QSAR STUDIES OF SURFACTANT TOXICITY TO *DAPHNIA MAGNA*

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Amendment 1:

ABSTRACT

The inherent nature of surfactants to aggregate at surfaces makes measurement of log P (octanol / water partition coefficient) for these substances extremely difficult. It is possible, however, to calculate a log P descriptor based on the method described by Hansch and Leo (1979).

Work presented in this thesis describes the study of the acute toxicity of sulphonated esters (FAES) of general formula R-CH($SO_3^-Na^+$)-CO_2-R' to *Daphnia magna*. Due to structural similarities of this class of anionic surfactant to linear alkylbenzene sulphonate (LAS), it was considered that the log P based QSAR originally developed to describe the toxicity of LAS to *D. magna* (Roberts, 1989) also would be a good predictor of the acute toxicity for FAES substances.

Results of the toxicity studies showed that FAES substances were less toxic than predicted. However, when plotted against log P calculated using the conventional fragment approach of Hansch and Leo with the addition of a position dependent branching factor (PDBF) to account for water sharing between hydrocarbon chains, the regression slope was parallel to but distinct from that of LAS. This indicated that either FAES substances were not acting as by the same mode of action as LAS or that modification of the log P calculation was required.

Further studies of the toxicity of binary mixtures of FAES with known polar and non-polar narcotics, established that FAES exhibited concentration addition with LAS and phenol. This indicated that they behaved with a similar mode of action and it would be expected that LAS and FAES would share the same QSAR. The difference of the regression slopes of FAES and LAS observed earlier, therefore, suggested the requirement of a modification to the original log P calculation.

The modified proximity factor developed in this thesis considers the effects of relative size of proximal polar fragments on log P. Spherical hydration sheaths surrounding each fragment were assumed and overlapping volumes calculated for fragments at different carbon separation. When incorporated into the log P calculation, the new log P values now allow toxicity values for LAS and FAES substances to be incorporated into the same QSAR.

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DAPHNIA MAGNA, SURFACTANTS AND QSARS

1.1 INTRODUCTION

It is estimated that anything from 70000 up to 100000 synthetic substances are in everyday use with thousands of new substances being added to the list each year. Many substances are released into the environment as a result of manufacture, distribution and disposal. The complex mixture of toxic substances potentially present in waste water and the environment is now being seen as an ever increasing problem (Blum and Speece, 1990; Hedgecott, 1994). There is concern that biological communities exposed to such mixtures may be affected as a result of acute and chronic exposure to such substances.

Concurrent with increased understanding of the importance of invertebrates to the functioning of aquatic ecosystems, the use of ecotoxicology in water quality assessment and management has greatly increased over the last few years. Toxicity tests are used as an indication of the effects of substances on biota, comparing the sensitivity of different species exposed to these substances. They are used in the determination of risk when discharging substances into the environment and formulating legislation on these discharges.

The assessment of risk of toxic effects is essentially a series of extrapolations each with associated variance (Suter *et al.*, 1985). For a comprehensive assessment it is necessary to assess possible effects on a wide range of species, chronic toxicity, life-cycle toxicity and changes in population size due to direct effects and due to the combined direct and indirect effects. Thus toxicity tests need to be predictive and reproducible to obtain reliable parameters for regulatory purposes. Test methodologies have been largely standardised to improve reliability of reproducibility of test results on both an intra- and interlaboratory level by such organisations as the EPA (US Environmental

Protection Agency), the ASTM (American Society for Testing and Materials), OECD (Organisation for Economic Co-operation and Development), the EU (European Union) and the ISO (International Organisation for Standardisation). The most discrete of measurable toxic responses is mortality which is usually standardised in aquatic toxicological studies as LC50. This refers to the concentration which is lethal to 50% of a population in a test system in a given time. To aid reproducibility, standardisation extends to cover not only the performing of tests but culturing techniques of the test organisms, analytical procedures etc. when appropriate.

Of the limited number of test species used in routine toxicity tests, one of the most widely used is *Daphnia magna* Straus (OECD, 1993). The use of *D. magna* as a test species has been encouraged as it is a wide ranging species, is relatively easily cultured in the laboratory, has high fecundity and reproduces parthenogenically. The wide use of this organism now generates large numbers of data which can be used to compare, under favourable conditions, the inherent toxicity of large numbers of substances. Commonly this data takes the form of EC50 values which refers to the concentration of test substance causing immobility in 50% of the test organisms. Data obtained from the standard *D. magna* acute toxicity test can be highly reproducible providing the standardised guidelines such as the EEC Commission Directive 92/69/EEC and OECD (1993) Guidelines, section 2, guideline 202, for production of the data are followed.

Despite the large numbers of accumulated data, however, there is no information on the toxic impacts of an estimated 79% of commercial substances. (Blum and Speece, 1990). With the ever increasing number of substances and their by-products there are large and expanding gaps in the data base and it is clearly an impossible task to evaluate the toxicity of every substance to every test species on an experimental basis. It is, therefore, necessary to develop alternative methods / tools for the assessment of toxicity. One such technique uses QSARs (Quantitative Structure Activity Relationships). QSARs have been developed to predict the toxicity of substances using physical and chemical

descriptors such as K_{ow} (octanol / water partition coefficient). These descriptors are obtained empirically or by calculations based on structures. The toxicological properties of substances which share common features can be seen to vary predictably in relation to these selected descriptors. Such relationships may contribute to a better understanding of the behaviour of substances and can serve as valuable screening tools in the initial assessment of the toxicity of substances to selected organisms.

The Ecotoxicology Unit of Unilever Research, Port Sunlight Laboratory assesses the environmental acceptability of substances used in consumer cleaning products e.g. detergents. The Unit comprises Biology, Biodegradation and Analytical Chemistry units. A key role of the Biology Unit is to assess the toxicity of detergent ingredients to aquatic organisms including *D. magna.* The research reported in this thesis was undertaken in conjunction with and using facilities at the Biology Unit of the Ecotoxicology Section at Unilever Research Port Sunlight Laboratory.

There are a large number of available acute toxicity data for surfactants but few QSARs which correlate these data with physicochemical parameters. The initial aim of this research was to develop a better understanding of the relationship between the toxicity of one class of anionic surfactant, namely fatty acid ester sulphonates (FAES) to *D. magna* and certain physicochemical parameters. Particular consideration was given to log P (octanol / water partition coefficient) since toxicity of surfactants is often directly proportional to hydrophobicity (Hermens, 1984; Könemann and Musch, 1981; Veith *et al.*, 1983).

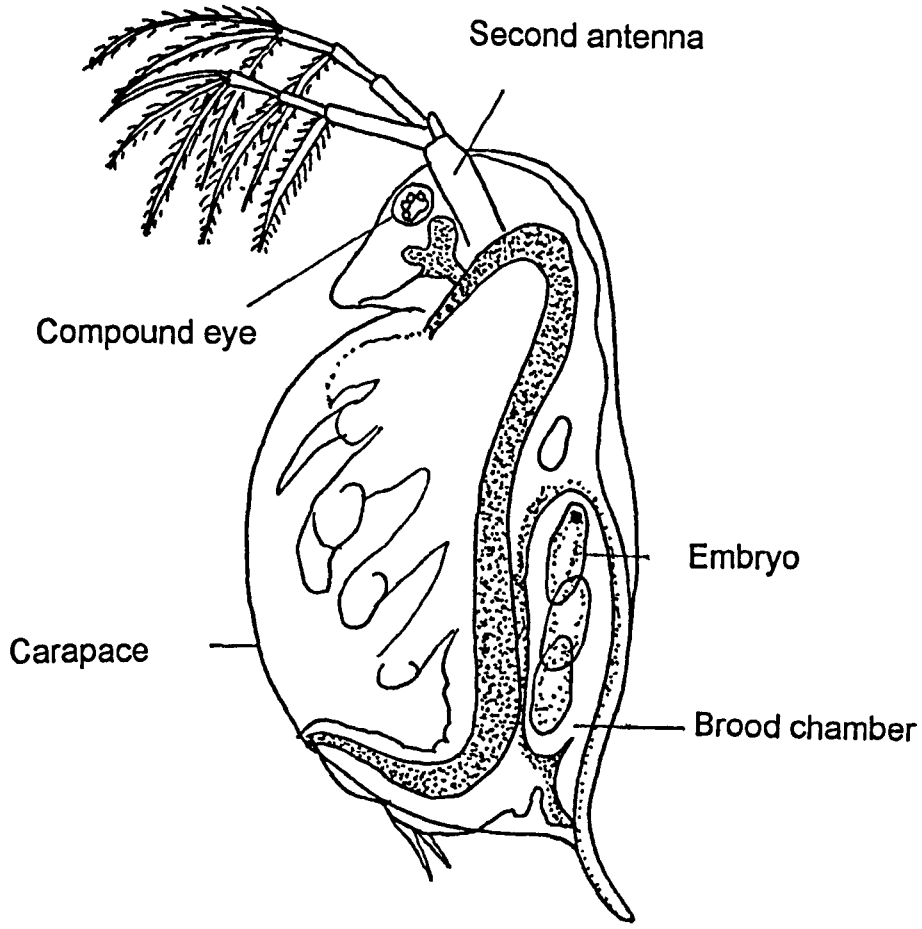
1.2 THE GENERAL BIOLOGY OF *DAPHNIA MAGNA* (STRAUS) (*CRUSTACEA, DAPHNIDAE*)

There are more than 42,000 known species of the sub-phylum *Crustacea* which include familiar arthropods such as crabs and lobsters. The crustaceans are primarily aquatic, the majority being marine although there are many freshwater species (Barnes,1987). The genus *Daphnia* includes some 50 species and occurs world-wide. There is sub-generic division which approximately subdivides the globe with *Ctenodaphnia* predominating in southern continents and *Daphnia* being mainly Holarctic (Hebert,1978).

The key to crustacean classification lies in the distribution and modification of the limbs and the presence of a carapace covering the thorax. Appendages are typically biramous. The limbs possess a basal joint, the protopod, from which arise two branches, the inner endopod and an outer exopod, each of which may consist of one to many segments. There are innumerable variations on this basic plan. Sometimes one branch is lost and the appendage becomes uniramous. In branchiopods all the diverse groups are characterised by trunk appendages which have a flattened, leaflike structure, the exopod and endopod each consisting of a single flattened lobe bearing setae. The trunk appendages are used in gas exchange and are usually adapted for suspension feeding and commonly for locomotion as in the case of *D. magna*. Along with other 'water fleas' the species is characterised by a bivalved carapace which encloses the trunk but not the head and terminates in a spine. It is the largest herbivorous cladoceran in the northern hemisphere and the adult can grow to 6mm in length. The single compound eye, although sessile, can be rotated and used partially to orientate the organism.

Branchiopods are almost entirely fresh water dwellers. Whilst there are many cladocerans with a benthic or near benthic existence, *D. magna* is a pelagic, mobile species which swims by downward strokes of the enlarged secondantennae, resulting in a largely vertical motion. The antennae also allow

Fig. 1. Schematic diagram of *Daphnia magna* (Magnification x 24)



slow descent, acting in the manner of a parachute whilst plumose setae on the abdomen act to stabilise movement.

Thoracic appendages carrying large filter-like screens constitute the feeding apparatus. Water, containing food particles, is pumped from head to tail through the gape in the carapace. The filtered particles are a heterogeneous mixture of algae, bacteria, protozoans and detritus. Feeding is inefficient at low and high food densities as either large volumes of water are required to be filtered to collect sufficient food or more food is collected than can be ingested respectively (McMahon, 1965; Enserink, 1995). Feeding is also affected by temperature and particle size (McMahon, 1965; Wulff, 1980).

Under non stressed laboratory conditions with a temperature of *ca.* 20°C with a controlled photoperiod and abundant food, the parthenogenic life cycle of *D. magna* can be expected to begin with egg development in 6 to 7 days. At 9 to 12 days, after a succession of 5 to 6 moults, the progeny, called neonates, are released as free swimming adults. Successive broods of genetically identical females are then released every 3 to 4 days followed by a moult of the carapace to allow growth of the adult. Growth of the individual continues in this fashion to mortality after *ca.* 40 - 50 days and reproduction continues until senescence. Under certain conditions of environmental stress such as low temperature or reduced food availability the appearance of males and fertilised eggs can occur in the population. The eggs are large and only two are produced per clutch. The walls of the brood chamber are now converted into a protective capsule called ephippia. These resting eggs are capable of floating, sinking and adhering to surfaces, are resistant to desiccation and freezing and will survive the passage through the gut of predators. By such means the population survives periods of environmental stress.

Most species in the genus *Daphnia* have a wide range and are found in lentic habitats varying in size from small ponds and freshwater rock pools to large freshwater lakes; some habitats containing more than one *Daphnia* species, particularly in temperate regions (Blazka, 1966; Ganning, 1971; Hall, 1964;

Hebert, 1978; Hrbácková-Esslová,1965; Jones *et al.*, 1979; Wright, 1965; Wulff,1980). In temperate North American and European lakes there are generally more species present in lakes than ponds, where two to three species are often present whilst ponds contain one or two species (Hebert, 1978).

Both interspecific competition and predation can cause drastic cladoceran population decline in its natural habitat although it is probably predation which is the most important limitation of population growth. *D. magna* is a large species which in a deoxygenated environment shows a manifold increase in the haemoglobin content of its blood (Fox *et al.*, 1951). This results in a marked change in colour and this, combined with its size, makes it vulnerable to fish predation (Koivisto,1995). As a result *D. magna* is rarely found in fish inhabited environments and tends to be restricted to smaller lakes, ponds and pools (Hebert, 1978).

Such environments are unpredictable and populations are subject to wide fluctuations in pH, temperature, oxygen concentration, salinity and other abiotic factors (Ganning, 1971). *D. magna* is, however, reasonably well adapted to survive such fluctuations, being tolerant to low oxygen levels, high pH, wide ranges in temperature and, under extreme stress, produces ephippia.

Investigations into the dynamics of natural daphnid populations have dealt largely with those inhabiting temperate areas of North America and Europe where populations are re-established from ephippia or else are capable of overwintering. These natural populations tend to reach a peak density in late spring / early summer of 20-100 adults per litre and peak again in late autumn, or alternatively the population stabilises after the initial spring peak (Hebert, 1978; Wulff, 1980; Hall, 1964). In either case there is a marked difference between the low density spring population with large brood size and the higher density summer population with lower egg production. These differences can be ascribed to the effects of temperature and the availability and type of food supply (Lewis and Maki, 1981; Wulff, 1980). Temperature affects the frequency of moulting and hence the frequency at which neonates are produced; observations supported by laboratory

studies (Hall, 1964; Hebert, 1978; Korinek, 1966; Wulff,1980; Wright, 1965). Feed level is strongly implicated in egg production (Hebert, 1978; Hall, 1964; Wright, 1965; Jones *et al.*, 1979). Both factors are highly important in egg production control; the particular environment in which the population exists determining which is more important. Other environmental factors such as oxygen concentration and pH affect egg production but it is likely that the effects are due to altering of the feeding rate and thus of food uptake (Fox *et al.*, 1951).

1.3 SURFACTANTS AND SURFACE ACTIVITY

1.3.1 HISTORICAL REVIEW

Surfactants are widely used in large tonnage in both in both domestic and industrial applications.

Historically the oldest man-made surfactant is soap which was already in use for the washing of clothes and for medicinal purposes in 2500 BC by the Sumerians. Soap became used in cosmetic application by the Gauls and Germans, a use later adopted by the Romans, and it was not until the 2nd century AD that the cleaning properties of soap were reintroduced by the Greeks. A soap is a carboxylic acid salt possessing surface active properties, formed from the reaction of fatty acids with an alkali metal or organic base. The first generally recognised attempt at a synthetic soap substitute is the alkylation and sulphonation of naphthalene by Günther of BASF in 1917 (Falbé, 1987). This produced a strongly wetting and foaming agent but as the short chain compound it lacked sufficient detergency satisfactorily to replace soap.

Synthesis of a castor oil fatty acid ester sulphonate by esterification with short chain alcohols of castor oil fatty acid, and sulphonation of the ester by Bertsch produced a compound with similar properties to the alkylnaphthalene sulphonates. It was not until 1928, however, with the sulphonation of fatty alcohols, that the first synthetic soap substitute with sufficient detergency properties was successfully

synthesised. However, this was not an economically viable route for larger scale production and it was not until the availability of low cost fatty alcohols produced from the reduction of fatty acid esters that low cost fatty alcohol sulphates were brought onto the market in 1932 by Henkel and in 1933 by Procter & Gamble (Falbé, 1987).

By the 1950s soap had virtually been replaced as the surfaces-active component in detergents, mostly by tetrapropylenebenzene sulphonate (TPS). TPS proved to be inadequately biodegradable and began to be replaced in the 1960s by linear alkylbenzene sulphonate (LAS) which today is the most widely used surfactant.

1.3.2 BEHAVIOUR OF SURFACTANTS AND GENERAL STRUCTURE

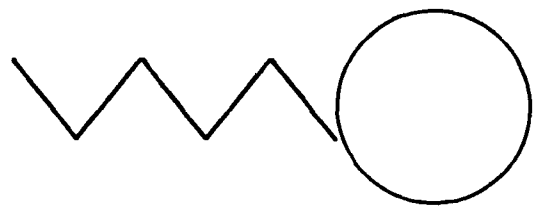
Surface activity is a phenomenon which arises from a non-symmetrical distribution of attractive forces in a surface layer. Molecules are attracted inwards away from the surface which, in liquids, results in contraction (Rosen, 1989). In terms of energy, the amount of work required to create the interface is called the interfacial free energy. Thus any measurement of surface tension is actually a measure of the interfacial free energy per unit area of the boundary between the liquid and air phases. Soluble substances which, when dissolved at low concentrations in the liquid phase, significantly alter the interfacial free energy are called surface-active agents (surfactants).

Surfactants are, therefore, materials which have a tendency to accumulate at surfaces and alter the properties of those surfaces by their presence. Usually they are active at interfaces in either solid / liquid, liquid / liquid or gas / liquid systems. Whilst the liquid phase may be any solvent, due to the widespread use of surfactants in natural, industrial and domestic situations, that of greatest interest is water.

Surfactants are amphiphilic molecules which are characterised by the presence of both a polar and a nonpolar moiety (Fig. 2). In aqueous solutions these are hydrophilic and hydrophobic regions. The hydrophobic region is commonly a flexible linear or branched hydrocarbon chain although there are a large number of surfactants which incorporate aromatic groups. The hydrophilic region consists of ionic or strongly polar groups which may carry either a positive or negative charge.

Detergents are products or formulations which contain surfactants, usually between 10% and 30% as the active ingredient, proportions of polyphosphate salts known as builders, although these are becoming less common, and a number of other subsidiaries all combined to promote the detergency of the product (Swisher, 1970)

Fig. 2. The basic structure of a surfactant molecule.



Hydrophobic tail Hydrophilic head group

Two processes are involved in affecting surface activity in aqueous solutions: the effect of the solute on the structure of water and the freedom of motion of the hydrophobic groups.

Current theories on water structure tend to favour the Frank - Wen 'flickering cluster' model which suggests that water has hydrogen bonded tetrahedral structured regions and also free unbound molecules (Némethy and Scheraga, 1962). Introduction of a solution of amphiphilic molecules into the aqueous medium results in an initial disruption of the ordered hydrogen bonded region. In the case of the hydrophobic region of the molecule there is usually no possibility of hydrogen bonding with water to compensate for bond disruption, and proton spin relaxation and elastic neutron scattering suggest that water molecules around the hydrocarbon chain restructure in a more ordered arrangement than the tetrahedral structured water region (Némethy and Scheraga, 1962). Hydrophobic hydration thus results in a decrease in entropy i.e. an unfavourable hydration of the hydrocarbon. The strong hydrogen bonding formed between the hydrophilic or polar moieties and water, however, compensates for the energy lost in the water disruption by this region of the molecule.

