that the manufacturer has submitted[378] (full details and guidance concerning the submission of a GRAS petition to the FDA is available at the internet site: http://vm.cfsan.fda.gov/~dms/opa-toc.html#ADF which contains FFDCA section 170.35 of title 21 of the Code of Federal Regulations).

Immediately after the passage of the FFDCA, the FDA published several proposals listing substances that they believed to be GRAS, and solicited comments from industry and academic food scientists[375]. Subsequently, in 1962 the FDA issued the

original 'FDA GRAS List' which contained several hundred common flavours such as spices, and only 27 well known synthetic agents[355]. At the time of the enactment of the 1958 Act, several thousand flavours and flavouring extracts were in use. This resulted in a large backlog of substances to be evaluated, and clearly indicated the problems faced by the FDA[357]. However, because flavourings comprise a minute proportion of a product's ingredients, thus consumer exposure is low, the GRAS review of flavours was considered by the FDA to be a relatively low priority[356].

Despite this, in 1965 the FDA and FEMA, disagreed upon the categorisation of a large number of substances as GRAS. This being a result of a difference of opinion in the interpretation of the GRAS criteria, and the fact that all of the information used in the classifications was not available to all food science experts[375]. An essential caveat in the legislation being that the scientific information necessary to determine GRAS status is required to be published in literature available to the public for review, (this is in contrast to a standard Food Additive Petition, in which data on an ingredient's safety may not appear in any published literature).

The difference of opinion over the interpretation of the GRAS criteria was essentially because FEMA were of the opinion that flavour ingredients are in-fact a special category and should be treated differently from other ingredients. Not only are they used in foods in low quantities, but safety data is often meagre as many do not have the commercial value to justify conducting extensive toxicity studies[356]. FEMA have as such, asserted that the flavour industry should have the right to decide whether a new flavouring substance is GRAS, and to continue adding to the FEMA GRAS List[357]. Many of the substances on the FEMA GRAS List are not on the FDA GRAS List, although the FDA have now informally acknowledged the use of substances on the FEMA GRAS List until they have themselves evaluated their safety[355].

During the passage of time, the development of toxicology lead to the questionable safety of a number of the members of the FDA GRAS List, principally among then was the sweetening agent cyclamate (which had been shown in an animal study to possibly increase the risk of developing bladder tumours)[375]. As such, in 1969, President Nixon directed the FDA to re-evaluate all items on the GRAS list for safety. This 'GRAS re-affirmation' process was managed by the Select Committee on Gras

Substances (SCOGS) and became the largest toxicological review in history. The aim of GRAS re-affirmation was to reassign compounds into categories which represented[8]:

1. GRAS status re-affirmed.
2. GRAS status maintained for a stated period of time while additional toxicity tests are made.
3. GRAS status rescinded (requiring the compound to undergo food additive pre-market approval) unless either conditions of safe usage can be established or additional toxicity data provided.

This process was enabled by a revision of the FFDCA in 1972 titled 'Eligibility for Classification as Generally Recognised As Safe' which clarified the classification of substances as GRAS, and codified the definition as being either[375]:

1. General recognition of safety through scientific procedures based upon published literature, requiring the same quality and quantity of scientific evidence that is required for approval of a food additive.
2. General recognition of safety based upon 'a history of common use in food' which does not require the same quality and quantity of scientific evidence as for a food additive, but should be based upon generally available data and information.

The first criteria for the definition was a dramatic change regarding new substances, in that the focus shifted from the 'general recognition' of safety by scientists, to an assessment of whether the substance met the FDA's standards for approval of a food additive. This change in effect limits the applicability of the GRAS exemption and lends greater formalisation to the affirmation process. However, between 1960 and the mid-1970's the legal criteria for determining a lack of 'general recognition' (essentially the FDA's position) was also being refined by case law. It was established that unanimity among experts was not required to illustrate that a substance is GRAS, but that the general reputation of the substance in the scientific community should be established[372]. Accordingly when it is recognised that there is a 'genuine dispute' among experts, the substance should not be recognised as being GRAS. As such, the position for a new substance would appear to be one where the determination of

GRAS status requires the same information input as for a *bona-fide* food additive, but can be based upon a less rigorous extrapolation of the data to human risk.

The second criteria for the definition caused considerable argument in that originally only substances with a history of consumption in the US were allowed to be considered[375]. This was remedied with an amendment to the FFDCA in 1988 such that the history of consumption in other nations may be considered as long as due regard is taken as to the quantity and reliability of the data[372; 375].

Furthermore, There was some continuing confusion over the 'Delaney Clause' which bans '*food additives that induce cancer in man or animals*'. Case law has now noted that the GRAS exemption from food additive classification, may allow the classification of GRAS of carcinogenic substances that carry trivial risk[374].