Due to the entropically favourable process of removal of the hydrophobic region of the amphiphile, the molecules aggregate at interfaces with the hydrophobic region orientated away from the aqueous phase. The presence of the hydrophilic group, however, prevents the complete expulsion of the surfactant from the solvent to form a separate layer as this would require the dehydration of the hydrophilic group. The aggregation of surfactant molecules at the surface results in displacement of water molecules. This reduces the surface tension because the intermolecular forces between the water and the non-polar regions of the surfactant molecule are weaker than those between the water molecules alone.

1.3.3 MICELLISATION

Removal of hydrophobic hydrocarbon regions of a molecule from aqueous medium results in entropy gains. As surfactant concentration increases, the interface becomes heavily crowded by molecules and small aggregations of molecules begin to form within the solution. These are termed micelles. The hydrophobic moieties align themselves and form a hydrocarbon core shielded by a surrounding hydrophilic layer.

A micelle may thus be thought of as having a liquid core formed by the hydrocarbon chains, which may contain appreciable amounts of water, with the ionised or polar heads of the molecules projecting outwards. It is the presence of the core which allows the solubilisation of non polar hydrophobic compounds which would otherwise be partially or fully insoluble.

The concentration at which the micelles appear is called the Critical Micelle Concentration (CMC) and is influenced by a number of factors. For ionic amphiphiles, increase in chain length of the unbranched hydrocarbon region reduces the CMC. This dependence on chain length (m) can be expressed as :

$\log CMC = A - Bm$	where A and B are constants for an homologous series.	Equation 1

As a general rule, with an increase in chain length of one methylene group the CMC is halved (Attwood and Florence, 1983) Above carbon chain lengths of 16 the relationship becomes invalid and further increase in chain length has no effect on CMC (Attwood and Florence, 1983). The effect of chain length is more apparent for non-ionic surfactants; the addition of one methylene group reduces CMC by two-thirds although the effect is reduced when branched chains are involved.

Other factors, such as the substitution of a phenyl or other aromatic group or substitution of CF_3 for the terminal CH_3, alter the CMC. Substitution of CF_3 has the effect of doubling the CMC, possibly by occupying positions in the micellar surface and reducing micellisation tendency.

CMC Is an important consideration when studying surfactant systems. At concentrations above the CMC, bioavailability of the compound ceases to increase appreciably, paralleled by the lack of increase in measurable soluble concentration.

1.3.4 SURFACTANT CATEGORISATION AND USES

The chemical structures of groups suitable as the hydrophilic and hydrophobic moieties of the surfactant molecule vary depending on its intended use and the nature of the solvent in which it is to be used. In aqueous solution it is usual to classify surfactants by the hydrophilic group. Four general groups are defined:

1) Anionic , where on dissociation in water the anion is the carrier of the surface-active properties, e.g. sulphonated esters ($R(SO_3^-)COOR' + M^+$), alkane sulphates ($ROSO_4^- + M^+$), alkylbenzene sulphonates ($RC_6H_4SO_3^- + M^+$).
2) Cationic, where on dissociation in water the cation is the carrier of the surface active properties, e.g. quaternary ammonium chlorides ($R_4N^+ Cl^-$).
3) Non-ionic, where no dissociation occurs in water and solubility is achieved by the presence of highly polar groups such as ethylene oxide (EO) ($-OCH_2CH_2-$) groups.
4) Amphoteric, where the molecule contains both a positive and a negative charge, e.g. alkyl sulphobetaines ($RN^+(CH_3)_2CH_2CH_2SO_3^-$). These molecules may show anionic or cationic properties depending on the pH of the solvent.

The world-wide output of surfactants in 1987 was about 15.2 million tonnes (Gillespie *et al.*, 1996). Of this over 50% was used in consumer products such as fabric washing powders / liquids, personal products, cleansing agents, and dishwasher powders. Industrially they are used extensively in the oil industry, as wetting agents in the mining industries, as dispersants in paints and lacquers, in pharmaceuticals, electronic printing and in many other applications. These uses result in discharges to the sewage system where treatment causes most to be degraded (Larson, 1990; Schöberl, 1989). Removal of all commercial anionic surfactants is high, mostly >90%, and all these substances are readily and ultimately biodegradable (Hennes-Morgan and de Oude, 1994; Maurer *et al.*, 1971; Masuda *et al.*, 1993; Painter, 1994; Steber *et al.*, 1989). However, there are small amounts which reach surface waters either via treatment plants or occasionally storm drains. Average measured concentrations in European and North American environments indicate levels of <0.04 mg/l LAS (Rapaport and Eckhoff, 1990) in rivers not highly polluted by sewage effluent. LAS concentrations can rise, however, to 1.6 mg/l in contaminated sites. Even though concentrations are generally low, since most surfactants have significant inherent toxicity to aquatic organisms they have been widely studied (Table 1). Linear alkylbenzene sulphonate (LAS), for example, is at present the most widely manufactured and applied surfactant and its toxicity to a variety of organisms has been studied extensively (Abel, 1974; Lal *et al.*, 1983; Lewis and Suprenant, 1983; Maki and Bishop, 1979; Roberts, 1991).

1.3.5 SURFACTANT AQUATIC TOXICITY

Hermens (1989) distinguishes four classes of chemicals which range from class I unreactive non-polar narcotics to class IV reactive chemicals exerting specific modes of action. Broadly speaking, toxicity can be divided into nonreactive (nonspecific) and reactive (specific) mechanisms. Reactive toxicity is that which is associated with a specific mechanism such as metabolic pathway inhibition e.g. inhibition of oxidative phosphorylation. Nonreactive toxicity on the other hand is not related to any specific reaction but is influenced by the quantity of toxicant acting

Table 1. Toxicity of some surfactants to various aquatic organisms

Surfactant	Species	Exposure (hours)	LC50 / EC50 (mg/l)	Hardness (mg/l $CaCO_3$)	Reference
C10 LAS	*D. magna*	48	30.0	120	Maki and Bishop, 1979
C12 LAS	*D. magna*	48	5.9	120	Maki and Bishop, 1979
C14 LAS	*D. magna*	48	0.68	120	Maki and Bishop, 1979
C11.8 LAS	*Gammarus* sp.	48	3.3	165	Lewis and Suprenant, 1983
4-C12 LAS	*D. magna*	48	18.0	25	Roberts, 1989
4-C12 LAS	*D. magna*	48	11.0	250	Roberts, 1989
C15-C18 ABS	*D. magna*	48	3.2	-	Shcherban, 1979
Sodium Laurate	*Oryzias latipes*	96	11	51	Onitsuka, 1989
SNP 9(EO)	*M. bahia*	48	24 - 30	90 - 130	Hall *et al.*, 1989
Lin. C13.4 AE 7(EO)	*D. magna*	48	1.3	150	Dorn *et al.*, 1993
Lin. C13.4 AE 7(EO)	*P. promelas*	96	1.6	150	Dorn *et al.*, 1993
Bra. C13.4 AE 9(EO)	*D. magna*	48	12	150	Dorn *et al.*, 1993
Bra. C13.4 AE 9(EO)	*P. promelas*	96	6	150	Dorn *et al.*, 1993
C14.5 AE 7(EO)	*D. magna*	48	0.62	120	Lewis, 1983
CTAC	*D.magna*	48	0.025 - 0.05	165	Lewis and Suprenant, 1983

Lin. : Linear, Bra. : Branched, CTAC : cetyl trimethyl ammonium chloride, SNP : sulphonated nonyl phenol, EC50 : Effective concentration causing immobility in 50% of test organisms

upon the membranes. Thus reactive toxicants are highly dependent on topological specificity often including size and shape of the molecule and nature and position of functional groups (Dearden *et al.*, 1994). These can be modelled with a range of electrical and steric parameters such as Hammett substituent constants (Dearden *et al.*, 1994; Hansch and Leo, 1979). In contrast nonreactive mechanisms are exhibited by a large array of chemicals including most surfactants.

Early explanations for nonreactive toxicity related the toxicity of chemicals to solubility in lipids (Blum and Speece, 1990). This theory was extended to assume that physiological responses not invoked by specific reactions were measurable when equilibrium was established between the concentration of the toxicant in solution and the concentration in the surface layer (Ferguson, 1939). Thus physiological effects could be related to the activity of the toxicant in solution. The activity could be estimated from the ratio of toxicant concentration to its saturation concentration and thus as solubility increased the concentration required to produce a given response also increased.

This does not always hold true, however, for compounds with lower solubility and a toxicity 'cut-off' is reached such that even in saturation the compound produces no or limited toxic effects. This may be a result of reduced chemical activity of compounds occupying large molecular volumes (Veith *et al.*, 1983). It was found, for example, when testing alcohol toxicity to fathead minnow (*Pimephales promelas*) that the relationship between mortality and saturation concentration was linear for homologues below 1-decanol and non-linear above.

Similar effects are reported for the toxicity of alkylhydroxamic acids to salmon (*Salmo salar*) fry (Addison and Coté, 1973). Increase in acute toxicity with chain length occurred up to C10 chains but not above. With the absence of precise analytical data it was concluded that the effect was due to higher chain insolubility and failure to reach high enough concentration.

Surfactants can be highly toxic to fish and aquatic invertebrates (Abel, 1974; Lal *et al.*,1983; Sloof *et al.*, 1983). Good linear relationships for increased toxicity to fish and aquatic invertebrates with progressive increase in carbon chain are well established for LAS (Gafa, 1974; Holman and Macek, 1980; Lundahl and

Cabridenc, 1978; Maki and Bishop, 1979; Roberts, 1989) and for branched alkylbenzene sulphonates (Gafa, 1974). Assessments of the nonionic surfactant linear alcohol ethoxylates (AE) show similarly that increase in hydrophobic chain length increases toxicity, but the addition of ethoxylate units (EO) decreases toxicity, an observation confirmed for the marine crustacean *Mysidopsis bahia* (Dorn *et al.*, 1993; Scott-Hall *et al.*, 1989). Reports also show that toxicity to *D. magna* and *P. promelas* is reduced by the addition of branching in the hydrophobic chain (Dorn *et al.*, 1993). This effect results from the reduction of water molecules required to solvate the hydrocarbon chains by the sharing of water molecules between the paired chains. The hydrocarbon thus becomes more hydrophilic and less lipophilic and hence less toxic.

Comparisons of the toxicities of the three main surfactant categories have shown that typically for both invertebrate and fish species cationics are most toxic followed by nonionics and then anionics (Lewis and Suprenant, 1983). Least variation in toxicity was observed for nonionics followed by anionics then cationics. Of the test species *D. magna* was typically the most sensitive. A similar conclusion was drawn in a study of alkyl benzene sulphonates (ABS) (Lal *et al.*, 1983). Unpublished work at Unilever Research, however, involving toxicity to *D. magna*, *Danio rerio* (zebra fish), and a number of algae species, indicates that algae are more sensitive to cationic surfactants than are daphnids, with fish being the least sensitive, the reverse is true for anionics. Sensitivities to alcohol ethoxylates are very similar between species.

Widely reported variations in diet, test species, test duration, water chemistry and loading density unfortunately often make comparisons of different studies difficult. Loading density (number of organisms per ml of test solution) does not appear to affect toxicity values to any significant degree as long as it does not reach a level where adsorption onto biomass reduces test solution concentration. Results from a study of surfactant toxicity to *D. magna*, for example, have shown that LC50 values determined for anionics, cationics and nonionics were similar for a range of loading densities (Lewis, 1983).

Increase in water hardness has been found to cause considerable variation in toxicity to aquatic organisms. For example, LAS has been found to have increased toxicity to fish species and *D. magna* in hard water (Holman and Macek, 1980; Roberts, 1988). Trout and goldfish appear to be markedly more sensitive to sodium lauryl sulphate in hard water than in soft. The toxicity of alcohol ethoxylates to fathead minnow seems unaffected by water hardness (Tovell *et al.*, 1974; Tovell, 1975).

The phenomenon of increased toxicity of surfactants to aquatic organisms with increasing chain length can be explained by increased hydrophobicity of longer chain homologues which increase their partitioning into lipid bilayers from the surrounding aqueous medium (Veith *et al.*, 1983). On partitioning into membranes the mode of action of surfactants is best illustrated from mammalian studies conducted mainly using anaesthetic drugs. Some of these ideas may be applicable to studies involving aquatic organisms.

Disruption of membrane structure and function is of importance in many biological effects caused by surfactants (Attwood and Florence, 1983). Studies with non-ionic surfactants have shown that degrees of solubilisation of the membrane can be obtained depending on the ratio of surfactant to membrane lipid. If only a small amount of surfactant is present then the molecules are incorporated into the membrane without any membrane disruption. At higher concentrations membranes are solubilised into micellar solution containing mixed protein-lipid-surfactant micelles with surfactant micelles and free surfactant molecules. A third stage may result if sufficient surfactant is present, with an equilibrium of protein-surfactant micelles, surfactant-lipid and surfactant micelles.

Other studies of non-ionic surfactants with kidney membranes suggest that membrane disruption involves binding of surfactant molecules to exposed polar segments of membrane proteins (Attwood and Florence, 1983). Protein binding appears to involve hydrophobic interaction of the hydrocarbon chain which leads to conformational changes in the protein molecule at low concentrations. Nonionic surfactants do not usually, however, denature proteins (Lapanje, 1978). When ionic

surfactants are involved there are obviously additional charge interactions between surfactant and biological surfaces.

Many surfactants promote permeability of membranes but the effects are complex and there is no simple explanation for them. Not all surfactants increase absorption into membranes. However, some exhibit an inhibiting effect; this is probably due to the poor ability of some surfactants to penetrate lipid membrane owing to shape factors (Attwood and Florence, 1983). Penetration of the surfactant into the lipid membrane and hence introduction of addition hydrocarbon chains would result in increased fluidity of the hydrocarbon interior of the membrane and lead to decreased passage of solutes through the membrane. Studies involving the goldfish (*Carassius auratus*) and ethoxylated nonionic surfactants show that the absorption of various barbiturates is dependent on the hydrophobic and hydrophilic chain lengths in the surfactant molecule and on its size (Attwood and Florence, 1983).

When attempting to understand the toxic effects of surfactants to fish, we must recognise that mode of penetration, uptake, metabolism and elimination of the surfactant are important. The main uptake site is the gill tissues (Tolls *et al.*, 1994). Tissue distribution studies of LAS and alkyl sulphates in goldfish have shown large proportions of surfactant associated with the gills after 6 hours exposure (Newsome *et al.*, 1995). The percentage is significantly decreased after 24 hours. The liver rapidly takes up the compound and the highest concentrations are eventually found in the gall bladder which shows a reverse trend in surfactant uptake to the gills. LAS is extensively metabolised in goldfish and little parent surfactant is secreted. The major metabolite has been identified as butyric acid-3-benzene p-sulphonate, possibly with some C6 and C8 intermediates which suggests metabolism by oxidation and formation of omega acids. Elimination of metabolites was found to be enhanced by feeding which is possibly due to the stimulation of bile secretion into the gut resulting in metabolite elimination after reabsorption from the intestine. Similar distribution and elimination patterns have been observed for alcohol ethoxylates and it would appear that anionic surfactants and alcohol ethoxylates are readily metabolised by fish to short chain, less lipophilic metabolites with relatively low toxicity to fish. Thus, apart from minor changes to the liver and kidney structure, histological damage to fish is

confined, so far as is known, to the gills and epidermis (Abel, 1974). The gill membrane shows signs of necrosis and swelling of the gill lamellae, and changes in the membrane permeability can cause asphyxiation. Reports have stressed that gill damage is at least in part non-specific (Abel and Skidmore, 1975). Alkylbenzene sulphonate damage to gills in Rainbow trout (*Oncorynchus mykiss*), for example, is reportedly concerned in part with epithelial tissues, adhesion of second lamellae and epithelium detachment (Brown *et al.*, 1968). Leucocytic infiltration, associated with epithelium detachment as in mammalian inflammatory response, has also been commonly cited as another non specific effect of surfactants (Abel and Skidmore, 1975; Brown *et al.*, 1968). Information on the biochemistry of other aquatic organisms is unfortunately scarce and it can be only speculated, by extrapolation from mammalian studies, as to what are the exact mechanisms involved in physiological effects. The most likely effect for these simpler organisms is membrane disruption, although swelling due to changes in membrane permeability causing either mechanical separation of enzymes or alterations in sodium uptake has been cited by some authors as the main cause (Albert, 1968; Blum and Speece, 1990). It is likely that there is more than one mode of action (Abel, 1974; Addison and Coté, 1973).

1.4 QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIPS

QSARs (Quantitative Structure Activity Relationships) have been developed as a technique for predicting the acute toxicity of substances using physical and chemical descriptors. Early contributions in the field were based on findings by Overton (1897) and Meyer (1899), in which partitioning of a substance between two phases could be related to narcosis in a variety of organisms including crustaceans and fish if the two phases were water and lipid (Connell, 1994) Further important developments were made by Ferguson (1939) who proposed that toxicity occurs at a constant thermodynamic activity (equation 2).

$C_r = kS_r^{1/n}$ Where C_r = Toxic concentration Equation 2.
and S_r = Solubility (mol / l) of the r^{th} member of the series

It is, however, in the last few decades, in particular the last ten or fifteen years, that there has been significant increase in the application of QSAR, largely as a response to the need for data combined with the growth in available computer facilities.

Hansch and Fujita (1964) succeeded in combining a series of parameters with a hydrophobicity substituent in the general equation :

$$\log 1/C = k_1 \log P - k_2(\log P)^2 + k_3 pKa + k_4 Es + k_5 \qquad \text{Equation 3.}$$

Where C = concentration required to produce an effect
P = partition coefficient
Ka = acid dissociation constant
Es = steric parameter
k1-k5 are constants

This provided the spur for the multiparameter approach which is now common in QSAR development where a large and increasing range of descriptors are used which cover three main molecular features (Cronin, 1991) :

1) electronic
2) steric
3) hydrophobic

1.4.1 ELECTRONIC PARAMETERS

Electronic parameters are important when the mode of action of the substance is specific, where intermolecular interaction forces control xenobiotic-receptor interactions (Dearden, 1990). A range of parameters has been developed to model these interactions which can be classified into: 1) classical substituent, 2) classical whole molecule and 3) quantum chemical parameters.

1) Classical substituent parameters include the Hammett sigma constant (σ_X) which has been used to correlate, for example, the toxicity of nitrobenzenes to fathead minnow (Roberts, 1988). The parameter assigns numerical values to the substitution of X on an aromatic ring (Dearden, 1990). Hammett (1937) defined the parameter σ as:

$$\sigma = \log K_X - \log K_H$$

where K_H is the ionisation constant for benzoic acid in water at 25°C and K_X is the ionisation constant for a meta or para derivative under the same conditions. Positive values of σ thus represent electron withdrawal from the aromatic ring, and negative values electron release to the ring, by the substituent. No consistent values could be obtained for ortho-substituents due to steric, hydrogen bonding and short range inductive effects.