Following the GRAS 're-affirmation process' of the 1970's, the FDA stated that[349]:

> *'It has been too often assumed that the GRAS substance may be used in any food, at any level, for any purpose. As a result, the uses of some GRAS food ingredients have proliferated to the point where the GRAS status has been brought into serious question.'*

Furthermore, many industries (for example, the fragrance industry) had come to consider GRAS designation as confirming safety via any exposure route[352]. As such, a rule was to be introduced to the end that a substance can only be considered to be GRAS with respect to particular stated uses[349]. However, this raised considerable consternation within the manufacturing industry, as possible future uses of substances would not be considered as GRAS. As such this rule was subsequently relaxed in 1982 so that the description of 'current good manufacturing practice conditions of use' need only be supplied at the request of the FDA[371; 375].

The FDA have in the past demonstrated that they are quite prepared to challenge independent GRAS determinations[372; 376]. It is their responsibility to ensure that the GRAS criteria are correctly applied so as not to circumvent the requirement of pre-market approval for new substances.

A.4.2 The Flavor and Extract Manufacturers' Association

Following the 1952 Delaney Report, without the GRAS concept, the FDA would have been faced with regulating as food additives not only those substances truly in need of prompt attention, but also thousands of other substances generally considered at the time to be of little public health concern. The cyclamate incident demonstrated that such substances could possibly be less benign than previously thought, and President Nixon's order of a major review changed significantly how the GRAS system was administered. The informal GRAS criteria gave way to more formalistic criteria which immediately made the GRAS concept more difficult to apply[372].

The Flavor and Extract Manufacturers Association Expert Panel (FEXPAN) conduct the only large-scale independent effort to evaluate substances under the GRAS criteria of the Food Additives Amendment to the 1958 FFDCA[372]. The principal objective of FEXPAN is the following[357]:

> *'To protect the consumer's health in the context of flavor use and to provide the scientific basis for helping industry maintain a pattern of self-regulation'*

Founded in 1959 by Bernard L. Oser, FEXPAN was established to determine the GRAS status of food flavourings[355]. FEXPAN Experts were selected not only because of their training and experience, but because they had no prior connections with the food or flavour industries[356] (it is also important to note that FEXPAN GRAS decisions are made in executive sessions, with no employees of the flavour or food industries present[355]). FEMA staff prepare literature reports which are presented to FEXPAN, following the review of the submissions, compounds are assigned either: GRAS status; not GRAS status; or given HOLD status pending the availability of further information. For GRAS compounds, upper and lower limits are given for the concentration range under specified conditions of use[357].

Current members of FEXPAN are: Professor John Doull (University of Kansas Medical Center); Dr. Ian Munroe (Canadian Centre for Toxicology); Professor Paul

Newberne (Co-Chairman of FEXPAN, Boston University School of Medicine); Professor Phil Portoghese (University of Minnesota, College of Pharmacy); Professor Robert Smith (Chairman of FEXPAN, St. Mary's Hospital Medical School, University of London); Professor Bernard Wagner (Deputy Chairman of FEXPAN, New York University School of Medicine); Dr. Carrol Weil (retired – Bushy Run Research Center, Mellon Institute) and Professor Lauren Woods (Medical College of Virginia, Virginia Commonwealth University). Professor Richard Ford is a Liaison Member of FEXPAN, and President of the Expert Panel of RIFM. Dr. Tim Adams serves as the FEXPAN Executive Secretary, and Director of Scientific Affairs[355]. FEXPAN also employ consultants on an *ad hoc* basis to provide special expertise as required[379].

With the FDA's informal approval, FEXPAN began the first comprehensive survey of the identity and uses of all known flavouring substances in the US[375]. The FDA even submitted it's own list of natural flavour substances for evaluation[355] (FEMA have to-date evaluated over 1800 flavouring agents to determine GRAS status, only 10 substances have been removed from the GRAS list[355], and only two challenges to FEXPAN's decisions have ever been made [for saccharin and cinnamyl anthranilate[133] (one synthetic and three natural flavouring compounds are currently prohibited in the US[372])]. Surveillance indicates that the only verifiable adverse reactions to regulated food flavourings currently in the market-place are due to immunological conditions[380-384].

FEXPAN is currently engaged on it's third full cycle (second GRAS re-affirmation) and is also currently organised to address the clearance of new substances[278; 379; 385; 386]. Furthermore, research is on-going towards the most efficacious safety assessment protocols[387; 388], particularly with respect to mixtures and new classes of compound, such as those, for example, produced via genetic manipulation techniques[389]. The safety assessment process involves evaluations of exposure, structural analogy, metabolism, pharmacokinetics and toxicology[349; 356; 357]. Ingredients are evaluated individually taking into account the available information on the group of structurally related substances[355]. The data related to the first GRAS assessment program was compiled in Scientific Literature Reviews (SLRs), (primary determinations were published in *Food Chemical News* with the FEMA program initially explained in

Food Technology, all subsequent FEMA GRAS determinations have been published in *Food Technology*). As part of GRAS re-affirmation, FEXPAN evaluate groups of structurally related compounds and produce Interpretative Summaries. Group and Interpretative Summaries are produced in order to supersede SLRs. The new format is intended to be more 'user friendly', and compatible with computerised relational data-base systems[278].

A.4.3 The FEMA Expert Panel Safety Assessment Of Flavouring Agents

First developed in the early 1960's, a series of 20 criteria are used to evaluate GRAS status[355]. Elements that are used include exposure, natural occurrence in food, structural analogy, metabolism, pharmacokinetics and toxicology[349; 356; 357].

For each GRAS application, FEXPAN calculates an exposure estimate, which together with the structural, toxicological and pharmacokinetic data, give the Panel perspective regarding the consumption and safety of the substance as a food constituent[278]. All Panel decisions must be unanimous, all GRAS determinations are published for comment, and all information is provided to the FDA[355].

FEMA GRAS publications include average, usual and maximum use levels of the flavouring compounds, in order to reflect the range of use levels considered by FEXPAN in the safety assessment[349]. As such, FEXPAN examines the use category of the material in question (for example, baked goods), including whether it is a natural constituent of foods. They then evaluate the proposed use levels in light of the total potential exposure to the substance given it's toxicological and pharmacokinetic characteristics, in addition to it's known or probable metabolism.

A.4.3.1 Prioritisation of Safety Assessment

Due to the large number of flavouring substances initially presented to FEXPAN for review, a method of prioritisation was developed based partly upon the work of Crammer *et al*, with respect to a 'Decision-Tree' procedure[390; 391]. This system, through a series of 33 questions, mostly about chemical structure, classifies substances into one of three classes, and thus ranks of concern; low, intermediate, and

high presumed toxicity. These classes when combined with exposure information lead to a clear priority classification.

It is interesting to note, that the decision tree scheme, as used by FEMA[349] and the *Codex Alimentarius* Commission[5] for priority setting, has also recently begun to be used by JECFA as a part of their flavouring additive safety assessment procedure[250] (see A.5.1.2).

A.4.3.2 Exposure

Exposure data are critical to the safety assessment of flavour ingredients. Flavour ingredients are rarely added to foods in excess of 100 ppm[284], and their usage for organoleptic purposes is generally self-limiting. In addition the actual concentration in food may be substantially less than that initially added due to volatilisation, and absorption from food may be limited in some cases[278]. Exposure to a flavouring ingredient from it's natural source also needs to be taken into account. As such, when quantitative data is available for naturally occurring substances, FEXPAN considers the 'Consumption Ratio' as part of their evaluation[349]. Of the 1815 currently classified as GRAS by FEMA, 1400 have been identified in nature, with a high probability that the majority of the remaining 415 will be found to be 'nature identical'[284].

The Consumption Ratio describes the ratio between the quantity of a flavour ingredient consumed as a natural component of basic and traditional foods, and the quantity consumed as a flavour added to foods when it is consumed by the same population over the same period of time[284]. The majority of flavour ingredients with available quantitative data, exhibit a Consumption Ratio greater than 1[6; 7], indicating that exposure occurs predominantly as a result of natural occurrence in traditional foods. The ratio is not intended to justify the safety of particular flavours, but is used to strengthen the conclusion that a substance is GRAS[349].

There are a number of methods of assessing human exposure to food additives as illustrated in table A.2:

Method	Description
Per Capita	Based on total annual production divided by the total population exposed
Budget or Use-related	Assumption that all food / drink or specific goods contains additive at maximum approved level and consumption of those goods by the population
Dietary record	Based on food consumption measurements, usually over a 7 day period
Retrospective	Analysis of concentrations in food and patterns of consumption

Table A.2: Methods for assessing human exposure to food additives[351].

Maximum exposure to flavour additives has traditionally been estimated using one of two principle methods: the possible average daily intake (PADI – a use-related estimate); and the daily *per capita* intake method[6; 278]. PADI is calculated using the concentration of a flavour ingredient added to foods within specific food categories, and the daily mean consumption of those food categories. It incorporates the assumption that all foods within a category are flavoured with the substance in question. However, in reality, most flavour ingredients are used in a limited number of foods within a category. As a result the PADI is often a substantial exaggeration of the potential exposure to individual flavour ingredients. This is seen by the fact that the annual production of flavours is often exceeded by PADI estimates by several orders of magnitude[349]. Daily *per capita* intake is determined by the disappearance of flavour ingredients from the marketplace, that is, the annual total quantity of substances reported to be sold by the flavour manufacturers for use in food and beverages[392]. This method is considered a conservative estimate because it incorporates the assumption that only 10% of the population consumes the entire annual volume reported by the industry. There is also an extra margin of safety in that it is assumed that only 60% of manufactured flavouring substances are reported in industry surveys.

The daily *per capita* intake is regarded as being more representative than the PADI methodology, and this has been borne out in volunteer controlled studies[357]. In one 12,000 participant study, the PADI was found to be in the range of 10 - 100,000 times greater than the consumption patterns observed, where-as the *per capita* method was found to be in the region of 0.2 - 30 times study observations[278]. FEXPAN consider

the *per capita* method to be the preferred exposure guide[356]. It is however, also valuable to consider exposures to specific age groups, and to address this R.L. Hall developed a 'probabilistic intake method' in 1976 based upon an industry-wide survey, and a extensive food additive survey sponsored by the FDA[3].