The general Hammett equation is expressed as:

$$\log k_X = p\sigma + \log k_H$$

where k may be a rate or equilibrium constant for reaction centres usually shielded from interaction with the aromatic ring and p is a series constant and is a measure of the sensitivity of a reaction to the effects of substitution. The value of p is thus affected by temperature, solvent changes etc.. σ constants have now been determined in solvents other than water, with a consequent variation in values. This, in part, has led to a confusing range of σ constants for which some authors advocate an average value (Hansch and Leo, 1979).

Another of the classical substituent paramters, molar refractivity (MR), is equal to the product of the specific refractivity and the molecular weight and is given by:

$$[(n^2 - 1) / (n^2 + 2)] \times (MW / d)$$

where n is the refractive index of the substance of density d and molecular weight MW. It correlates well with electron polarisability and can be considered to be a measure of the ability of electrons to be polarised in the presence of an electric field. However, MR can be equally considered as a steric parameter as it also correlates well with van der Waals volume and other volume terms (Dearden, 1990). MR is an additive property and can readily derived be from fragment values which have been calculated for many common groups (Hansch and Leo,1979). Despite this, it has not found such wide application as log P partly because in whole animal systems, lipophilic sites are so numerous that log P effects tends to mask those of MR.

Hydrogen bonding is an important parameter, affecting processes such as solubility and receptor binding, for which it is difficult to obtain a quantitative measure (Dearden, 1990). It has been suggested by some authors that a hydrogen bonding parameter should be better able to model electronic influence of substituents on a receptor than does the Hammett constant (Dearden, 1990).

2) Classical whole molecule are widely used in QSAR as it is not always possible to derive substituent constants to model effects. These include values such as the acid dissociation constant, dipole moment and solvatochromic parameters.

The acid dissociation constant (pKa), is closely related to the Hammett constant and reflects electron-directing effects as well as controlling ionisation of substances. It is in the latter capacity that it has been used to correlate the toxicity of substances such as phenols to fish (Saarikoski and Viluksela, 1981). Values for pKa are largely determined experimentally and these should be used in preference to calculated values in QSAR analysis as they are far more accurate.

Dipole moments (μ) have been used extensively in QSAR as many drug-receptor and drug-solvent interactions require dipoles. These are generally determined experimentally with a magnitude of $\mu = qd$, where q is the charge (both the positive and negative) and d is the distance of separation of the charges (Sharp, 1983). As μ is difficult to determine and varies with solvent used, there is great interest in calculating a value from molecular orbit theory (Dearden, 1990).

Solvatochromic parameters have been found accurately to correlate many diverse chemical properties, including toxicity, which depend on solute-solvent interactions (Blum and Speece, 1990; Hickey and Passino-Reader, 1991). These

parameters are based on the assumption that such interactions are controlled by three factors, a volume term, a dipole term (π^*) and hydrogen bond donor (α) and acceptor (β) terms. However, the fact that numerical values for such parameters are available for only a limited number of substances allows them only limited application even though they have the potential to produce very accurate QSARs (Blum and Speece, 1990).

3)Quantum chemical parameters. Properties of a molecule are related to its electron distribution and behaviour and thus parameters based on quantum chemistry have been used in QSAR analyses. Such parameters include HOMO and LUMO energies and superdelocalisability.

HOMO (highest occupied molecular orbit) and LUMO (lowest unoccupied molecular orbit) energies are concerned with localised interactions. HOMO represents the ease with which an electron can be donated and thus is related to ionisation. LUMO represents the ease of acceptance of an electron. They have been widely used in correlating the toxicity of substances such as polycyclic aromatic hydrocarbons (PAHs) to aquatic organisms (Mekenyan *et al.*, 1994; van Vlaardingen, 1996).

Superdelocalisability is defined as the sum of the HOMO and LUMO densities divided by the sum of the HOMO and LUMO energies and is representative of reactivities or interactive abilities of molecules (Dearden, 1990).

1.4.2 STERIC PARAMETERS

The size and shape of molecules may be of importance in the control of biological activity by preventing receptor binding or hindering metabolism (Dearden, 1990). A large substituent group may shield a polar group and reduce a substance's hydrophilicity, and size may prevent a substances from passing through membranes. Size and shape are also highly important in receptor binding. The simplest of such descriptors are molecular weight and volume.

Molecular weight and volume have been used fairly extensively in QSAR due to their relative simplicity. Molar volume is defined as molecular weight / density. Measurements in solution are required for solids, which require extrapolation to infinite dilution. Calculation of molecular volume can involve summation of the individual van

der Waals volumes of the constituent atoms. A number of computer programs are available which can calculate a value using more realistic methods involving the rolling of a water molecule over the molecular surface (Dearden, 1990).

Van der Waals radius is suitable only for substituents and, more specifically, for near spherical substituents and is, therefore, of limited use as a parameter for use in QSAR. It can, however, be used in the calculation of atomic surface area which can be used in a summation calculation for the estimation of molecular surface area. Again there are computer programs available which provide a more accurate estimation.

The Taft steric constant (E_s) as defined by:

$$E_s = \log (k_X / k_H)$$

where k is the rate constant for the acid hydrolysis of esters of type $X\text{-}CH_2COOR$ (Hansch and Leo,1979). Unfortunately variation of this type cannot be used to obtain E_s values for many common substituents which are unstable under the conditions of acid hydrolysis. The Charton steric parameter overcomes this problem by relating E_s to van der Waals radius by:

$$E_s = -1.839 r_v \text{ (average)} + 3.484$$
$$(n = 6, r = 0.996, s = 0.132)$$

where r_v (average) is the average number of the minimum and maximum van der Waals radii estimated by Charlton (Hansch and Leo, 1979).

Shape parameters are relatively few in number and cover Sterimol parameters, Molecular Shape analysis and 3D parameters (Dearden, 1990).

The Verloop-Hoogenstraaten sterimol parameter describes the dimensions of a molecule or substituent in five directions; by length, maximum and minimum widths and two intermediate widths at right angles to the main axis of the energy-minimised molecule (Dearden, 1990; Hansch and Leo, 1979).

Molecular shape analysis derives a value for common overlap steric volume (Vo). The substances are energy minimised to determine stable conformations and a reference substance selected with which to compare the training set substances. The resulting Vo can also be used to calculate an overlap surface.

It is now possible to calculate 3D parameters using computer programs, and these may be as simple as intermolecular distances. However, as this is a fairly recent development in parameter derivation, their use has been relatively limited so far.

1.4.3 HYDROPHOBIC PARAMETERS

Hydrophobicity is the most important chemical feature of an organic molecule in determining its behaviour in aquatic systems (Donkin, 1992). For many biological effects, partition coefficient alone is sufficient to explain the toxic response to a substance. This is because it represents the tendency of a substance to partition itself between the organic phase such as membranes and the aqueous medium. The most common measure of hydrophobicity is log P (octanol / water partition coefficient), which can be measured or calculated with relative ease for a large variety of substances (Hansch and Leo, 1979). In reality any solvent which forms an immiscible phase with water and has a large solubility for hydrophobic substances can be used as a reference solvent (Valsaraj and Thibodeaux, 1990). Octanol , however, best resembles the oily fats used in the original partition coefficient work of Overton and Meyer in that it possesses a long alkyl chain and a polar group. It is also readily available in pure form and is considered a reasonable substitute for the lipoid phase (Lyman, 1982).

Often, when working with a set of derivatives of a parent compound where a large portion of the structure remains constant, knowing the relative hydrophobicity of substituents is sufficient for correlation analysis. The parameter π , analogous to σ, has been defined to work with relative hydrophobicity of substituents:

$$\pi_X = \log P_X - \log P_H$$

where P_X is the partition coefficient of the derivative and P_H is the partition coefficient of the parent compound (Hansch and Leo, 1979). A positive value of π means that the substituent favours the octanol phase relative to H. A negative value of π means that the substituent favours the aqueous phase relative to H. Inert groups such as CH_3 have a relatively constant π value whereas some other groups, such as the halogens, are more sensitive. Most sensitive are those substituents carrying a lone pair of electrons on the atom attached directly to the ring, such as NH_2.

When two substituents are placed on an aromatic ring, the mutual electronic effects of one upon the other changes the value of π for each, although one substituent is often dominant. π values applicable to the system can be estimated after a few values have been measured.

Hansch and Leo (1979) concluded that the use of π values was preferable to the fragment approach for the calculation of log P of aromatic substances and the reverse true of aliphatic substances.

1.4.3.1 LOG P

The octanol / water partition coefficient can be defined as 'the ratio of a substance's concentration in the octanol phase to its concentration in the aqueous phase of a two phase octanol / water system' (Lyman, 1982). P or K_{ow}, therefore, is dimensionless and given by :

$$P = \frac{C_o}{C_w} \qquad \text{Equation 4}$$

where C_o and C_w are concentrations of the substance in the octanol and water phases respectively when the phases are in equilibrium.

It must be stressed that this value is not the same as the ratio of a substance's solubility in octanol to its solubility in water, since at equilibrium the octanol and aqueous phases are not pure; the octanol phase contains 2.3 mol/l

water and the aqueous phase contains 4.5 x 10^{-3} mol/l octanol (Connell, 1994). Another reason it that at the sometimes high concentrations found at saturation, self-association of the solute, particularly in the non-polar phase, can affect solubility (Dearden and Bresnen, 1988). In addition, at high solute concentrations, the solvent phase can no longer be considered to be pure solvent irrespective of the mutual solubility with the other solvent (Dearden and Bresnen, 1988). Measured P values for substances which are readily soluble in lipids cover an extensive range of ten orders of magnitude. They have been measured as low as 10^{-3} and as high as 10^{8} and so it is usual to express data as logarithms; thus log P values have been measured between -3 and 8 although lipophilic substances usually fall in the range of 2 to approximately 6.

1.4.3.2 MEASUREMENT OF LOG P VALUES

OECD specify the shake-flask approach to log P evaluation. The method involves adding a small concentration of the solute to a mixture of mutually saturated octanol and water which is volume adjusted according to the expected log P. The system is shaken until equilibrium and the phases reseparated, a process usually requiring centrifugation. An appropriate analytical technique such as gas chromatography (GC) or high performance liquid chromatography (HPLC) is then used to determine solute concentrations (Chiou and Schmedding, 1982). The method does have limitations which make it unsuitable for measurement of log P > 4 or 5 (Brooke *et al.*, 1990). When the concentration of the substance in the aqueous layer is low, i.e. log P is high, small amounts of contaminant in the ~~octanol~~ octanol can lead to large errors. Glass and other surface adsorption causes problems and requires minimisation for good results and there is a tendency to form emulsions during shaking in the aqueous layer for all substances. In addition the results can be affected by contaminants in the equilibrium vessel. Modified shake-flask methods such as the stir-flask method (Brooke *et al.*, 1990), which allow equilibration without agitation, reduce the

possibility of phase contamination but the problems of substance adsorption remain.

Dearden and Bresnen (1988) recommended the use of a stir flask method, in which both phases are continuously mechanically stirred or the filter probe method which continually draws off one phase, passes it through an on-line analytical instrument and then returns it to the vessel. They also recommended constant temperature of all steps, careful choice of solute concentration to reduce or eliminate problems of self-association or phase composition, buffering for ionisable substances, sufficient time for equilibrium and complete pre-saturation of each phase.

All the methods rely upon substances separating satisfactorily into the two phases and adsorbing minimally to surfaces. Surfactants, therefore, give rise to a unique problem in the measurement of log P. The inherent nature of these substances to aggregate at interfaces prevents them from clearly separating into either phase. Even more sophisticated techniques using generator columns containing porous polymers such as Chromosorb, or measurements involving retention times in HPLC systems (Krop *et al*,.1997; Veith *et al.*, 1979) cannot escape the problems of surface activity.

Octanol / water measurement techniques for surfactants are, therefore, not a practical solution for the determination of log P. It is possible, however, to calculate a log P descriptor. Such estimation methods provide log P values calculated from either regression equations or from fragment constants (Hansch and Leo, 1979; Lyman, 1982).

1.4.3.3 CALCULATION OF LOG P

Calculation of log P from regression models is a common method for obtaining quick values. Parachor, as a measure of molar volume, has been related to P along with various other electronic and steric parameters such as hydrogen bonding, dipolarity / polarisability terms, free energy of solvation and, using more advanced regression analysis, autocorrelation vectors (Balalban and

Catana, 1994; Connell, 1992; Devillers, 1995; Kamlet *et al.*, 1988). The linear correlation between partition coefficients and aqueous solubilities is well established, such as that described by Banerjee *et al.* (1980) :

$$\log P = 5.2 - 0.68 \log S \qquad \text{Equation 5.}$$
$$(r = 0.94)$$

Where P and S are the partition coefficient and solubility respectively (no other statistics provided).

Measurement of solubility is more difficult than of log P, particularly when solubility of a substance is low, so although the correlation of log P with solubility is fairly good, due to the larger method errors involved, preference should be given to either calculation from other solvation descriptors or to the approach using fragment constants such as that developed by Hansch and Leo (1979).

The fragment approach of Hansch and Leo (1979) to estimate octanol / water partition coefficients uses empirically derived group fragments (f) and structural factors (F) and combines these in a relatively simple summation algorithm :

$$\log P = \text{fragments (f)} + \text{factors (F)} \qquad \text{Equation 6.}$$

Fragment values are available for a large number of atoms and groups of atoms. These values vary for identical fragments depending on whether the fragment is part of an aliphatic or aromatic structure, which results in a data set of over 200 fragment values. 14 structural factors exist to account for branching, unsaturation, multiple halogenation, polar fragment interactions (proximity), bonds and rotation around bonds although obviously it is not necessary to consider all of these for every substance. The method has been formalised in the 'ClogP' program developed by the Pomona Medicinal Chemistry Project.

The large data set of f and F values allows log P values to be calculated for most man-made substances and so is an extremely useful method for this purpose. Log P values for substances with large complex molecules can be calculated completely or modification of an available measured value for a structurally similar substance can be made by simple addition or subtraction of f and F values.

Errors in the method emanate from the assumption that important structural effects are adequately described by the available F values and that log P is dependent on an additive structural approach. Thus the error in the method will reflect the degree of uncertainty in the fragment and factor values. There are some substances for which these assumptions are not justified. Where the method fails there are indications that it is conformational information which is at fault (Leo, 1993). However, the assumptions do hold true for the majority of substances. The results of one set of 76 test calculations showed that the average absolute error was 0.14 log P unit. 66% of the substances had error <0.1 log P unit and 83% had error < 0.2 log P unit (Lyman, 1982). The tested substances were, however, of fairly simple structure e.g. ethanol, cyclohexane, 2-chloroethanol, and it is probable that more complex structures would show greater error.

The fragment method was pioneered by Rekker *et al.* (1977). Using a database of measured values, statistical techniques were employed to determine the average contribution of fragments. However, there is no indication as to what constitutes a fragment. For some aromatic hydrocarbon groups, for example, only combined fragments are listed e.g. C_6H_4 and C_6H_5. However, it was appreciated that a 'proximity' value was required between polar groups which were closely positioned in the molecule. The method relies upon the discretion of the operator for the breakdown of the molecule in question into its composite fragments. The outcome of an additive method should not be affected by such an operator variable, providing the correct fragment values are available. The necessary fragment values are unfortunately not always available for correct calculation of

log P particularly when considering proximity. This obviously reduces the flexibility of the method.

The Rekker method is now computerised as 'ProLogP', from CompuDrug Ltd., Budapest which removes a degree of operator error. Other computerised fragment methods are also available, such as KOWWIN, KLOGP, Σf-SYBYL and SANALOGP_ER. Some computerised methods for log P calculation use atom-based procedures, such as SMILOGP and CHEMICALC-2 and conformation-dependent procedures such as ASCLOGP. Accuracy of calculated values using these procedures varies although the fragment based approaches are generally superior to atom-based and conformation-dependent methods (Mannhold and Dross, 1996).

The limitations of such computerised methods are that they require no fundamental understanding of the calculation method and also that they have limited availability, compared to the well established manual method of Hansch and Leo, for calculation of log P values .

Surfactant structures are relatively simple and calculation of log P values should be straightforward using the Hansch and Leo method. It is a fairly accurate method combined with a relative ease of calculation and a preferred method of calculation over regression models (Lyman, 1982).

1.4.4 TOXICITY QSARS

Whilst the log P is not a perfect model for biological membranes it has proved of extensive value in QSAR development for the toxicity of surfactants, because for many non-reactive substances it is often directly proportional to hydrophobicity (Könemann and Munsch, 1981; Veith *et al*, 1983).

Most such linear relationships take a similar format (Zitko, 1975) :

$$\log 1/C = a \log P + b \qquad \text{Equation 7.}$$

Where C = active concentration causing narcosis (mol/l)

P = partition coefficient

a,b = constants

Probably the most well known log P based toxicity QSAR is that developed by Könemann (1981) for toxicity to guppies which has been widely applied for predicting baseline toxicity (see below). In its original form it was presented as :

$$\log (1/LC50) = 0.87 \log P - 4.87 \qquad \text{Equation 8.}$$

$(n = 50, r = 0.988, s = 0.237)$

As the units of toxicity were μmol / l, conversion to mol / l allows presentation of the relationship in its more commonly used form :

$$\log (1/LC50) = 0.87 \log P + 1.13 \qquad \text{Equation 9.}$$

$(n = 50, r = 0.988, s = 0.237)$

Although developed for predicting the toxicity of aliphatic and aromatic substances to guppies, the equation has been shown to predict toxicity of many non-ionic, unreactive substances which appear to have a general narcotic mode of action. This includes non-ionic surfactants (Roberts, 1991). Equations with similar slopes and intercepts have been found to apply to various fish and invertebrate species including *D. magna* (Table 2). Very few unreactive substances have been found to be significantly less toxic than predicted by equation 9 (Sloof *et al.*, 1983). Those cases which are, tend to be due to solubility or evaporation problems (Addison and Coté, 1973; Roberts, 1991). Equation 9 can, therefore, be considered to represent baseline toxicity.

The related polar narcosis mechanism (Schultz *et al.*, 1986) has been proposed to account for polar contributions to binding to membranes (Saarikoski and Viluksela, 1982):

Table 2. Regression equations for nonreactive narcosis of organic compounds to aquatic species

Species	Regression equation : log (1/C)[a]	Reference
Baseline Narcosis		
D. magna	0.91 log P +1.28	Hermens *et al.*, 1984
P. promelas	0.94 log P - 0.94 log (0.000068P + 1) +1.25	Veith *et al.*, 1983
D. rerio (Early life stage)	0.94 log P + 1.38	van Leeuwen *et al.*, 1990
14 species [b]	0.97 log P + 0.89	Sloof *et al.*, 1983
O. latipes	0.81 log P + 0.97	Ikemoto *et al.*, 1992
D. magna	0.94 log P + 0.93	Ikemoto *et al.*, 1992
Tetrahymena pyriformis	0.80 log P + 0.96	Schultz and Tichy, 1993
Polar Narcosis		
D. magna (HW)	0.70 log P + 2.23	Roberts, 1989
D. magna (SW)	0.64 log P + 2.44	Roberts, 1989
D. magna	0.56 log P + 2.79	Vehaar *et al.*, 1995

[a] : C = EC50, LC50 or IGC50 (growth inhibitory concentration)(mol/l)

[b] : average QSAR of all species (include crustaceans, molluscs and fish)

HW / SW : Hard / Soft water

$$\log (1/LC50) = 0.63 \log P + 2.52 \qquad \text{Equation 10.}$$

$(n = 17, r = 0.982, s = 0.16)$

Although the equation was developed to predict toxicity of phenols as mol/l to guppies, it has been applied successfully to predict toxicity of anionic surfactants such as LAS, PAS and alpha olefin sulphonates to *D. magna* and other aquatic species (Roberts, 1991; Unpublished Unilever data, 1991)

Various requirements must be met for a QSAR to be of use. Firstly it is valid only for substances of the same type as those used in its development i.e. the domain of the model should be defined. The definition should also cover ranges of parameters for which the model is valid. The exact endpoint being modelledshould be described as well as the details of the test method, e.g. test species, age etc., involved in obtaining the data. A derived relationship must be of sufficient accuracy for the purpose for which it is intended. The required accuracy may vary depending on the use and endpoint under consideration but correlation coefficients and overall statistics including standard deviation of predicted errors at least should be given when reporting a QSAR. Ideally the relationship should be able to cope with inherent variability in toxicity data. For wide applicability the parameters used in the QSAR should be fairly easy to obtain. When the topological descriptor molecular connectivity (MC) was used to describe the toxicity of chlorinated compounds to sheepshead minnow (*Cyprinodon variegatus*), improved correlations over log P were demonstrated (Sabljic,1983). Solvatochromic parameters are also considered to produce accurate QSARs. However, it is reasonable to assume, bearing in mind the nonspecific nature of surfactants, that log P alone can explain their physiological effects. Accuracy of log P based QSARs has been shown (Könemann and Musch, 1981; Roberts, 1991; Schultz *et al.*, 1986) and its relative ease of calculation promotes its use in surfactant QSARs.