As can be seen from figure A.1, using the *per capita* methodology, in an US survey, most flavouring substances were found to be consumed in amounts of less than 1000 μg/person/day.

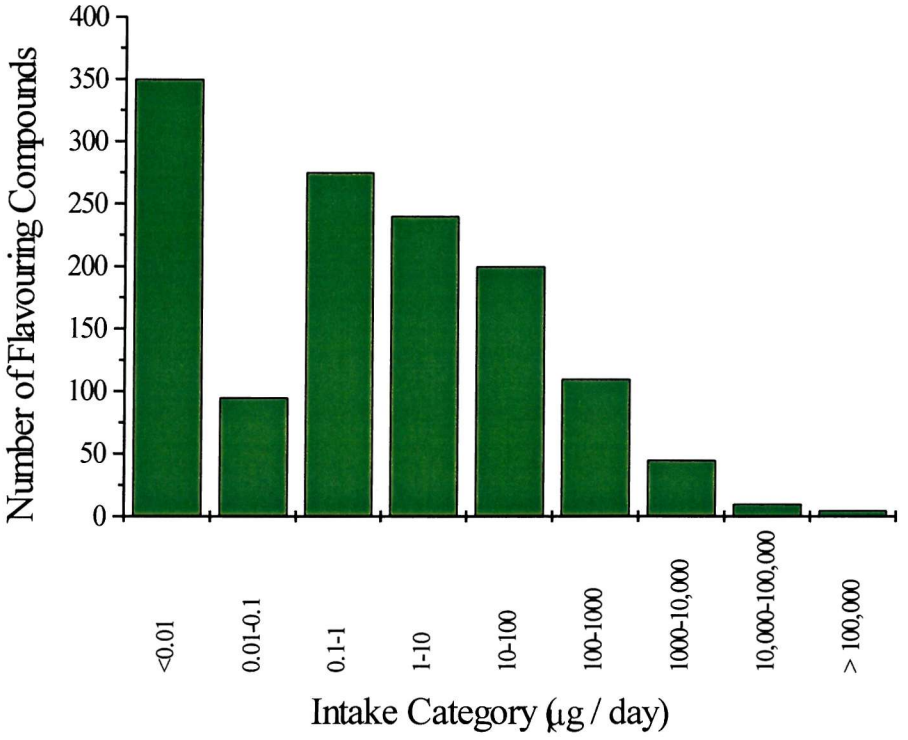


Figure A.1: Number of flavouring substances within various intake categories[3].

A.4.3.3 Structural Analogy

The FEMA GRAS program is partly based on the philosophy that for substances for which the exposure is trivial, predictions regarding their safety can be reliably evaluated within the context of their structurally related group[349; 356; 357]. For substances with significant exposure, more extensive data on the individual substance may be required. It is generally regarded that structurally related flavour ingredients exhibit similar routes of metabolism[355]. In the absence of metabolic information, bio-

transformations can usually be predicted on the basis of the structural groups present. For the majority of flavour ingredients, no-observed-adverse-effect-levels (NOAELs) from animal studies of structurally related compounds are more than 100,000 times their exposure levels from use as flavour ingredients. It is assumed that such large margins of safety more than compensate for toxicological differences seen between different members of a structural group[278].

A.4.3.4 Metabolic Fate

Metabolic data has a critical role in the GRAS assessment process, however, the availability of comprehensive metabolic and toxicological profiles is limited. Most metabolism studies identify only major excretion metabolites in non-human animals under high dose conditions. It is assumed that such studies do however, help identify the primary detoxification pathways. Some *in vitro* information is available (see 1.3 on hydrolytic metabolism), and with the advancement of modelling techniques toward possible *in vivo* human outcomes this data may become more valuable[351; 393].

A.4.3.5 Toxicology

In conjunction with their views on structural analogy, FEXPAN believe that it is unwarranted to require toxicological studies for every flavour ingredient[278]. Metabolic fate can often be predicted if it is not known. Relevant toxicological studies of flavour ingredients typically include oral acute toxicity tests, and sub-chronic or chronic animal feeding studies. Chronic studies reporting adverse effects are evaluated in greater detail than similar studies in which minor or no effects were observed[349; 356; 357]. The relationship between toxic potential and exposure is evaluated by comparing the estimated daily *per capita* intake to the NOAEL reported in an adequate sub-chronic or chronic feeding study, and multiplied by a uncertainty factor (usually 100 to account for toxicodynamic and toxicokinetic differences amongst and between species[394]), for either the flavour ingredient or a structurally related substance[278].

It is important to note that the extrapolation of high dose animal studies to low dose human exposure is potentially problematic in the case of flavouring substances which are usually consumed at very low levels. Metabolism and toxicokinetics may be

significantly different, other sources of error may include: nutritional effects (such as palatability, digestion and absorption); biochemical effects (such as enzyme inhibition / induction or co-factor depletion); physiological effects (such as hormonal, organ or cellular functioning); distribution effects (due to plasma / tissue protein binding); excretion effects (due to saturation of capacity); and vascular effects (leading to decreased distribution and clearance)[351].

There are instances when, even though some information on safety and metabolic fate is available for other members of the chemical group, FEXPAN conclude that some toxicological data should be obtained. The classical acute LD_{50} is no longer used, and instead, a Primary Toxicity Screen is employed. This calls for a 14-day feeding study in rats at a level 1000 times the daily *per capita* intake calculated in mg/kg body weight / day[357]. No substance is administered to rats at a daily level of less than 10 mg/kg body weight. If no adverse effects are observed, the Panel may consider the substance safe for human consumption at the proposed level.

In cases where adequate toxicity data is not available, the history of use is considered of primary importance[278]. Although several general principles developed by FEXPAN are also valuable when used with caution[355]:

- The toxicity of a member of a homologous series of compounds, can be predicted from the toxicity of the immediately adjacent congeners.
- The introduction of double or triple bonds in an homologous series of compounds, increases the toxicity.
- Increasing the molecular weight tends to decrease the toxicity.
- The functional group or groups of a compound determines not only the nature of toxic action, but also the structure of bio-transformation products.

Considerations of these criteria, along with the low levels of exposure to the compounds led to the definition of Toxicologically Insignificant Usage (TIU). TIU signifies simultaneous fulfilment of all of the following criteria[357]:

- Use for at least 10 years by more than one company.
- Average maximum use level in any finished food not in excess of 10 ppm.
- Annual national consumption of less than 1000 lb.
- Absence of any unfavourable indications from the structure, composition, or experience in use, which would cast doubt upon safety.

In an effort to produce a more appropriate designation of TIU, FEXPAN adopted the term 'priority low – usage minimum' (PLUM), which relates the maximum intake of a flavouring substance to both food consumption and average use levels in each food category. The PLUM designation requires that the substance must be[357]:

- Of simple structure and of known composition and purity.
- Its structure must suggest that it will be readily handled through known metabolic pathways and excreted without toxic effect.
- It must be a member of a structurally related group that, without known exception, is or can be presumed to be of low toxicity.
- It must not be used at levels that would result in an average daily intake of more than 2.0 mg.

The first of the publications dealing with new flavouring substances appeared in 1970[395]. In 1962, at the request of the FDA, FEXPAN began to review botanically derived flavouring substances[355], which constitute often complex mixtures of several compounds. To this end, a set of criteria were developed for use alongside the normal assessment process for this class of flavours. Substances were required to[355]:

- Have been in use by at least two firms, one of which must have used it for at least 20 years, and there must have been at least 40 'firm-years' (number of firms multiplied by the number of years of common use).
- Be used in a range of food categories, presumably resulting in relatively wide consumption or in specific products widely used.
- Be absent of any information suggesting possible hazard under the conditions of use.

A.4.3.6 Mode of Administration

For the purposes of GRAS assessment, the most relevant mode of administration of flavour ingredients is that which mimics human exposure, ingestion in the diet. Some flavour ingredients are sensitive to the acidic and basic conditions of the alimentary canal, and thus undergo spontaneous rearrangement. Enzymes in the gastric juices, intestinal fluid and intestinal mucosa may catalyse hydrolysis, and in addition, intestinal flora may provide a source for active reduction. Intravenous, inhalation and intraperitoneal routes of exposure preclude these important biochemical transformations which occur prior to absorption. As such, the results of studies in which flavour ingredients are ingested as part of a normal diet are most relevant for safety assessment. Data from studies other than dietary administration are given less weight in the GRAS assessment[278; 396].

In the absence of adequate oral studies, results of inhalation studies may be used for low molecular weight, volatile substances that exhibit similar pathways of metabolism and excretion regardless of the mode of administration. As a crude approximation, NOAELs in inhalation studies are converted into equivalent-absorbed concentrations in order to estimate a safety factor[349]. The Expert Panel emphasise that these estimates are based on many assumptions that may not be accurate and should only be considered when exposure from use as a flavour ingredient is many orders of magnitude less than exposure in inhalation studies[278].

A.4.3.7 Relevance of Other Toxicity Studies

In addition to sub-chronic and chronic toxicity studies, other available studies may be considered by the FEMA Expert Panel during GRAS assessment if considered to be relevant[355]. For example, genotoxicity tests have been performed for a wide variety of flavour ingredients, and some reproductive toxicity information is available[349; 397; 398]. These tests, although not required, are considered useful by the Expert Panel for interpreting adverse effects in chronic feeding or carcinogenicity studies[278; 385]. In general it is considered by FEXPAN that in respect of *in vitro* studies, the effects observed for high concentrations of a substance suspended in an artificial cellular environment have limited relevance to the human consumption of low concentrations

of flavour ingredients. The interpretation of reproductive studies takes into consideration factors such as: dose-response information; the relationship of the minimum effective dose to the typically low exposure to flavour ingredients; the relevance of findings at maternally toxic dose levels to human exposure levels; and pharmacokinetic and metabolic differences between experimental animals and humans[357].