1.4.5 QSAR APPLICATION

One criticism against the use of QSARs is that they may not be based on an understanding of the mechanisms involved in toxicity (Blum and Speece, 1990). When considering nonspecific toxicity, however, log P based QSARs offer a reasonable intuitive understanding of the properties affecting toxicity. Ultimately of course better understanding of mechanisms will help solve many toxicity questions. However, in the short term QSARs do help provide some guide to toxicity prediction. They themselves can provide insight into mechanisms. A series of substances fitting a QSAR with high correlation indicates that they may behave in a similar way; such information can then be applied to the prediction of mixture toxicity. (Hermens *et al.*, 1985; Könemann, 1981).

Biological QSARs can also aid in the development of modelling of whole ecosystems and the distribution of chemicals and mixtures of chemicals within and between the various phases of the environment (Donkin and Widdows, 1990). QSARs have thus become prolific tools for priority setting and risk assessment (Feijtel, 1995). The Toxic Substances Control Act in the U.S. and the European Commission Regulation 1488/94/EEC and the Council Regulation 793/93/EEC on the evaluation and control of the risks of existing substances has provided the impetus for the wide scale use of QSARs. Substances are ranked according to relative risk by an Informal Priority Setting method which identifies substances high on the priority list due to lack of data regarding the physico-chemical, ecotoxicological and toxicological properties of the substance. QSARs can most appropriately be applied in priority setting in two ways. Firstly they can be used to fill gaps in data sets and secondly to highlight non-valid data.

Risk assessment results at each stage of the notification process are used in decision making. QSARs provide support for such decisions regarding further testing, selection of parameters and areas of other concern. Therefore, used appropriately QSARs can save time and money and may lead to reduced experimental testing of animals in accordance with Council Directive 86/609/EEC. QSARs used in risk assessment, however, should not be the only basis for

making decisions; measured data are always preferable. Any QSAR models used in risk assessment need to be thoroughly evaluated and are considered to be acceptable for a particular use if the QSAR is valid and if the resulting estimate is sufficiently accurate for the intended use.

QSARs are thus important tools in providing data on substances where there is little or no experimental information. They provide considerable confidence in decision making and have the potential to bridge the gap between environmental and laboratory investigations of toxicology (Donkin and Widdows, 1990).

CHAPTER 2

PREDICTION OF THE ACUTE TOXICITY OF FAES TO *DAPHNIA MAGNA*

2.1 INTRODUCTION

2.1.1 FATTY ACID ESTER SULPHONATES

Fatty Acid Ester Sulphonates (FAES) are anionic surfactants of general formula :

$$R - \underset{\displaystyle SO_3^-}{\underset{|}{CH}} - COO - R'$$

They can be found in the literature under various acronyms. Most commonly they are termed MES (sulphomethyl esters) as commercially they are generally available with the terminal methyl R' group. For clarity, in this thesis FAES will be taken to mean sodium FAES and not the acid form unless otherwise stated. Where chain length is mentioned e.g. C12 methyl FAES, C12 will refer to the R chain plus the two carbon atoms leading up to and including the ester carbon and methyl will refer to the R' chain.

They represent an interesting group of oleochemical based surfactants. They are used in the far east for their excellent detergency properties in washing detergents (Masuda *et al.*, 1993; Masuda *et al.*, 1994; Stirton *et al.*, 1954) and as lime dispersing and wetting agents (Stirton *et al.*, 1965) but have found limited application in Europe and the United States. As palm oil and tallow derivatives (Pittinger *et al.*, 1993) commercial FAES usually possess C16 / C18 R chains. Whilst these longer chain molecules have reduced water solubility, they do possess enhanced detergency qualities compared to their shorter chain counterparts (Stirton *et al.*, 1954).

Difficulties in production of FAES to a consistent quality on a large scale have prevented their widespread commercial application. New technology

developed by Lion Corp., Japan, has now overcome these problems; this fact, combined with a reasonably priced, renewable feedstock, means that products containing FAES as the active ingredient are now becoming more widely available.

Synthesis of FAES can usually be achieved by one of two pathways. The first, by the sulphonation of fatty acid esters, was investigated by Henkel in the 1960s, who produced various patents. Up until this time the main problem concerning the direct sulphonation of fatty esters was that the α-hydrogen atoms were only weakly activated due to the presence of the ester group (Stein and Baumann, 1975; Stein *et al.*, 1970). Stronger sulphonating agents were required under more drastic reaction conditions which tended to lead to dark sulphonation and decomposition products at the higher temperatures (Stein *et al.*, 1970). The sulphonated products were still serviceable, however, if they were bleached with a suitable bleaching agent such as H_2O_2 (Stein and Baumann, 1975; Stein *et al.*, 1970). As a result a technically useful manufacturing process was unavailable without the need for further purification steps.

Due to the commercial availabilty and quality of gaseous SO_3 mixed with an inert gas such as air or nitrogen, this has been the favoured agent for the commercial sulphonation of fatty acid esters (Stein and Baumann, 1975). For small scale sulphonation, however, it is more usual to use liquid SO_3 (Smith and Stirton, 1967). The reaction is a complex one but appears to take the following course: 1) the initial ester / SO_3 reaction produces an intermediate species, I , regarded as an ester-SO_3 complex; 2) intermediate I reacts more slowly to produce intermediate II, the FAES acid precursor; 3) on ageing the acidic reaction mixture produces the FAES acid form which can then be neutralised to form the FAES sodium salt (Stein *et al.*, 1970).

One of the best methods to date for the commercial production of FAES appears to be a process patented by LION (US 3,969,375, 1976) which involves a two stage sulphonation of the ester, the first at *ca.* 70°C and the second at *ca.*

130°C with continuous neutralisation to pH 8 -10. The method involves further treatment with methanol, bleaching with H_2O_2 and neutralisation with NaOH.

A second method involves direct sulphonation in the α-position of the fatty acid using liquid SO_3, which is then esterified with the appropriate chain alcohol. This is the route approached by various authors for 'bench-top' preparation of FAES compounds (Bistline *et al.*, 1956; Micich *et al.*, 1972; Stirton *et al.*, 1962a; Stirton *et al.*, 1962b; Stirton *et al.*, 1965; Weil *et al.*, 1955). The ease of preparation of FAES compounds by this method promotes its use in the synthesis of homologous series of compounds where choice of R and R' chains is extremely flexible.

From an environmental standpoint only small amounts of FAES compounds are required to obtain good detergency (Schwartz and Rader, 1965) resulting in a reduction in loading in waste discharges (Masuda *et al.*, 1993). It would be expected that primary elimination of fatty acid sulphomethyl esters in sewage treatment would be *ca.* 20% of the influent concentration as a result of precipitation or adsorption processes and the majority would enter the main biological stage of the plant. Here they are readily biodegradable in terms of both primary and ultimate degradation (Masuda *et al.*, 1993), with a resulting >99% primary degradation and ≥90% ultimate degradation (Steber and Wierich, 1989). Using the Methylene Blue Active Substances (MBAS) method to measure surfactant concentration, which will only detect undegraded parent surfactant, 100% loss in methylene blue activity has been seen after 2 days for the C14-C16 FAES compared to a little over 5 days for LAS (Masuda *et al.*, 1993). This observation is confirmed for C18 methyl FAES and C18 isopropyl FAES by other studies where 100% loss in MBAS is seen in 3 days under aerobic conditions (Maurer *et al.*, 1971, Maurer *et al.*, 1977).

Toxicity data for these substances are far from complete, and few data are to be found in the literature. They are, however, as with other surfactants, nonreactive toxicants and show the familiar increase in toxicity with increasing chain length. The 96 hour LC50s of C12, C14 and C16 methyl FAES to the

Japanese killifish (*Oryzias latipes*), for example, have been observed at 298 mg/l, 24 mg/l and 1.3 mg/l respectively. The 96 hour LC50 for C14 / C16 methyl FAES mix was 2.4 mg/l which is similar to that of LAS (4.0 mg/l)(Onitsuka *et al.*, 1989).

FAES compounds lend themselves to QSAR analysis for several reasons. Firstly, their relative ease of synthesis allows creation of a homologous series of high purity compounds with a range of log P values. Secondly, they possess good solubility and can be synthesised with suitable chain length to elicit a measurable and reproducible toxic response below the limit of solubility. Thirdly, as anionic surfactants with structural similarities to certain other substances in the same class, their behaviour and mode of action are of interest in the development of surfactant toxicity prediction.

2.1.2 ACUTE TOXICITY

A bioassay in its widest sense is the measurement of any stimulus, be it physical, chemical, biological etc., by means of the reaction which it produces on living matter (Finney,1971).

In aquatic toxicology, acute toxicity tests are the most widely used. They are used to assess the numerical value of toxicity and to compare the potencies of toxicants to species.

The purpose of a toxicity test, therefore, is to determine the toxicity of a given material on a target organism, by the determination of the concentration of test material which produces a specific effect on a specified number of the test organisms in a given time. In practice for acute toxicity tests, this involves exposing a group of organisms to several concentrations of the toxicant, usually in a geometric series (based on a logarithmic scale) in conjunction with a control treatment in which no toxicant is present, and measuring mortality over a specific time period. Most commonly this is to determine the median lethal concentration (LC50) of the test material to the test organism.

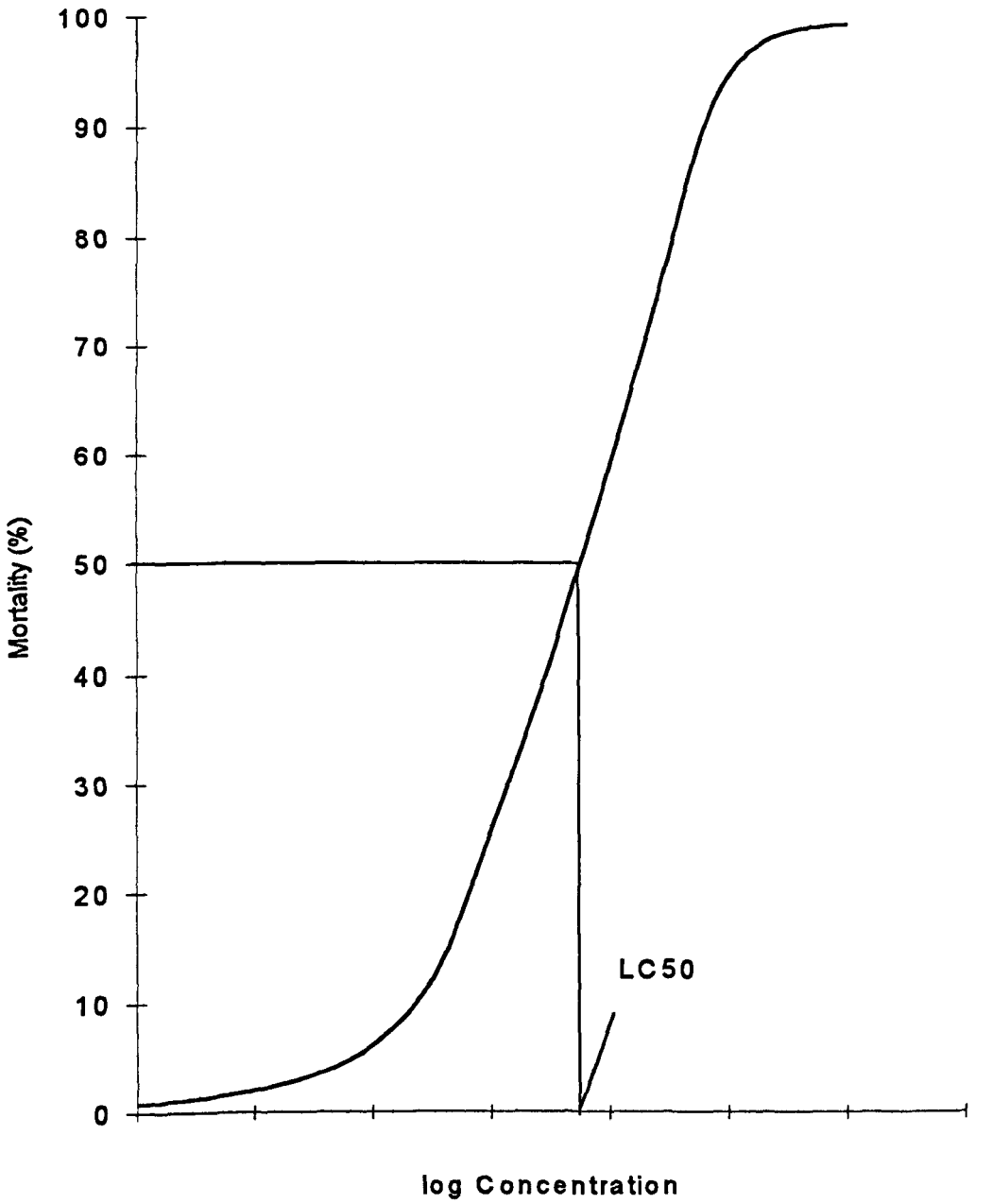
In any population of test organisms there is a normally distributed range of tolerances to a given toxicant. The resulting plot of percent mortality versus $\log_{10}$ of toxicant concentration produces a sigmoidal curve (Fig. 3). Mortality of sensitive individuals accounts for the lower tail of the curve whilst more resistant individuals are represented in the upper portion. The centre is almost rectilinear and represents an average response rate of the test population. The concentration corresponding to 50% mortality in this region is the LC50. There will always be some associated variability with these estimates. The most common measures of these are the standard error, variance, standard deviation and 95% confidence limits which define an interval such that on 95% of occasions when these limits are calculated the LC50 will fall inside the calculated limits.

When performing acute toxicity studies, it is assumed that observed mortality is a direct result of the exposure to the toxicant. This is dependent on inherent toxicity of the toxicant, length of exposure, exposure concentration and sensitivity of test species. However, test organisms are subject to many other environmental stresses such as handling, temperature variation, lack of food etc., which may contribute to observed mortality in the test system. In order to minimise these non-toxicant effects a maximum allowable mortality is defined for organisms in the contol treatment. Standard guidelines of the EEC Commission Directive 92/69/EEC part C.2., OECD (1993) section 2, guideline 202 and EN ISO 6341: 1996, for conducting acute toxicity tests on *D. magna*, for example, specify a 10% maximum mortality rate in the control treatment.

Operator discretion is required for applying a particular statistical method for estimation of the LC50. The most common transformation is probit which creates a linear relationship between response and $\log_{10}$ concentration. Other common analyses include linear interpolation, moving average methods, Litchfield- Wilcoxon and Spearman-Karber.

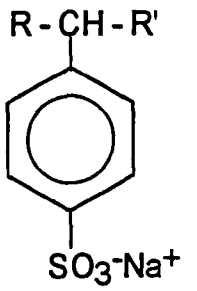
The tests performed in this chapter assess the inherent toxicity of FAES compounds to *D. magna*. The generated data have been used in log P-based QSAR analysis of these compounds. FAES compounds share structural

Fig. 3. Schematic percentage mortality versus $\log_{10}$ exposure concentration. LC50 corresponds to 50% mortality



similarities with LAS in that they possess a long hydrocarbon chain and a negative by charge sulpho group (Fig. 4). It was expected that due to these structural similarities the toxicity of these compounds to *D. magna* could be reasonably well predicted using the polar narcosis equation (equation 10) which has previously been shown to generate reliable predictions of the toxicity of LAS to *D. magna* (Roberts, 1989).

Fig. 4. Basic structure of LAS



2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

Twenty-one FAES compounds were used in total. The inital assessment used ten compounds obtained internally at Unilever Research. Another eleven compounds were then synthesised for further toxicity assessment (Tables 3 and 5). Six LAS compounds were used of chain length C9 - C14, each with known isomer distribution (Table 7), obtained internally at Unilever Research.

2.2.2 FAES SYNTHESIS

FAES compounds were synthesised by direct sulphonation of carboxylic fatty acids and esterification of the sulphonated product as follows:

$$R\text{-}CH_2\text{-}COOH \longrightarrow \underset{\displaystyle SO_3H}{R\text{-}CH\text{-}COOH} \longrightarrow \underset{\displaystyle SO_3^-Na^+}{R\text{-}CH\text{-}COOH} \longrightarrow \underset{\displaystyle SO_3^-Na^+}{R\text{-}CH\text{-}COOR'}$$

All FAES compounds were prepared using the same basic method. Differences occurred, however, in weights of product synthesised and thus of weights and volumes of reagents, although molar ratios of reagents were the same in all cases. Other small differences manifested themselves as a result of changes in aqueous solubilities of intermediates, reaction and refluxing times, boiling points and relative ease of volatilisation of some reagents etc.. These are dealt with in general terms in this chapter and were subject to a degree of operator discretion. The following example for the synthesis of C12 ethyl FAES illustrates the method.

2.2.2.1 SULPHONATION OF C12 CARBOXYLIC ACID

$$CH_3\text{-}(CH_2)_9\text{-}CH_2\text{-}COOH + SO_3 \longrightarrow CH_3\text{-}(CH_2)_9\text{-}CH_2\text{-}\underset{\displaystyle SO_3H}{CH}\text{-}COOH$$

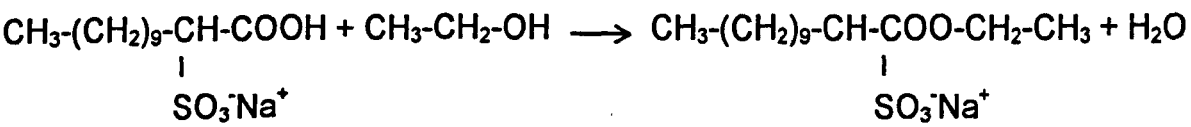
SO_3 was supplied by Hayes Chemicals, U.K.. All other reagents were supplied by Aldrich Chemicals, U.K.. All reagents were of ≥98% purity.