A.5 International Safety Assessment

The World Health Organisation (WHO) activities concerned with the safety assessment of food chemicals were, in 1980, incorporated into the International Programme on Chemical Safety (IPCS; a co-operative organisation sponsored by the International Labour Organisation, the United Nations Environment Programme and WHO). Within this structure, the Joint Food and Agriculture Organisation (FAO) / WHO Expert Committee on Food Additives (JECFA), was established in 1955 to report to the *Codex Alimentarius* Commission and provide a unique assessment of food chemicals, including food additives, food contaminants, and in some cases residues of veterinary drugs[250; 380]. JECFA is purely advisory and has no formal regulatory function.

A number of other organisations have elaborated criteria toward the safety assessment of flavouring compounds, these include: the Committee of Experts on Flavouring Substances of the Council of Europe; the Commission of the European Communities' Scientific Committee for Food; and BIBRA International[5]. However, JECFA maintain the most active role in safety evaluation.

JECFA recommendations are based upon scientific judgement and safety assessment models which are widely accepted. Through reports, toxicological monographs and profiles of chemical specifications, national food regulatory authorities and the *Codex Alimentarius* Commission are provided with the necessary elements for making decisions on the rational use of chemicals in food.

The tenth meeting of JECFA in 1967 recommended that '*further meetings should be convened to draw up specifications for flavouring substances used as food additives to evaluate the toxicological hazards involved in their use*'[399], this being further stated during the eleventh meeting in 1968[400] and re-iterated during fifteen subsequent meetings over a period of 20 years [from the 15th meeting in 1972 to the 39th meeting in 1992 (meeting reports have continued to be published by the WHO in its Technical Report Series)]. Furthermore it has been suggested that a working group be established in order to help evaluate flavouring additives[5]. However, despite the safety assessment of a limited number of flavouring chemicals by JECFA and assignment of full and temporary 'acceptable daily intake' (ADI) values since 1968, little was achieved towards formalising an assessment process until 1995[401].

A.5.1 The JECFA Safety Assessment Of Flavouring Substances

JECFA have stated that much of the difficulty in the safety assessment of flavouring compounds arises from the large number of substances marketed, many of which occur in natural products, however their level of use is generally low and self-limiting. JECFA classify flavouring compounds into four groups[402]:

1. Artificial substances unlikely to occur naturally in food.
2. Natural materials not normally consumed as food, their derived products, and the equivalent nature-identical flavourings.
3. Herbs and spices, their derived products, and the equivalent nature-identical flavourings.
4. Natural flavouring substances obtained from vegetable and animal products and normally consumed as food whether processed or not, and their synthetic equivalents.

Like the US FDA, JECFA recognise that although most flavouring compounds have not been subjected to detailed toxicity tests, long histories of use may be available, including possible adverse reactions[402]. However, it became clear that such 'histories of use' may not enable an adequate measure of safety. For example, oil of sassafras had an extended history of use before it was discovered to be hepatotoxic and carcinogenic[402]. As such, at the twentieth meeting of JECFA in 1976[403], it was

recognised that the safety assessment of food flavouring compounds should be similar for that of other food additives. However the Committee also concluded that the evaluation should be flexible and could be conducted simply from 'available data' thus negating the requirement for extensive toxicity testing. For example, in 1987 JECFA concluded that[402]:

> *'A substantial number of esters used as food flavouring agents would warrant the allocation of a low priority for testing and acceptance for an ADI on the basis of metabolic studies alone. This would be true in cases in which flavouring compounds are shown to be rapidly hydrolysed to toxicologically known alcohols and acids, the safety of which have been known.'*

ADI values covering a range of compounds in a structurally related group are often allocated in this way[133] (with metabolic predictions for one compound being based upon data available for another). However, other factors such as the general lack of data concerning the hydrolysis of ester-type compounds, are currently resulting in increased caution in applying group ADI values (this was the main concern which lead to the instigation of the research project of N.R. Buck). It is interesting to note that since the initial allocation of a group ADI for the antioxidant esters propyl, octyl and dodecyl galate, the individual esters have been found to illicit significantly different toxicities[404].

The use of short-term mutagenicity and clastogenicity tests for the evaluation of flavouring compounds is not intended as a substitute for more exhaustive procedures, but merely as additional information upon which to base safety determinations[402]. Similarly, experience and information concerning human usage is intended to add weight to the safety assessment process[133]. In effect, as with the GRAS system, a number of criteria are used to collectively add proof to the safety of flavouring compounds, and thus avoid to a large extent the requirement for more exhaustive and expensive studies.

Despite the fundamental difference of JECFA's advisory role and the regulatory powers enabling FEXPAN in North America, and the general paradigms that JECFA

aim to assess compounds for *evidence of safety* whilst FEXPAN assess often for *lack of evidence of hazard*, a similar general policy towards the safety assessment of flavouring compounds has been adopted by both. The approaches taken by JECFA and FEXPAN have been further moved towards consolidation by the International Committee on Flavour Priority Setting (of *Codex Alimentarius*) which may advise JECFA on the prioritisation of safety assessment[5].