To the reaction flask equipped with a condenser and pressure equalising funnel was added 100ml (1.3 mol) dry 1,2-dichloromethane and 20g (0.1 mol) C12 fatty acid. The solution was heated to 55°C stirring continuously. 5ml (0.12 mol) SO_3 was added dropwise to be in slight excess and the reaction mixture heated under reflux for one to two hours at 55°C. The resulting solution possessed a black 'tar' appearance. For shorter chain length acids it proved necessary to retard the addition of SO_3 and increase the reflux time to achieve this appearance. The excess solvent was removed by evaparation to leave a black solid. The absence of the black solid at the end of the reaction was indicative of a large excess of solvent. Removal of the solvent by evaporation produced the black solid.

2.2.2.2 SYNTHESIS OF SODIUM FATTY ACID SULPHONATE (SFAS)

The black sulphonated acid was dissolved in approximately 600-700ml distilled water (sufficient to dissolve the solid). The occurrence of a 'milky' appearance at this stage indicated the presence of unreacted fatty acid. To the solution was added an excess of saturated NaCl solution until no more precipitation occurred. Precipitation resulted rapidly. Shorter chain length compounds tended to have greater aqueous solubility and cooling overnight was required to induce precipitation. The resulting C12 SFAS precipitate was filtered off, washed with acetone and dried.

2.2.2.3 ESTERIFICATION OF SFAS



To a reaction flask equipped with a reflux condenser was added 100 ml (1.76 mol) ethanol so as to be in vast excess. To this was added 10g (0.03mol) of C12 SFAS and 2-3g of acid-washed Amberlite IR120 (plus) ion exchange resin. The solution was heated under reflux for 24 - 48 hours. The end of the reaction was marked by a clear solution indicating complete SFAS reaction. The excess alcohol was removed to leave the white crystalline C12 ethyl FAES which was then dissolved in distilled water and freeze dried. The presence of a viscous liquid with some of the products indicated the presence of water. Redissolving in water and freeze drying removed water leaving the white solid. The longer chain, less volatile alcohols used in some of the esterification steps proved difficult to remove by evaporation. These were removed by repeated extractions with nonpolar solvents such as hexane or removal under heat and higher vacuum.

Analysis by nuclear magnetic resonance (NMR) spectroscopy revealed >96% of active product (Fig. 53, Appendix III). Similar activity was found for the other ten synthesised compounds and for the initial FAES compounds (Table 133, Appendix III).

2.2.3 WEIGHT PERCENT PRODUCT

Using NMR spectroscopy the absorption of energy by certain spinning nuclei in a strong magnetic field allows the identification of molecular configurations. Quantitative analysis of a compound can be established from NMR by inclusion of an internal standard. For most purposes, NMR spectra are described in terms of chemical shifts and coupling constants (Willard *et al.*, 1988) In different chemical environments a particular nucleus is shielded from the applied magnetic field in a way which varies depending on the distribution of the surrounding electrons. The specific positions of the shifted resonance frequencies (δ in ppm), represented by peaks on the spectrum, are used to characterise the neighbours of a given nucleus and are constant for given conditions. For example, a proton next to a sulpho group appears at approximately 3.7 - 3.8δ. Interacting nuclei cause mutual splitting of the otherwise sharp resonance lines into multiplet split peaks, called spin-spin coupling. The signal peak for use in quantitative analysis preferably contains a sharp singlet peak to allow maximum accuracy which is typically $\pm$ 2%.

Analysis of a given spectrum reveals an absorption band for a given sample, the area under which is proportional to the number of nuclei responsible for the absorption. The spectrometer electronically integrates the signal which is represented on the spectrum as a step function, the height of each step being proportional to the number of nuclei in that region of the spectrum. As the empirical formula is known for the analysed samples, the total height of the step function divided by the number of protons associated with the step yields the increment of height per proton. Thus, from the peak area of the standard (A_{std}) and

one other for the sample (A_{sam}), the weight of the standard per ml of solvent (W_{std}) and the weight of the sample (W_{sam}), a weight percent of the product can be calculated by :

$$\text{Weight \% Product} = \frac{W_{std} \cdot A_{sam} \cdot N_{std} \cdot MW_{sam} \cdot 100}{W_{sam} \cdot A_{std} \cdot N_{sam} \cdot MW_{std}} \qquad \text{Equation 11.}$$

where N is the number of protons in the group giving rise to the absorption peak and MW is the molecular weight of the compound.

Approximately 0.015 g of the sample was accurately weighed into a vial to which was accurately added 1ml of a suitable solvent of deuterated water previously prepared to contain an internal Trioxan standard at a concentration of 2.8×10^{-3} g/ml. The solvent must be deuterated so that it does not absorb in the proton spectral region. The solution was added to an NMR tube via a glass fibre plug to remove any particulates.

The weight percent product was calculated from the resulting trace using the above formula (equation 11) selecting the peak heights associated with the presence of the sulpho group and Trioxan standard (Appendix III).

2.2.4 CULTURING CONDITIONS OF *DAPHNIA MAGNA*

Culturing conditions followed were those of standard culture procedure used for all *D. magna* cultures at Unilever Research. The organisms were maintained in hard Elendt M7 medium (Table 107, Appendix II), at 20°C ± 2°C under a 16 hour light, 8 hour dark photoperiod. Adult cultures were maintained at six adults per 800 ml of medium per culture vessel with a total of twelve culture vessels. This was contrary to the 5 adults per vessel suggested in the Standard Operating Procedure (SOP) for culture mainenance but was initiated in order to increase neonate production. Culture vessels were arranged such that there were four groups of three vessels, each group at a different age. Each group of three

vessels contained adults of the same age. There was a seven day separation of age between each group of vessels and the group adjacent to it. Adults were discarded at 28 days and the group of three vessels which contained these senescent adults were reset with neonates removed from any of the culture vessels. Neonates were removed daily from all cultures even if no toxicity testing was carried out on that day. Feeding was on a daily basis with the alga *Chlorella vulgaris,* at a level of 1.7×10^5 per ml of culture medium. When the feeding level was seen to promote excessive algal growth in the cultures, feeding frequency was reduced. *C.vulgaris* was cultured on site as part of the *D. magna* culturing system.

2.2.5 PREPARATION OF STOCK / TEST SOLUTIONS

Stock solutions of each of the FAES compounds were prepared by dissolving the test material in Elendt M7 medium as the preferred solvent. Distilled water was an acceptable alternative solvent but was allowed only if the stock solution was present as ≤10% of the final test solution. Typical stock concentrations were 1000 mg/l or 100 mg/l depending on the range of test solution concentrations. In some cases the stock was required to be used as the highest test concentration and, therefore, was required to be prepared in Elendt M7 medium.

Test solutions were prepared by serial dilution of stock solutions with Elendt M7 medium, typically on the progressive scale of ...1.0, 0.56, 0.32, 0.18, 0.1mg/l.... Solutions were prepared at the start of the test and renewed at 24 hours to help maintain test solution concentrations by minimising losses due to biodegradation.

2.2.6 TOXICITY TESTING

All acute toxicity data were determined by exposing less than 24 hour old *Daphnia magna* neonates to a geometric series of concentrations of each test material on a logarithmic scale according to the standard 48 hour acute toxicity test procedure defined in the EEC Commission Directive 92/69/EEC part C, C2. Concentration ranges were chosen ideally to induce 100% effect in the highest concentration and 0% effect in the lowest, with a range of responses in between. Neonates were carefully removed from the cultures using a fine mesh net at the beginning of the day in which the test was to start and isolated temporarily in a 250ml crystallising dish containing Elendt M7 medium. The standard testing procedure recommends exposure of 5 individuals to 100ml test solution, where sufficient volume is available, in 100ml crystallising vessels which are covered to reduce evaporation. Four replicates were used per test concentration. Test solutions were replaced at 24 hours when immobile individuals were counted and removed. Final immobility assessment was made at 48 hours. All toxicity data are expressed as EC50 values in mg/l unless otherwise stated. EC50 is based upon the concentration of toxicant which causes immobility, as opposed to mortality, in 50% of the test organisms.

At 0 , 24 and 48 hours samples were taken from old and new solutions for analysis to determine dissolved oxygen concentration, pH, total hardness and temperature at time of neonate addition / transfer. Samples were also taken from the control, lowest and highest test solution concentrations from selected tests and preserved with approximately 3% formalin for analysis of test solution concentration by MBAS.

2.2.7 STATISTICAL ANALYSIS OF MORTALITY DATA

The mortality of neonates after 48 hours was analysed using the computer program BMPDIN (Ecotoxicology SOP 124). This applies three statistical methods

for the estimation of EC50 / LC50; binomial, moving averages and probit.

The binomial method is a non-linear interpolation method, which can be used to analyse data containing less than two treatments which elicit a response of less than 100%. It makes no approximation of, or assumptions regarding, the data. The probability of an individual being immobilised at the EC50 is 0.5 and hence the probability of all individuals being immobilised at the EC50 is 0.5^n, where 'n' is the number of exposed individuals. Similarly the probability that the EC50 lies between a concentration in which 100% response is seen and a concentration in which no response is seen will be $2(0.5)^n$. If no organisms respond at concentration 'A' and all individuals repond at concentration 'B', then the estimated EC50 = $(AB)^{0.5}$. The method is useful for the analysis of data sets which are predominantly comprised of values of < 50% response.

The moving averages method is an interpolation method which is applicable to a wider range of data sets. It assumes that the data show a monotonic response in which all exposure concentrations produce a higher response than the adjacent lower concentrations. This method should not be used when the data are non-monotonic, when there are fewer than two concentrations in which there is partial response and when the data predominantly comprise values which show < 50% response.

The probit method applies a transformation to the response data to produce a linear relationship between response and log concentration. It can be used to analyse non-monotonic data but should not be used when there are fewer than two concentrations at which there is partial response.

2.2.8 METHYLENE BLUE ACTIVE SUBSTANCES (MBAS) FOR ANALYSIS OF TEST SOLUTION CONCENTRATIONS

The determination of low levels (typically 0-20 mg/l) of anionic surface active materials by the manual methylene blue technique is used in the Ecotoxicology Unit for the analysis of a wide range of samples including surface

and potable waters. Higher concentrations can be diluted for analysis. The Abbot method (1962) is an improved version of that recommended by the Committee on Synthetic Detergents (1956) for the determination of anionic surface active materials. Pre-extraction of the reagents has led to results being far less subject to positive interference from naturally occurring materials that form extractable complexes with methylene blue. Decreasing the optical density of the blanks and use of smaller volumes to reduce emulsification difficulties has also overcome some of the problems of the original method.

The anionic surfactant associates with the methylene blue cation to form a chloroform-extractable ion-association complex, whereas the unassociated cation has very low solubility in chloroform. The ion-association complex in alkaline solution, to avoid proteinaceous interference, is partitioned into a chloroform phase. This is then back extracted with an acidified methylene blue solution in order to remove inorganic anions which form ion-association complexes with the methylene blue dye but have low chloroform solubility.

Five reagents were required for the procedure in addition to the sample :

1) Alkaline borate buffer solution (pH10.4), was prepared as a combined 19.0 ± 0.1g (0.05 M) sodium tetraborate decahydrate and 4.00 ± 0.05g (0.1 M) sodium hydroxide in distilled / deionised water.

2) Sulphuric acid was prepared as approximately 0.5 M solution in distilled / deionised water.

3) Chloroform. A.R. chloroform (containing 2% w / v ethanol) provided suitable low blank absorbance readings.

4) Methylene blue solution, was prepared as a 0.250 ± 0.005 g/l solution in distilled / deionised water.

5) Manoxol OT, anionic reference surfactant (or alternative reference anionic surfactant), was prepared as 1.0 ± 0.005 g/l solution in distilled / deionised water.

A solution of 10ml methylene blue, 10ml alkaline borate and 100ml distilled /deionised water for each sample determination was prepared and four extractions

made with chloroform, discarding the chloroform and washing with 10 - 20 ml chloroform between extractions.

To a 250 ml separating flask was added a suitable volume of sample containing 10 - 100 μg of surfactant, which was made up to 100 ml with distilled / deionised water. (A blank and 80 μg standard were prepared in the same manner). 60 ml of extracted alkaline methylene blue solution and 15 ml chloroform were added to the flask for each sample. Extraction was then performed with a steady shake for 1 minute.

The lower chloroform layer was then run into a second 250 ml separating funnel containing 50 ml distilled / deionised water, 3 ml 1M sulphuric acid and 60ml alkaline methylene blue solution. Extraction was again carried out as above and the chloroform layer run into a 50 ml volumetric flask via a small filter funnel plugged with cotton wool which had been pre-wetted with chloroform.

The above steps were repeated twice more from the addition of 15 ml chloroform onwards. Following the final chloroform extraction, the cotton wool was washed with chloroform and the volume made up to the mark in the volumetric flask with chloroform.

The absorbance of the final chloroform phase containing the methylene blue - surfactant complex was then read at 650 nm using 20 mm glass cells.

Sample analyses were carried out at eight or nine per run

The calibration curves were prepared and extracted as for samples with 0,2,4,6,8 and 10 μg of the test material.

2.2.9 LOG P CALCULATION

The fragment approach of Hansch and Leo (1979) for the calculation of a hydrophobic parameter synonymous with log P is a relatively simple summation algorithm as previously discussed (equation 6). The method uses fragment values (f) which remain constant and allows the application of factors (F) to account for more complex molecular interactions which affect the partitioning equilibrium e.g.

proximity between polar groups which reduces the hydrophilicity of each of the polar groups involved. The method described in this section is merely a summary of the most salient features of log P calculation for most structures in this thesis as reported by Hansch and Leo (1979) and is not an attempt fully to describe the theory behind its derivation.

One of the fundamental differences between this method and that of Rekker *et al.* (1977) is that it is able to define fundamental groups and does not rely upon operator discretion for the division of the molecule into its constituent groups.

In order to define a fundamental fragment it is firstly necessary to define two classes of carbon atoms. Isolating carbon atoms (IC) are those which possess four single bonds, at least two of which are to nonhetero atoms, or are multiply bonded to other carbon atoms. Nonisolating carbon atoms (NIC) are those which are multiply bonded to hetero atom containing groups such as nitriles or carbonyls.

A single fundamental fragment can, therefore, only be an IC or a hydrogen or hetero atom bonded entirely to ICs.

A multiple atom fundamental fragment can be created by joining either a NIC, a hydrogen or a hetero atom and all remaining bonds are joined to ICs.

H-polar fragments are those which participate in hydrogen bonding as the acceptor or donor, such as -OH or $-NH_2$.

S-polar fragments are those with strong electron-withdrawing potential but have little tendency to H-bond i.e. the halogens.

Two of the 'cornerstone' fragment values are those for hydrogen and for carbon atoms in alkyl chains. The value for hydrogen is calculated from a carefully measured value for log P_{H2} = +0.45, giving a value for log P_H = +0.225 (rounded up to 0.23). The value for carbon (f_C) in alkyl chains is calculated from an average of two values for f_{CH3} which are calculated from measured values of log P_{CH4} = 1.09 and log P_{CH3CH3} = 1.81.

$f_{CH3} = \log P_{CH4} - f_H = 0.865$

$f_{CH3} = 0.5 \log P_{CH3CH3} = 0.905$

Averaging these two values gives $f_{CH3} = 0.89$. From this it is possible to calculate a value for f_C :

$f_C = f_{CH3} - 3f_H = 0.20.$

These two values can be used to construct a log P value for any simple alkane structure.

f_C alters, however, if the fragment is part of an aromatic ring. The measured log P for benzene = 2.13. The value of f_{CH}, therefore, is calculated as 2.13 / 6 = 0.355 (compared to 0.43 in an alkyl chain). This results in a value of : f_C (aromatic) = f_{CH}(aromatic) - f_H = 0.355 - 0.225 = 0.13. Care must be taken when using this aromatic value as it is calculated to take account of bonds within the ring whereas this is not done when considering nonaromatic structures. On a practical basis it is preferable when calculating aromatic structures to use whole fragments for rings as opposed to building them up from scratch i.e.:

$f_{C6H5} = \log P_{C6H6} - f_H = 1.90$

$f_{C6H4} = \log P_{C6H5} - f_H = 1.67$

Polar groups are also less hydrophilic when attached to aromatic rings e.g. f_{SO3-} = -5.87 when attached to an alkyl chain but -4.53 when attached to an aromatic ring.

Factors are applied to 'maintain the integrity of the fundamental constants'. Factors recognised for log P calculations for structures in this chapter are as follows:

a) Fb = bond factor = -0.12 (n-1) for alkyl chains, -0.09 (n-1) for alicyclic rings, where n is the number of bonds between fragments.

b) FgBr = group branch factor = -0.22 per non chain group.

c) Fp-1 = proximity factor for H-polar fragment, one carbon separation between polar fragments = -0.42 (f1 + f2), where f1 and f2 are fragment values for the two fragments.

It should be noted at this point that other fragments are recognised in the log P calculation but are not appropriate to structures in this chapter and will be applied, where appropriate, for other structures in later chapters. These are as follows:

d) Fdou = double bond factor.

e) Ftri = triple bond factor.

f) Fp-2 = proximity factor for H-polar fragment, two carbon separation.
=-0.26 (f1 + f2).

g) Fp-3 = proximity factor for H-polar fragment, three carbon separation.
= -0.10 (f1 + f2)

h) FmhG = multiple halogenation, geminal substitution.

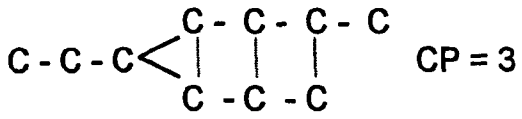
I) FmhV = multiple halogenation, vicinal substitution.

The method also incorporates FcBr = chain branch factor. This has not been applied to the calculations perfomed on any of the structures in this thesis. In place of this factor the Position Dependent Branching Factor (PDBF) has been applied (Roberts, 1991). Many surfactants possess branched chains which affect their toxicological properties depending on the branching position in the molecules (Dorn *et al.*, 1993). Branched chain molecules are more water soluble than the straight chain isomers. With no compensation factor in the lipid layer in log P measurement then the partition coefficient is reduced. This is attributed by

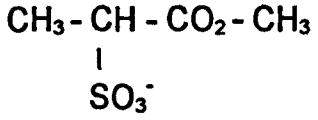
Roberts (1991) to the need for fewer water molecules to solvate the branched chain due to the sharing of water molecules between the chains. Roberts generated the PDBF by extending this argument to assume that if both branches are long then water sharing will continue along the branches for as long as the chains can be paired.

The modification to log P is calculated as -1.44 log (CP + 1) where CP is the number of carbon pairings between chains, for example :

Fig.5. Calculation of Carbon Pairing (CP)



2.2.9.1 EXAMPLE CALCULATION FOR FAES



$$\log P = 2f_{CH3} + f_{CH} + f_{SO3^-} + f_{COO} + Fp\text{-}1 + FgBr + 4\text{-}1(Fb)$$

$$= 2(0.89) + 0.43 + (-5.87) + (-1.49) + (-0.42(-5.87 + -1.49)) + (-0.22) + 3(-0.12)$$

$$= -2.64$$

Having calculated the basic FAES structure it is a simple process to calculate larger FAES structures. For each additional CH_2 fragment (fragment value 0.66) there will always be an increase in log P of f_{CH2} + Fb (0.66 - 0.12) to account for the additional fragment and associated bond. Thus for C12 methyl FAES a further 9 CH_2 fragments are required, resulting in an increase in log P of 4.86. The calculated log P for C12 methyl FAES is thus 2.22. Allowances must be made also for any chain branching which will require the application of the PDBF.