A.5.1.1 Prioritisation of Safety Assessment

JECFA determined that due to the large number of flavouring compounds, and the impracticality of extensive toxicological evaluations, compounds should be prioritised for safety evaluation and the extent of toxicity testing required. It is not the role of JECFA to assign such priorities to compounds, JECFA perform safety assessments upon substances on the advice of the *Codex Alimentarius* Commission and others. However, JECFA have made recommendations upon the prioritisation of compounds for safety assessment based on methodologies employed by FEMA and the FDA[10], and others have proposed mechanisms[3; 391]. Such prioritisation is based upon: short-term mutagenicity tests; previous human exposure; current and future exposure levels; and investigated and predicted metabolism and toxicity of the substance (for example from structure / activity relationships)[133; 391]. For example, where the chemical structure of a simple ester-type flavouring compound has been shown to be rapidly hydrolysed to its constituent acid and alcohol moieties, a low priority for testing may be established[5].

In considering the extent of exposure, JECFA determined that substances with an estimated *per capita* consumption exceeding 3.65 mg / annum (10 μg/person/day), and / or in use in food at a level higher than 10 mg / kg, should in the light of the other available information (such as toxicity studies and history of use), be given priority status[402]. In the absence of other available information, a combination of the exposure and chemical structure / activity relationships are used to establish priority status[391]. Priority setting is intended to be based upon summary information and is not intended to replace the second step of risk assessment proper[10].

A.5.1.2 The Integrated Decision Tree Safety Evaluation Procedure

In 1978 G.M. Cramer and R.A. Ford published the 'decision tree' approach for the estimation of the toxic hazard of substances[390]. This methodology was subsequently altered (principally by the inclusion of exposure data) and adopted by the US FDA for safety evaluation purposes in it's 'redbook'[5]. The technique is an effort to aid the risk assessment of the large number of chemical substances to which humans are exposed, and for which little safety information exists.

The decision tree consists of a number of branches in which questions are asked to which a positive or negative response leads to a further question. Finally, the compound being evaluated is identified as being in either 'class of concern' I, II or III. The construction of the tree is formulated around known metabolic pathways and toxicity; and the questions are based mainly upon the compound's chemical structure, although knowledge of the compound's occurrence in nature, body fluids and tissues is also required. The final classifications represent:

Class I : Substances of simple chemical structure and efficient modes of metabolism which would suggest a low order of oral toxicity.

Class II : Substances that are in a structural class in which there is lack of sufficient knowledge on the metabolism, pharmacology and toxicology for the class in general, but for which there is no clear indication of toxicity.

Class III : Substances of a chemical structure that permit no strong initial presumption of safety, or may even suggest significant toxicity.

During the thirty third JECFA meeting[405], these structural class criteria were reviewed as a means of ranking flavouring substances in terms of levels of general concern over potential inherent toxicity.

In 1994, I.C. Munro produced a proposal for JECFA describing a formalised procedure for the safety evaluation of flavouring substances[3], see figure A.2. This proposal was discussed during the forty forth JECFA meeting in 1995[401], further modified during the forty sixth[406] and forty ninth[250] meetings and published in-part in

1998[5]. The procedure has, to-date, been utilised by JECFA for the safety assessment of a number of flavouring compounds. Included in the procedure is the concept of a *threshold of toxicological concern* for each of the three structural classes above[5; 407].

The threshold of toxicological concern is an intake value which was derived from the examination and interpretation of a large database of compounds. Each compound was assigned to it's structural class and the upper 95th percentile value from the combined No-Adverse-Effect-Levels for all the compounds within each structural class was determined. From this, the thresholds of concern were calculated for a 60kg individual taking into account a 100 fold safety factor (to account for inter-species and inter-individual variation). Therefore, the threshold of concern, for each structural class, is intended to be an exposure value which is reasonably certain to be lower than the exposure to humans which would result in any adverse effects from any compound determined to be within that structural class. There is currently a considerable amount of work being undertaken to evaluate the concept of thresholds of toxicological concern for wider application to chemicals which are present in the diet at low levels and for which limited toxicological data is available[408].

A further *threshold of concern* is employed in the JECFA safety assessment scheme[250], a threshold of 1.5 μg/day (figure A.2 step B5), this is intended to reduce to acceptable levels any possible risks from carcinogenic compounds. This value has been calculated from a carcinogen potency database of approximately 3700 long-term animal studies of 975 chemicals[3]. The value represents an estimate, with considerable confidence, that a substance which has not been tested for carcinogenicity and that is consumed in an amount below this threshold, will not present a risk of human cancer greater than one in one million.

In summary, the recently implemented JECFA procedure for the safety assessment of flavouring compounds is based upon a structured framework which enables and focuses the work of experts in applying their judgement. The assumptions made within the scheme are conservative and based soundly upon real toxicological data from a large number of diverse compounds. The procedure is subsequent to the decision tree of Cramer *et al*[390] and includes the concept of thresholds of toxicological

concern[5]. In essence, the procedure combines metabolic, toxicological and exposure criteria to determine the actions or outcomes for the safety assessment of a particular compound. Flavouring compounds are often evaluated in groups, taking into account combined concentrations of predicted common metabolites, however the right to use alternative assessment approaches has been maintained.