2.3 RESULTS AND DISCUSSION

2.3.1 FAES TOXICITY

All initial compounds (Table 3) were expected to produce EC50 values below the limit of solubility in the test medium. The data for this group yield a good range of calculated log P values and a correspondingly wide range of EC50 values. These are presented in Table 3 as mean values for varying numbers of replicate tests. All test replicate data and associated confidence limits are presented in Table 71, Appendix I. Water quality data are presented in Tables 108 and 109, Appendix II.

Table 3 also shows predicted EC50 values calculated using both the polar narcosis (equation 10) and the general narcosis (equation 9) equations. Whilst measured EC50 values were expected to be reasonably well predicted by the polar narcosis equation, experience with other anionic surfactants has shown this equation to overpredict toxicity slightly. To provide a second, possibly underpredictive marker for comparison, EC50 values were also predicted using the general narcosis equation.

In general, measured EC50 values were higher (less toxic) than predicted values calculated using the polar narcosis equation. Predicted values using the general narcosis equation were closer to the measured values than those predicted using the polar narcosis equation although the equation still overestimated toxicity generally. The toxicities of C12 methyl, C14 methyl, C16 methyl, C14 isopropyl and C14 ethyl, however, were all underpredicted by the general narcosis equation.

Three possibilities exist which could explain the lower than predicted observed toxicities. The first is that observed toxicity is influenced by factors which are not being accounted for, such as biodegradation. Loss of test substance over the test period due to biodegradation would mean that the neonates were not being exposed to nominal or near nominal concentrations of

Table 3. Observed and predicted EC50 values and log P values for initial FAES substances

Substance	MW	log P*[c]	Observed EC50 #[a] (mg/l)	Predicted EC50 # (mg/l)[b]	
				A	B
C12 methyl	316	2.22	140	38	270
C12 butyl	358	3.84	16	4.1	12
C12 sec butyl	358	3.41	36	7.7	29
C12 amyl	372	4.38	7.2	2.0	4.3
C14 methyl	344	3.30	8.5	8.7	34
C14 ethyl	358	3.84	8.0	4.1	12
C14 isopropyl	372	3.95	7.8	3.6	10
C14 butyl	386	4.92	3.7	0.92	1.5
C14 amyl	400	5.46	1.3	0.44	0.53
C16 methyl	372	4.38	2.8	2.0	4.3

A : Calculated using 0.63 log P + 2.52 (equation 10).

B : Calculated using 0.87 log P + 1.13 (equation 9).

* Values to 3 s.f..

Values to 2 s.f..

[a] Mean values.

[b] Values predicted as mol/l and converted to mg/l.

[c] log P values calculated using Hansch and Leo method with PDBF where appropriate.

the test material for the duration of the test. Thus observed toxicity would not be a good representation of the true inherent toxicity of the test substance. The second is that the method of calculating log P is contributing to the apparent differences. The third is that these compounds are not behaving as expected i.e. they are not behaving as polar narcotics. It may be that there is a combination of the above reasons.

2.3.2 QUALITY OF TOXICITY DATA

To address the possibility that observed toxicity is in error, whilst there is inherent variability in any biological system, good repeatability of the *Daphnia* tests was found, with the majority of compounds being tested at least twice. Lack of test material was the reason for lack of repetition in the few cases where this occurred. For most of the compounds, the maximum and minimum EC50 values were within ± 14% of the mean (Table 83, Appendix I).

In addition, MBAS analysis of test solutions from selected tests shows that mean measured concentrations over the 48 hour test period have <20% disagreement with nominal concentrations (Table 91, Appendix I). There was no appreciable drop in concentration over a 24 hour period, indicating that there was no loss of test material due to degradation or adsorption on vessel surfaces for the duration of the test. Apparent reduced toxicity would also be observed if the EC50s occurred above the CMC of the test materials. Available CMC values indicate, however, that observed toxicity for each compound occurs well below the corresponding CMC (Table 4). It can be assumed, therefore, that test organisms were being exposed to concentrations close to nominal and that observed EC50 values are an accurate description of the inherent toxicity of the FAES compounds. It can be assumed also, therefore, that it is not the test system or operator error which is accounting for reduced observed toxicity.

It has been observed also for many years that the toxicity of esters can be correlated with their rates of hydrolysis (Overton, 1901). It has, however, always

Table 4. CMC values for FAES and LAS substances with corresponding EC50 values expressed as mmol/l

* Reference : Roberts, 1989; Stirton *et al.*, 1962a.

[a] Weighted average values based on isomer distribution in Table 7 and measured CMC values for individual isomers.

Values to 2 s.f..

been assumed that whilst esters are non-specific they are also non polar in action and it is the observed additional toxicity to that predicted by the general narcosis equation which requires the inclusion of a descriptor for hydolysis rate (Kamlet *et al.*, 1987). However, FAES compounds have been found to be surprisingly resistant to hydrolysis, due probably to the adjacent sulpho group protecting the carboxylate linkage through steric hindrance (Bistline *et al.*, 1956; Stein *et al.*, 1970; Stirton *et al.*, 1954; Stirton *et al.*, 1962a; Stirton *et al.*,1965; Weil *et al.*, 1955). This is particularly the case for acid hydrolysis although the nature of the R' group contibutes additional stability to alkaline hydrolysis; suphonated esters of secondary alcohols are remarkably stable to both acid and alkaline hydrolysis (Stirton *et al.*, 1954). Measured values for hydrolysis rates tend to be generated at rather more extreme conditions than would be experienced in any toxicity test. The rate of acid hydrolysis, for example, has been measured by heating 0.01mol of the ester in 100 ml of N/3 H_2SO_4 or N/10 NaOH at 100°C (Stirton *et al.*, 1965). The rate of hydrolysis is, however, temperature and pH dependent. Stein and Baumann (1975) related hydrolysis rate with temperature and pH for palm kernel methyl FAES (C12-C14) at a concentration of 3.4g / l (Fig. 6). The hydolysis rate can be seen to be extremely low over a broad pH range (3 - 9.5) even at 80°C. It can be concluded that at the lower temperature and near neutral pH conditions consistent with *D. magna* toxicity testing, ester sulphonates suffer practically no hydrolysis.

It can, therefore, be assumed that the observed EC50 values accurately describe the inherent toxicity of the FAES compounds.

2.3.3 POTENTIAL WATER SHARING BETWEEN HYDROPHOBES

The second possibility for the overprediction of toxicity by the polar narcosis equation is that there is an error in log P calculation method. An initial theory to explain this was based on the extension of the PDBF (Roberts, 1991) to include water sharing between remote hydrophobes which are capable of bending

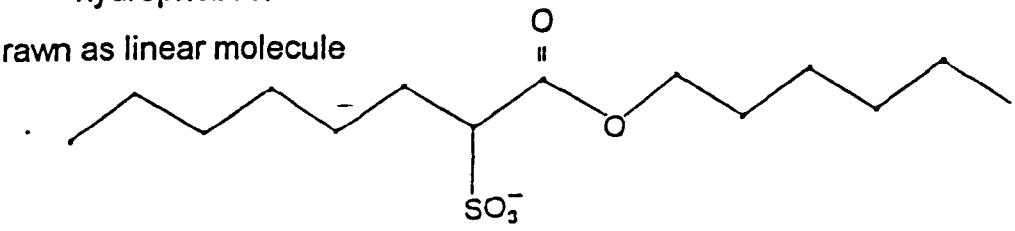
Fig.6. Rate of hydrolysis of palm kernel methyl ester sulphonate at concentration 3.4g/l in relation to temperature and pH

Reference : Stein and Baumann, 1975.

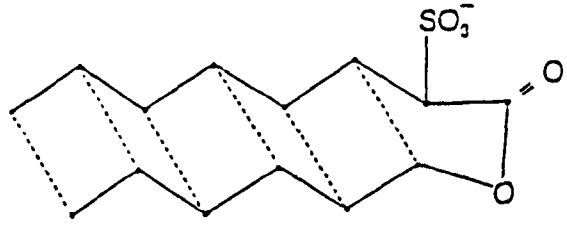
to form close alignment resulting in reduction of log P. This would result in a reduction of predicted toxicity. As this was not included in predicted EC50 calculation, toxicity is overpredicted.

Fig. 7. Schematic diagram of C8 Hexyl FAES to show possible application of PDBF theory to water sharing between topologically remote hydrophobes.

1. Drawn as linear molecule



2. Drawn to show possible conformation of chains with concomitant water sharing



CP = 6

The short chain methyl and ethyl groups may not have sufficient length for this effect to occur and thus would be reasonably predicted by conventional log P calculation. This set of data was too limited to determine if there was any systematic trend in the magnitude of under or overprediction and, therefore, a second set of compounds was synthesised.

Table 5 shows the second series of FAES with their respective calculated log P values. The set was synthesised to contain compounds with larger R' groups, both linear and branched. They were all synthesised to contain a total of 14 carbon atoms and thus have the same molecular weight. If it is assumed that there is no additional watersharing modification to log P other than that described by the PDBF then the consequent calculated log P values would have one of two values depending on whether the compound was linear or branched as denoted in Table 5. Since all compounds have the same molecular weight then the resulting

Table 5. Observed and predicted EC50 values and log P values for the second series of FAES substances.

Substance	MW	log P*	Observed EC50 #[a] (mg/l)	Predicted EC50 # (mg/l)[b]	
				A	B
C7 heptyl	330	2.76	140	18	97
C8 hexyl	330	2.76	180	18	97
C8 sec hexyl	330	2.33	400	34	230
C9 amyl	330	2.76	140	18	97
C9 sec amyl	330	2.33	270	34	230
C10 butyl	330	2.76	170	18	97
C10 sec butyl	330	2.33	220	34	230
C10 iso butyl	330	2.33	150	34	230
C11 propyl	330	2.76	120	18	97
C12 ethyl	330	2.76	150	18	97
C13 methyl	330	2.76	41	18	97

A : Calculated using 0.63 log P + 2.52 (equation 10).

B : Calculated using 0.87 log P + 1.13 (equation 9).

* Values to 3 s.f..

Values to 2 s.f..

[a] Mean values.

[b] Values predicted as mol/l and converted to mg/l.

predicted EC50 values will have one of two values also. Table 5 also shows measured EC50 values for this second set of compounds with predicted EC50 values calculated using the polar and general narcosis equations. All test replicate data and associated confidence limits are presented in Table 72, Appendix I. Water quality data are presented in Tables 110 and 111, Appendix II.

Again the best predictor of toxicity would appear to be the general narcosis equation. Allowing for inherent variability in the EC50 data there appear to be only two EC50 values as expected, with the exception of C13 methyl. As before toxicity is overpredicted using the polar narcosis equation for compounds with R' chains greater than methyl and underpredicted for the methylated compound.

As for the first set of compounds, all showed response below the limit of solubility. Test repeatability was again good; all maximum and minimum EC50 values were within ±10% of the mean (Table 84, Appendix I). Available CMC values indicated that EC50 values were below the corresponding CMC (Table 4). MBAS analysis showed good agreement between mean measured and nominal concentrations (Table 92, Appendix I) and minimal to no hydrolysis is expected to have occurred under the conditions in the test system, for reasons previously discussed (section 2.3.2).

Most compounds show reasonable fit to the general narcosis equation whilst methylated compounds appear to be outliers (Fig. 8). In an attempt to highlight further the differences between the outliers and the other points the data were expressed as ratios between measured EC50 values and predicted EC50 values calculated using the general narcosis equation, which seems best to predict toxicity (Table 6). Ratios of 1 would show perfect correlation, less than 1 underprediction and greater than 1 overprediction. Calculating carbon pairings (CP) between overlapping hydrocarbon R and R' chains and ranking compounds according to their toxicity ratios shows that compounds with CP = 1 (those with methyl R' groups) are aggregated and underpredicted (ratios < 0). The remaining compounds, however, show no clear ranked order of CP with increasing overprediction by the general narcosis equation.

Fig. 8. Log P versus log (1/EC50) for all FAES substances

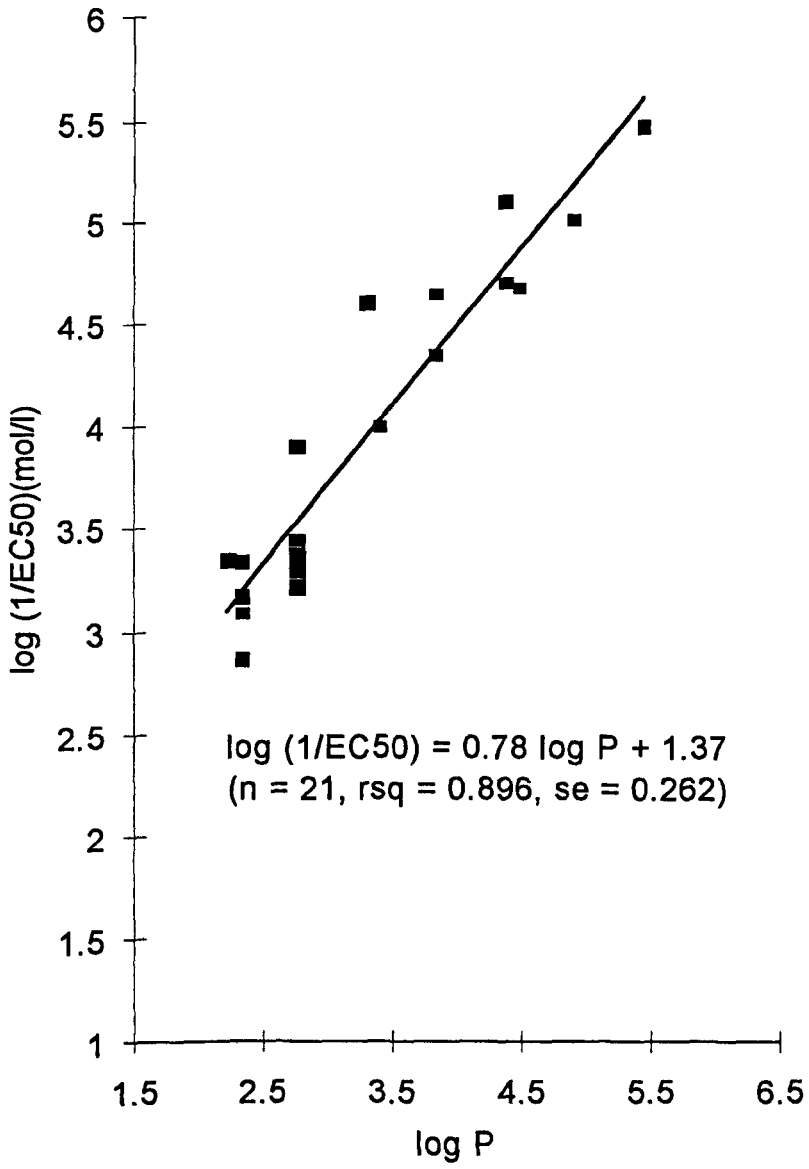


Table 6. FAES substances ranked according to observed EC50 / predicted EC50 ratio

Substance	Ratio (Observed EC50 / Predicted EC50)*	Potential CP
C14 butyl	2.47	4
C14 amyl	2.45	5
C8 hexyl	1.86	6
C10 butyl	1.75	4
C8 sec hexyl	1.74	5
C12 amyl	1.67	5
C12 ethyl	1.55	2
C7 heptyl	1.44	5
C9 amyl	1.44	5
C12 butyl	1.33	4
C12 sec butyl	1.24	3
C11 propyl	1.23	3
C9 sec amyl	1.17	4
C10 sec butyl	0.956	3
C14 iso propyl	0.772	2
C14 ethyl	0.667	2
C10 iso butyl	0.652	3
C16 methyl	0.651	1
C12 methyl	0.519	1
C13 methyl	0.423	1
C14 methyl	0.250	1

* Values to 3.s.f..

The lack of any defined ranking above CP = 1 would imply that if water sharing does occur between overlapping chains of CP ≥ 2 then the effect is small and indistinguishable from the scatter in measured EC50 values.

It would appear that the additional water sharing is not the most likely explanation for the overpredicition of the majority of FAES compounds by the polar narcosis equation and it may be that apparent differences between various chain length R' chains are too small so as to be masked by other influences such as inherent variability within the test.

2.3.4 GENERAL VERSUS POLAR NARCOSIS

It may be that FAES compounds are behaving with a general narcotic mode of action. The data were incorporated and compared with surfactants of known mode of action.

Initially a plot was made combining FAES data with alcohol ethoxylated (AE) data (in-house Unilever data). AEs are non-ionic surfactants and their EC50 values are accurately predicted by the baseline narcosis QSAR of Könemann (equation 9).

Fig. 9 shows a plot of log EC50 (predicted) versus log EC50 (measured) combining FAES data with those of AEs, where predicted FAES data are calculated using the general narcosis equation. The plot shows good correlation between predicted and measured values.

To highlight the correlation of FAES compounds predicted using equation 6 with AE compounds, the regression equation (equation 12) of log EC50 (predicted) versus log EC50 (measured) for FAES and AE data (Fig.9), can be converted as follows :

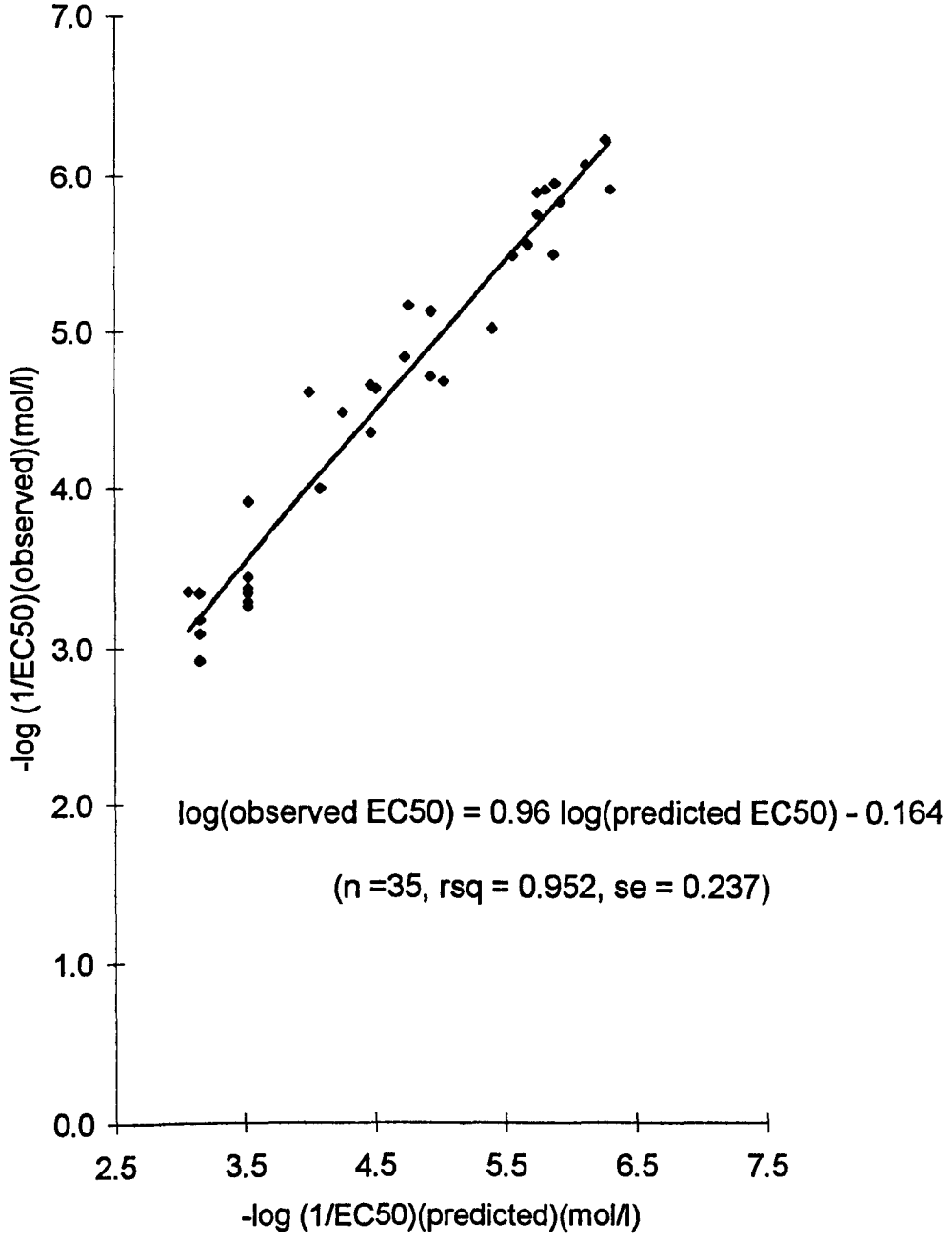
log EC50 (measured) = 0.96 log EC50 (predicted) - 0.16 Equation 12.

-log EC50 (predicted) = 0.87 log P + 1.13 Equation 9

Combining equations 12 and 9 results in :

log 1/EC50 = 0.84 log P + 1.24 Equation 13.

Fig. 9. Log EC50 (observed) versus log EC50 (predicted) for FAES and AE substances. Predicted FAES values calculated using equation 9



Equation 13 is very similar to the general narcosis equation (equation 9) and suggests that FAES compounds may behave as general narcotics.

If the predicted FAES are now calculated using the polar narcosis equation and the data re-plotted with AE data (Fig. 10) it can be seen that correlation is reduced by this treatment of the data and it would appear, therefore that FAES compounds correlate well with known non-ionic surfactants predicted using the general narcosis equation (equation 9).

If, however, the FAES data are now plotted with data for a known polar narcotic LAS a different conclusion may be inferred. Table 7 shows mean EC50 values for LAS compounds of known isomer distribution with log P values calculated to include PDBF for chains either side of the benzene ring. LAS compounds were chosen to have log P values in a similar range to those of the FAES compounds. All test replicate data and associated confidence limits are presented in Table 73, Appendix I. Water quality data are presented in Tables 112 and 113, Appendix II. Good repeatability was observed between test replicates. The maximum and minimum EC50 values were ± 12% of the mean in most cases with the exception of C12 LAS (Table 84, Appendix I). No explanation can be given for this result. However, the mean value is consistent with other mean values and is not influential in the determination of slope of the regression line.

MBAS analysis of test solutions in the compounds with lowest and highest log P values shows that confidence can be had in nominal concentrations for C9 LAS (Table 92, Appendix I). MBAS analysis for C14 LAS indicated >20% mean disagreement between nominal and measured concentrations and the mean EC50 value was corrected to account for the discrepancy by reduction of the EC50 value by the percentage equivalent to the mean disagreement in nominal and measured concentrations.

Fig. 10. Log EC50 (observed) versus log EC50 (predicted) for FAES and AE substances. Predicted FAES values calculated using equation 10

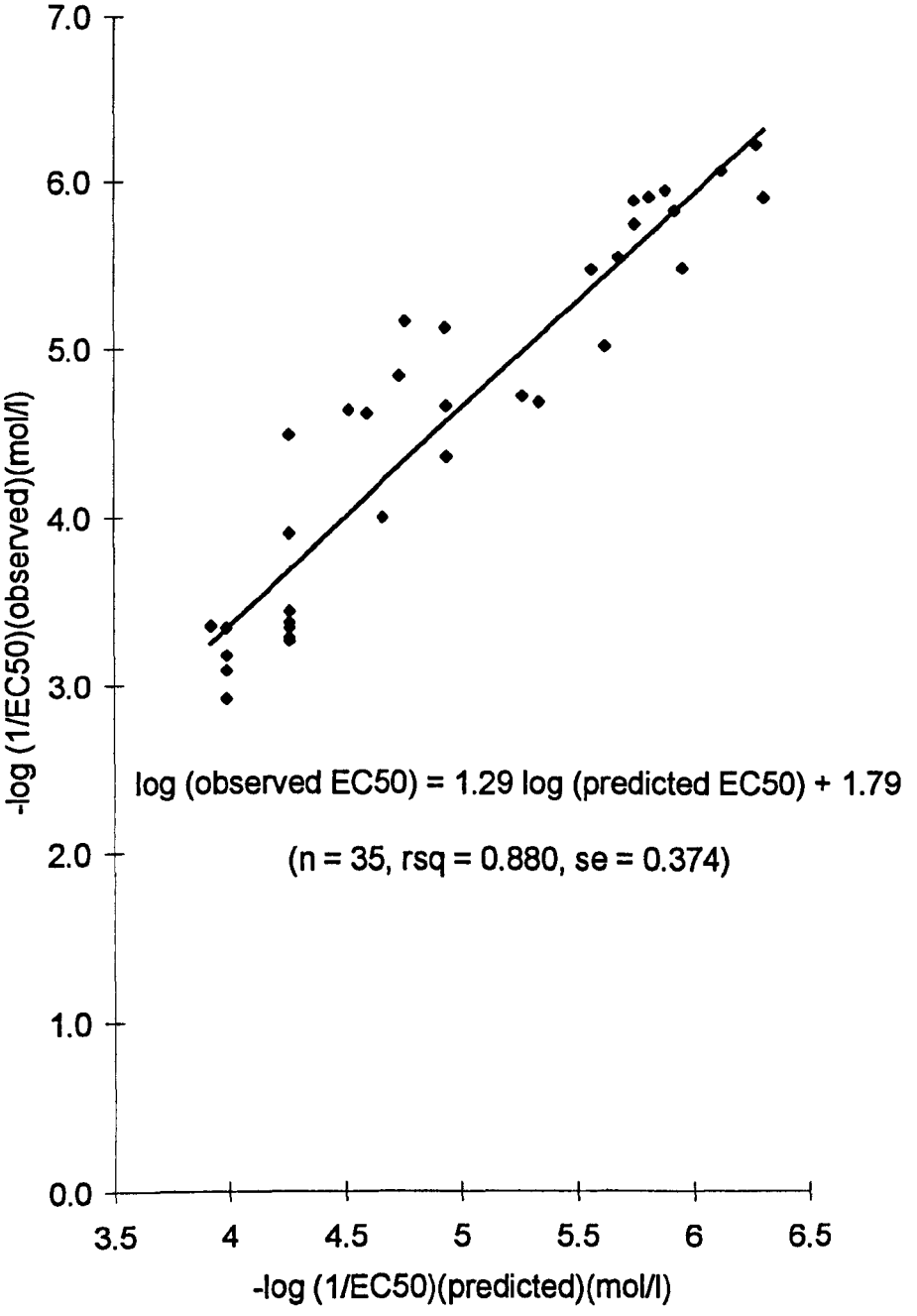


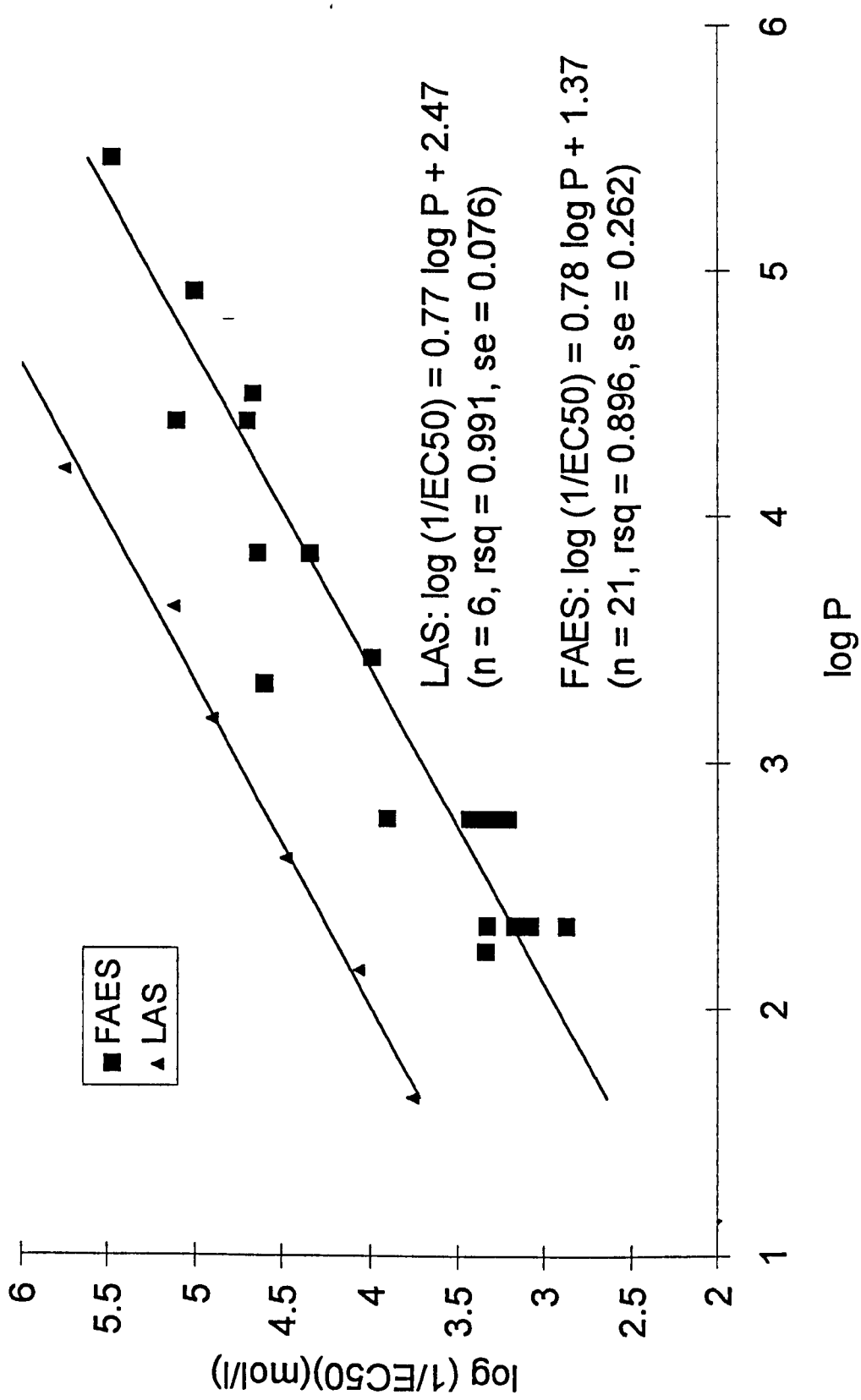
Table 7. Mean observed EC50 values and log P values for LAS

Chain Length	Isomer Distribution		MW	log P*	Observed EC50 #[a] (mg/l)
	Isomer	%			
C9	2	27.94	306	1.63	53
	3	28.25			
	4,5	42.57			
C10	2	28.50	320	2.15	28
	3	23.80			
	4	23.00			
	5	24.60			
C11	2	19.71	334	2.60	11
	3	20.09			
	4,5,6	60.17			
C12	2	22.36	348	3.17	4.3
	3	21.98			
	4	17.47			
	5,6	36.91			
C13	2	16.10	362	3.62	2.7
	3	15.70			
	4	16.70			
	5	19.80			
	6	31.80			
C14	2	20.77	376	4.19	0.95
	3	16.17			(0.67[b])
	4,5,6,7	61.20			

* Weighted average values to 3 s.f.. # Values to 2 s.f..

[a] Mean values. [b] Corrected due to low measured test medium concentrations.

Fig. 11. Log P versus log (1/EC50) for FAES and LAS substances



Although it was originally developed for toxicity of phenolic compounds to fish, application of the polar narcosis equation to acute toxicity of LAS *to D. magna* has been shown previously to provide good correlation with log P with the inclusion of the PDBF (Roberts, 1991).

Fig. 11 shows that both types of surfactant have separate regression equations. The slopes of these two lines, however, are highly similar and the equations can really only be differentiated from each other by the y-axis intercept i.e. they are separated by a constant factor. Given that the slopes are effectively parallel, allowing for inherent error in the system, then from this plot FAES compounds appear to be acting by a similar mechanism to LAS i.e. polar narcosis. This would be consistent with initial expectations but contrary to other findings. It is also possible that they may be acting part way between the polar and general narcosis mechanisms.

In order to assess mode of action with a greater degree of certainty, a series of mixture toxicity studies were proposed (Chapter 3).

CHAPTER 3

THE JOINT TOXICITY OF MIXTURES

3.1 INTRODUCTION

In the aquatic environment, organisms may be exposed to a large number of chemicals which may collectively induce a toxic response significantly different from that of the individual components (Cairns, 1968). Commercial surfactants are rarely pure compounds, but consist of mixtures of isomers and homologues and sometimes of different chemical types (Roberts and Marshall, 1995). Consequently these active components have the potential to act in the same way as other complex mixtures. Yet, despite the commonplace nature of chemical mixtures, there are relatively few biological effects data for such exposure compared to those for single chemicals (Shirazi and Linder, 1991). One of the reasons for this is undoubtedly the large amount of testing required for the complete analysis of even fairly simple mixtures. Defining the toxicity of a complex mixture is a problem at both a theoretical and a practical level and is recognised as an unsolved problem.

Central to the development of predictive methodology of the joint toxicity of mixtures is the distinction of substances of similar mode of action and those of dissimilar action. Plackett and Hewlett (1952) divided the joint action of two toxicants into four classes by considering the similarity and interaction of the substances in the mixture. When defining models of joint action, each substance may produce a number of qualitatively different responses and may act upon different biological systems in so doing. The classes of similarity and dissimilarity are defined as follows:

1) Similar joint action. If two toxicants, administered together or separately, create a response by causing the same physiological systems to react, the joint action is said to be similar with respect to the response.

2) Dissimilar joint action. If two toxicants, administered together or separately, create a response by causing different and distinct physiological systems to react then joint action is said to be dissimilar with respect to the response.

These two joint actions are further divided according to whether interaction occurs or not. Two toxicants A and B are said to interact if the presence of A influences the biological activity of B by either influencing:

a) the amount of B reaching the site of action of B or

b) the effects of B at the site of action of B, and vice versa. The classes can be summarised as follows:

No interaction	Similar joint action	A. Simple similar action[a].
	Dissimilar joint action	B. Independent action[b].
Interaction	Similar joint action	C. Complex similar action[c].
	Dissimilar joint action	D. Dependent action[c].

[a] Also known as concentration addition or simple additive toxicity.

[b] Also known as response addition.

[c] Synergisitic (more than additive toxicity) or antagonistic (less than additive toxicity).

Where interactions occur between substances it becomes impossible directly to predict toxicity of the mixture based on the toxicities of the individual components. For example, one substance may modify the absorption or elimination of other substances or may enhance the penetration to the target receptor. In addition, under environmental conditions where complex mixtures of substances occur, other chemical and physical factors may influence one or more of the substances in the mixture (Lewis, 1992). As a result most evaluations of mixture toxicity consider only non-interactive action, a focus which has been justified by its applicability to most laboratory data (Broderius *et al.*, 1995; Broderius and Kahl, 1985; De Wolf *et al.*, 1988; Hermens *et al.*, 1984; Hermens *et*

al., 1985; Konemann, 1981; Lewis and Perry, 1981; Logan and Wilson, 1995; Roberts and Marshall, 1995; Shirazi and Linder, 1991).

The implication from the concentration addition model is that the substances act independently but produce similar effects such that one substance can be expressed in terms of the other (Broderius, 1991). It is characterised by the ability to replace a certain proportion of the LC50 of a substance without changing the response (Anderson and Weber, 1975). Since the substances act upon very similar systems within an organism, positive correlation would be expected between the susceptibility of individual organisms with each of the substances. For homogenous populations the concentration / response curves for individuals exposed to individual substances and mixtures of similarly acting substances would be expected to be similar in shape.

The response addition model predicts a situation in which the components of the mixture act upon different sites or have different modes of action and either act upon different biological systems or upon the same system in a different way. Each component contributes to the toxicity of the mixture only if its concentration is at least at a threshold level. The concentration / response curves for individual substances and mixtures may not be similar in shape. Response to a combination of substances will depend upon the response to the individual substances and the tolerance of the organisms.

Various models are used in the evaluation of mixture toxicity. The most common of these is the toxic unit approach which is the basis for most of the widely used methods. Its use in the evaluation of additive toxicity has been described by Sprague (1970). The method is based upon the end point of an acute or chronic toxicity study, commonly an LC50, of the individual substances of a mixture (Broderius *et al.*, 1995; Hermens and Leeuwangh, 1982; Logan and Wilson, 1995). Each substance is expressed as a toxic unit (TU) in which the concentration is expressed as a proportion of the endpoint, and the TUs are summed. If the TU value: 1) equals 1, the toxicity is assumed to be additive, 2) is

greater than 1, then less than additivity is assumed and 3) is less than 1, then greater than additivity is assumed. Thus TUs can be calculated as follows :

$$TU = C_i / EC50_i \qquad \text{Equation 14}$$

where Ci = concentration of component at the EC50 of mixture.

Könemann (1981) used a mixture toxicity index, based on the TU approach, to calculate toxicity of mixtures of large numbers of components, although it can be used for smaller numbers of substances :

$$\text{Mixture toxicity index (MTI)} = 1 - \log M / \log M_o \qquad \text{Equation 15.}$$

where $M = \Sigma$ (concentration of substances i / LC50i) $= \Sigma f_i$

$M_o = M/f_{max}$ (f_{max} is the largest f_i in the mixture).

The toxicity scale is defined as :

MTI	
<0	Antagonism
=0	No additivity
0 - 1	Partial additivity
=1	Concentration addition
>1	Supra addition (synergism)

The MTI results are more reliable when mixtures are composed of substances each present at an equally toxic concentration (equitoxic ratio) and also with larger numbers of substances.

Marking (1977) proposed a method which allowed linear distribution of concentration addition about zero. The additive index thus assumes additivity at a value of zero and greater or less than additivity for positive or negative values.

Illustration of joint action can also be presented in the form of an isobole (Broderius, 1991). Isoboles are defined as lines of equal response and can be determined by plotting the concentration of a response of one substance against that of another (Fig. 12). Combinations of the two toxicants represented within the square correspond to joint toxicity responses. Areas outside the square represent

Fig. 12. Isobole diagram depicting various lethal responses of a mixture of two substances

Reference: Broderius, 1991

antagonistic responses. Where the substances exist at different ratios within the mixture, lines are illustrated which radiate from the origin.

The additivity model is possible only for a mixture of compounds greater than two where there is absence of interaction. Problems may occur when applying these non-interactive classifications to mixtures of more than two substances as different models may apply to joint action which may occur between groups. There is evidence, however, that any mixture of chemicals at low environmental concentrations, such that each substance is below its threshold for causing non-baseline toxicity, will induce non-polar narcosis by acting additively (Könemann, 1981). It has been tentatively proposed to assume that strictly additive toxicity occurs for all common toxicants (Alabaster, 1981). Similarly for anilines, mixture toxicities were consistent with concentration addition (Hermens *et al.*, 1985). A method for estimating toxicity of hydrocarbon mixtures based on the concentration of individual components in water could be used with non-polar QSARs to predict toxicity to a number of aquatic species and was consistent with additivity of toxic units (Peterson, 1994). Results generally show that complex mixtures exhibit responses from concentration addition to partial addition (Broderius *et al.*, 1995).