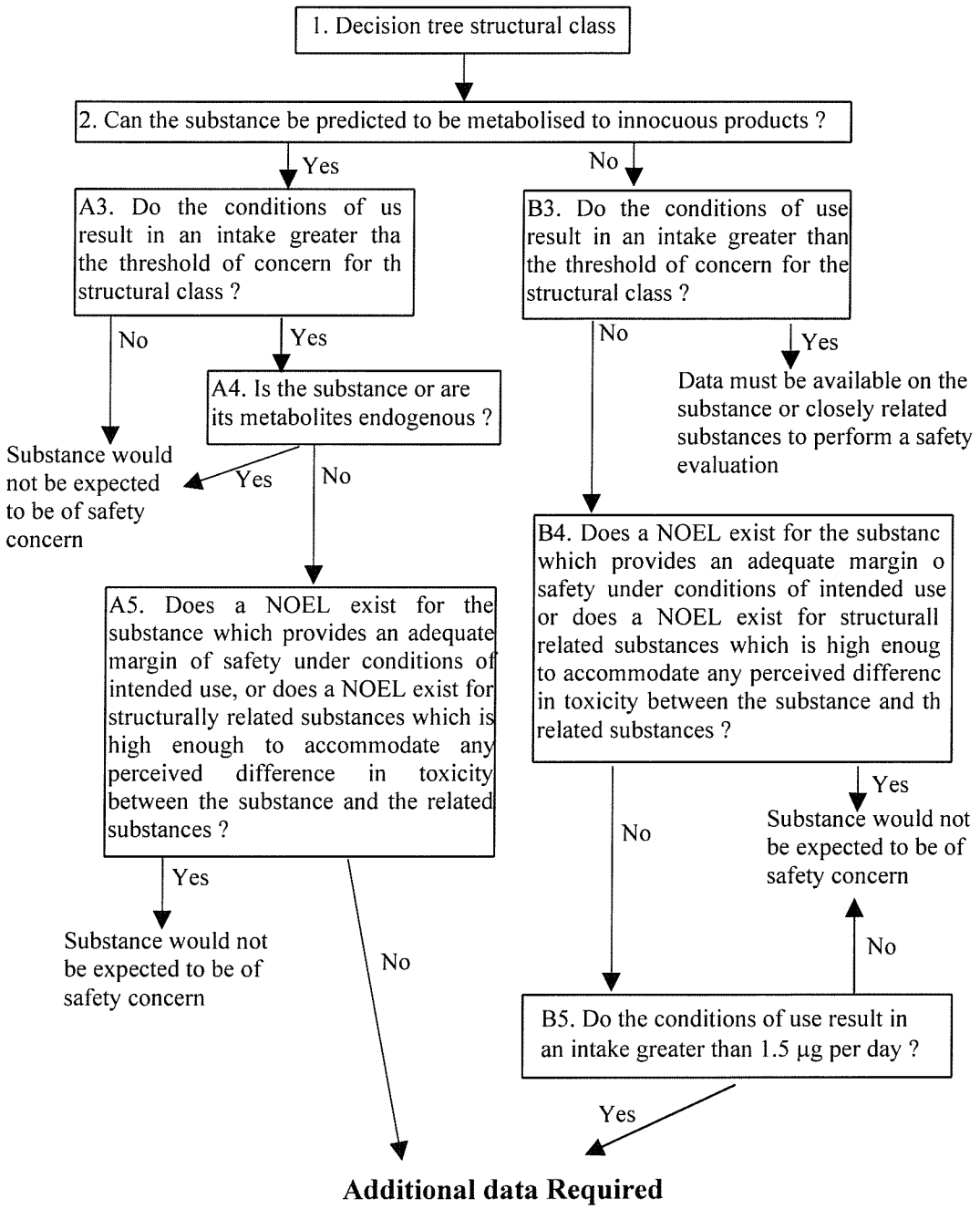


Figure A.2: The JECFA safety evaluation procedure for food flavouring compounds[341].

A.6 Conclusions

Food flavouring additives present a unique challenge to organisations tasked with their safety evaluation. Nearly 2000 are currently present in the market and for most little toxicological data are available (and it is usually not economically viable to instigate toxicological tests for individual flavouring compounds). For these reasons it is clear that flavouring additives cannot be evaluated via protocols designed to assess the safety of other types of food additives. However, most of the compounds are known to be consumed as natural components of the diet, they often have extended histories of use as food additives and are consumed in limited quantities. Consequently, safety evaluation protocols have evolved within national and international expert organisations which best utilise the available data within a framework of the current understanding of the disciplines of xenobiotic metabolism and toxicity. Work which strengthens this framework, such as that presented in this Ph.D. Thesis Report, is continuous and increases the knowledge available to make the predictions necessary to substitute for the lack of data concerning individual food flavouring additives.

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