Broderius and Kahl (1985) have shown it possible to use joint toxicity theory to establish strict additivity toxicity of mixtures of non-polar narcotics. Strict observed additivity is required in such circumstances in order for the mode of action to be considered similar for each of the components of the mixtures. Such assumptions involved when observing additivity can be considered to discriminate between the non-polar and polar narcosis mechanisms (Veith and Broderius, 1990). It follows, therefore, that QSARs can be used to select substances with simple similar action if used in conjunction with measured values (Könemann, 1980). Concentration addition would be expected for all substances which fall under the same relationship (Hermens *et al.*, 1985). Similarly if one component of a binary mixture is selected for known mechanism of action as a reference

substance and concentration addition is observed for the mixture, then the second component can be assumed to act by a similar mechanism.

It is the purpose of this chapter to establish mode of action of FAES compounds by testing these substances in mixtures with specific reference substances of known mode of action with *D. magna*. Responses were evaluated in terms of TUs as concentration addition and response addition.

In order to discriminate between non-polar and polar narcosis it was necessary to select reference substances described by these log P based models as defined by the Könemann (equation 9) and Saarikoski and Viluksela (equation 10) equations respectively. Veith *et al.* (1983) used aliphatic alcohols as standard non-polar narcotics and n-octanol was used as the non-polar reference substance by Broderius and Kahl (1985). Phenols were selected as the main reference polar narcotic material in accordance with Saarikoski and Viluksela (1982) and Veith and Broderius(1990).

3.1.1 PHENOLS

As a result of their widespread distribution in the environment phenols, in particular chlorophenols, have been studied extensively (Penttinen, 1995). They are released into natural waters from various industrial and agricultural processes and are recognised as being toxic to aquatic organisms (Devillers and Chambon, 1986; Kishino and Kobayashi, 1996a,1996b; Mitrovic *et al.*, 1968; Penttinen, 1995; Walker, 1988). The toxicity of chlorophenols varies depending upon the position and degree of chlorination; toxicity increases with increasing numbers of chlorine atoms (Devillers and Chambon, 1986; Kopperman *et al.*, 1974; Penttinen, 1995), although toxicity is reduced by substitution at the *ortho*-position (Mcleese *et al.*,1979).

It has been recognised over the last few years that phenols are significantly more toxic than predicted by baseline narcosis and it is proposed that they behave as either polar narcotics or respiratory uncouplers (Penttinen, 1995; Pirselova *et al.*, 1996; Saarikoski and Viluksela, 1982; Schultz *et al.*, 1986). The lower substituted chlorophenols are generally proposed to have non-specific mode of action but are more toxic than baseline narcosis due to increase dipolarity and/or hydrogen bond donor acidity (Penttinen, 1995). It is the tetra- and pentachlorophenols which have been classified as uncoupling agents. Whilst these act specifically to induce the uncoupling of oxidative phosphorylation on the inner mitochondrial membrane they have no specific binding site and interaction with the membrane is nonspecific (Terada, 1990).

However, the main complication in the study of the toxicity of phenols to aquatic organisms is the issue of ionisation. Toxicity has been shown to correlate with the dissociation constant pK_a (Kishino and Kobayashi, 1996a; Saarikoski and Viluksela, 1982). Thus toxicity of phenols, as with other ionisable substances, is highly dependent on the pH of the medium (Kishino and Kobayashi, 1995; Kishino and Kobayashi, 1996a; Könemann and Musch, 1981; Saarikoski and Viluksela, 1981). The undissociated form of these substances is more toxic than the dissociated form. The main reason for this is that the transfer of chlorophenol from medium to organism is principally a result of passive diffusion of the undissociated form through membranes. The main reason for the greater toxicity of the undissociated form, therefore, is the higher potential for it to bioaccumulate (Kishino and Kobayashi, 1995) although the dissociated form does partially contribute to the overall toxicity (Saarikoski and Viluksela, 1981). Thus, where pH of the medium exceeds the pK_a, dissociation of the phenol reduces bioaccumulation and consequently the toxicity. In view of this the toxicity of chlorophenols can be considered to be directly related to lipophilicity (LeBlanc *et al.*, 1988), and QSARs involving these substances are essentially based upon log P. These may contain log P as the sole descriptor (Saarikoski and Viluksela,

1982; Jaworska and Schultz, 1994) or include additional descriptors (Devillers and Chambon, 1986).

It is essential when performing toxicity tests with phenolic substances that, in order to identify inherent toxicity, pH and pK_a be considered such that pH(medium) << pK_a, providing that the pH is not so low as to cause a response itself.

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

In accordance with methods of Veith *et al.* (1983) and Broderius and Kahl (1985) a series of n-alcohols were selected for this study as reference baseline narcotic substances : 1-decanol, 1-nonanol, 1-octanol, 1-hexanol, 1-pentanol.

Several substances were selected as the reference polar narcotic substances. An inital series of studies were carried out using LAS as the reference material. These were followed by studies using 2-chlorophenol (Saarikoski and Viluksela, 1982), and phenol (Veith and Broderius, 1990; Saarikoski and Viluksela, 1982) as the standard polar narcotic material.

All alcohols and phenols were obtained from Aldrich, UK Ltd.. All alcohols were of >98% purity. Phenol and 2-chlorophenol were of ≥99% and 98% purity respectively. 2,4,6-Trichlorophenol and p-cresol were also obtained and were of 98% and 99% purity respectively.

LAS was obtained from Unilever Research. It comprised a mixed isomer and average chain length C11.8 (C10-C14) material, 52.5% active w/w and was tested on the basis of active content. The percentage remaining was mostly water with small amounts of inorganic material (< 0.3%). All nonactive constituents were nontoxic.

The FAES compounds used were as obtained or synthesised as described in chapter 2. Purity was as described (Table 133, Appendix III).

3.2.2 CULTURING CONDITIONS OF *DAPHNIA MAGNA*

Culturing conditions were as described in section 2.2.4.

3.2.3 PREPARATION OF STOCK / TEST SOLUTIONS

3.2.3.1 INDIVIDUAL SUBSTANCE TOXICITY STUDIES

All substances to be used in mixture toxicity studies were tested initially as individual compounds to assess inherent toxicity. LAS and alcohol stocks were prepared by dissolving the test material in Elendt M7 medium as the preferred solvent or in distilled water to a concentration of 1000 mg/l or 100 mg/l as described in section 2.2.5.

In order to ensure that the toxicity of the undissociated form of phenol and 2-chlorophenol was observed, it was necessary to prepare stock solutions at a pH below the pKa of each substance. The median values of seven pKa values reported in the literature were 9.9 and 8.5 respectively (Table 8). To ensure that only the undissociated forms were present in solution and to provide direct comparison with the results of Saarikoski and Viluksela (1982), stock solutions were prepared with Elendt M7 medium which had been adjusted to pH 7 using 1M HCl and 1M NaOH prior to use. Reported pKa values for p-cresol and 2,4,6-trichlorophenol are 10.26 and 6.35 respectively (Pirselova *et al.*, 1996). Stock solutions of p-cresol and 2,4,6-trichlorophenol were prepared with Elendt M7 medium which had previously been adjusted to pH7 and pH6 respectively. The pH of the final stock solutions was checked before preparation of test solutions.

3.2.3.2 MIXTURE TOXICITY STUDIES

Most stock solutions were prepared as binary mixtures comprising equitoxic amounts of each of the two components. In addition a few stocks were prepared as 9:1, 8:2 and 7:3 ratios of the two components. The various mixtures

Table 8. Reported pK_a values for phenol and 2-chlorophenol

Phenol	2-chlorophenol	Reference
9.9	8.3	Kishino and Kobayashi, 1996
9.9	8.5	McLeese *et al.*, 1979
9.8	8.3	Shigeoka *et al.*, 1988
10.05	8.65	Saarikoski and Viluksela, 1982
9.99	8.55	Pirselova *et al.*, 1996
9.92	8.52	Könemann and Musch, 1981
9.9	8.495	Devillers and Chambon, 1986

Table 9. Binary mixtures of substances used in toxicity tests

Mixture	Mixture
LAS / C13 methyl FAES	Pentanol / C8 sec hexyl FAES
LAS / C12 ethyl FAES	Hexanol / C12 ethyl FAES
LAS / C10 butyl FAES	Hexanol / C10 butyl FAES
LAS / C8 hexyl FAES	Hexanol / C8 hexyl FAES
LAS / C7 heptyl FAES	Hexanol / C7 heptyl FAES
Phenol / C14 methyl FAES	Octanol / C12 butyl FAES
Phenol / C12 amyl FAES	Octanol / C13 methyl FAES
Phenol / C14 ethyl FAES	Nonanol / C12 amyl FAES
Phenol / C12 butyl FAES	Nonanol / C14 methyl FAES
Phenol / LAS	Decanol / C12 ethyl FAES
2-chlorophenol / C14 butyl FAES	Decanol / C13 methyl FAES
LAS / Nonanol	Decanol / C8 Hexyl FAES
Phenol / Nonanol	Decanol / C7 heptyl FAES

prepared for testing comprising FAES, LAS, phenol, 2-chlorophenol and alcohol components are presented in Table 9. Each test mixture was prepared to test the assumption of additivity where FAES substances were mixed with LAS or phenols or where two known polar narcotics were mixed. Test mixtures were prepared to test the assumption of non-additivity where the reference substance was a non-polar alcohol. Stock and test solutions were prepared such that each component of the mixture nominally contributed half the total TU at the observed EC50 of the mixture. In order to achieve equitoxic proportions of the individual components in the mixtures it was necessary to account for the difference in toxicity of the components which was calculated simply by:

$$\% \text{ component A} = \frac{(\text{EC50 component A}) \cdot 100}{\text{EC50 component A} + \text{EC50 component B}} \qquad \text{Equation 16}$$

For each mixture the contribution of each of the two components to the EC50 is presented in the tables where EC50 and TU values are presented.

Where one component of the mixture was fluid, as was the case for the alcohols, 2-chlorophenol and LAS mixture studies, the two components were mixed prior to dissolving and making up to volume in Elendt M7 medium. Where both components were solids, to ensure complete mixing, one component was dissolved in a few drops of Elendt M7 medium before the second component was added and mixed, and then made up to volume with Elendt M7 medium. All mixtures proved soluble. Where stock preparation involved phenolic substances, all Elendt M7 medium was adjusted to pH7 with 1M HCl and 1M NaOH prior to use. The pH of the final stock solution was checked before preparation of test solutions.

3.2.3.3 TEST SOLUTION PREPARATION

Test solutions were prepared by serial dilution of stock solutions with Elendt M7 medium, typically on a progressive scale of ...1.0, 0.56, 0.32, 0.18, 0.1 mg/l.... Where test solution preparation involved phenol and 2-chlorophenol, all Elendt M7 medium was adjusted to pH7 with M HCl and M NaOH prior to use. Where test solution preparation involved p-cresol and 2,4,6-trichlorophenol, Elendt M7 medium was adjusted to pH7 and pH6 respectively with HCl and NaOH. Solutions were prepared at the start of the test and renewed at 24 hours.

3.2.4 TOXICITY TESTING

All acute toxicity data were determined by exposing less than 24 hour old *D. magna* neonates to a series of concentrations of the test substance / mixture as described in chapter 2. Concentration ranges for mixtures of components expected to act by similar mode of action were chosen by assuming additivity and for substances expected to act by different mode of action by assuming non-additivity.

At 0, 24 and 48 hours samples were taken from the control, lowest and highest test solution concentrations and preserved with approximately 3% formalin for analysis of test solution concentration by MBAS and GC-MS of anionic substances and phenols / alcohols respectively.

3.2.5 STATISTICAL ANALYSIS OF MORTALITY DATA

The mortality of neonates at 48 hours was analysed using the computer program BMPDIN as described in section 2.2.7.

Amendment 2: p 88 - addition of paragraph to section 3.2.6

The GC-MS method required the extraction of aqueous phenol samples with chloroform A.R. (containing 2% w / v ethanol), at a 10 : 1 aqueous : chloroform volume ratio. Using a Perkin Elmer GC 8700 instrument with an MS ion trap detector, 3µl volumes of the chloroform extracted phenol samples were manually injected onto the non-polar BPX-5 column, (Column length = 25m, film thickness = 0.25µm and pressure = 3psi helium) with p-cresol as an internal standard. Temperature profile was 50°C - 250°C, set to 10°C / min. Samples for calibration was prepared by serial dilution of phenol stock prepared in Elendt. Further samples were prepared with constant concentration of p-cresol and calibration plots prepared as phenol concentration versus phenol : cresol ratio as peak area. The detection limit was 0.04 ppm with an accuracy of 5 - 10%.

The fragment approach of Hansch and Leo (1979) was used for log P calculations of all substances in the chapter where not already calculated in section 2.2.9.

3.2.8 STORAGE OF SAMPLES CONTAINING PHENOL

Samples containing phenol were stored with 3% formalin and retained at 20°C ± 2°C for periods of between 2 - 9 weeks before analysis. The majority of samples were stored in the dark. Lack of space was the reason for not storing samples in the dark where this occurred. No difference in loss of phenol over the storage period due to storage in dark or light conditions was observed (section 3.3.3)

Positive correlation can be found between loss of phenolic substances and microbiological activity which suggests that this has considerable impact on the stability of phenolic samples (Carter and Huston, 1978; Ettinger, 1943). Such

studies of phenol concentration have been generally concerned with low environmental concentrations in the ppb range, where phenol was exposed to high levels of bacterial degradation. However, concerns that phenol samples from the mixture toxicity tests were being lost during the period of storage before analysis, prompted two studies into phenol concentration stability under the storage conditions imposed upon the toxicity test samples.

3.2.8.1 STUDY 1. THE STABILITY OF PHENOL IN ELENDT M7 MEDIUM OVER FIVE WEEKS

Approximately 1.0 g/l stock phenol was accurately prepared by dissolving 0.1003g phenol in 100ml Elendt M7 medium previously adjusted to pH7 with M HCl and M NaOH. Test solutions were prepared by the serial dilution of the stock solution with pH7 adjusted Elendt M7 medium. Solution concentrations of 0.0, 1.8 and 18 mg/l phenol were prepared to emulate approximate concentrations of toxicity test samples under storage. The prepared solutions were stored in 100ml screw top bottles in duplicate at 20°C ± 2°C under all potential preserved and unpreserved conditions imposed upon the stored toxicity test solutions (Table 10).

Samples were removed from each of the study vessels at time t = 0 and then approximately at weekly intervals for a period of 5 weeks in order to emulate approximate storage time of toxicity test samples. Samples were analysed for phenol concentration using GC-MS by the analytical chemistry units at Unilever Research.

3.2.8.2 STUDY 2. THE STABILITY OF PHENOL IN ELENDT M7 MEDIUM OVER EIGHT DAYS

Results from phenol stability study 1 indicated loss of unpreserved lower concentrations samples. Study 2 was, therefore, conducted under the same conditions as study 1 by accurately preparing approximately 1.0 g/l stock phenol by dissolving 0.1017g phenol in 100ml Elendt M7 medium previously adjusted to

Table 10. Storage conditions for phenol stability study 1

Sample (mg phenol/l)	3% Formalin		No Formalin	
0.0	In light	In dark	In light	In dark
1.8	In light	In dark	In light	In dark
18.0	In light	In dark	In light	In dark

Table 11. Storage conditions for phenol stability study 2

Sample (mg phenol/l)	3% Formalin		No Formalin	
0.0	In light	In dark	In light	In dark
1.8	In light	In dark	In light	In dark
5.6	In light	In dark	In light	In dark
18.0	In light	In dark	In light	In dark

N.B. All samples stored in same position under same conditions of temperature etc..

pH7 with M HCl and M NaOH. Test solutions were prepared as for study 1 with an additional concentration of 5.6 mg/l phenol and with the exception that samples were removed from the sample vessels at time t = 0 and then daily for the following four days with a final sample to be removed at the final t + 8 days. Conditions imposed upon the stored toxicity test solutions are summarised in Table 11.

Samples were analysed using GC-MS by the analytical chemistry units at Unilever Research.

3.3 RESULTS AND DISCUSSION

3.3.1 STABILITY OF PHENOL

3.3.1.1 STUDY 1

Tables 12 - 15 show mean measured phenol concentrations over the six week test period. All replicate data are presented in Tables 99 and 100, Appendix I. Replicate values showed good consistency, all being within ±14% of the mean with the majority < ±5%(Tables 101 and 102, Appendix I). The values for unpreserved 1.8mg/l samples showed complete loss of phenol sample over the first week and results for both concentrations in both unpreserved and preserved samples (Fig. 13) suggested that sample concentrations were lost over the first two weeks of the study. With unpreserved samples at 1.8mg/l as the exception, however, there proved no consistency in results over the whole six week period. Even removing the most evident outlier of apparent increase of 725% on start concentration for preserved 18mg/l samples, it is obvious that fluctuations in phenol concentration over the test period were paralleled in all samples with the exception of unpreserved 1.8mg/l samples. This indicated errors associated with the analysis technique rather than with the phenol samples. Whilst some values may represent an accurate measurement of phenol concentration, it is not possible to distinguish these from the inaccurate values. Results cannot

Table 12. Mean measured phenol concentrations with 3% formalin in the light and dark by GC-MS for 6 week phenol stability study 1

Sample*	Measured concentration of phenol (mg/l) at time t (days)#						
	t = 0	t + 7	t + 15	t + 22	t + 28	t + 35	t + 43
0.0 L	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0 D	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.8 L	1.1	0.97	0.0	0.0	0.99	1.2	0.56
1.8 D	1.2	0.99	0.0	0.0	8.7[a]	1.4	0.52
18 L	10	12	1.0	2.9	8.2	14	4.8
18 D	9.3	13	1.2	3.1	8.1	15	4.8

Table 13. Mean measured phenol concentrations stored with 3% formalin as % mean measured start concentrations for stability study 1

Sample*	% remaining of start concentration at time t (days)						
	t = 0	t + 7	t + 15	t + 22	t + 28	t + 35	t + 43
0.0 L	-	-	-	-	-	-	-
0.0 D	-	-	-	-	-	-	-
1.8 L	100	91	0	0	90	109	51
1.8 D	100	83	0	0	725[a]	117	43
18 L	100	120	11	29	82	140	48
18 D	100	140	13	33	87	161	52

*Approximate nominal concentration phenol (mg/l). # Values to 2 s.f.

L: Sample stored in light, D: Sample stored in dark.

[a] Spurious value

Table 14. Mean measured phenol concentrations with no added formalin in the light and dark by GC-MS for 6 week phenol stability study 1

Sample*	Measured concentration of phenol (mg/l) at time t (days)#						
	t = 0	t + 7	t + 15	t + 22	t + 28	t + 35	t + 43
0.0 L	0.0	0.0	0.0	0.0	0.0[a]	0.0	0.0
0.0 D	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.8 L	1.0	0.0	0.0	0.0	0.0	0.0	0.0
1.8 D	1.1[b]	0.0	0.0	0.0	0.0	0.0	0.0
18 L	9.6	10	1.0	2.9	7.2	12	4.3
18 D	8.4	11	0.95	2.7	6.3	11	3.4

Table 15. Mean measured phenol concentrations stored with no formalin as % mean measured start concentrations for stability study 1

Sample*	% remaining of start concentration at time t (days)						
	t = 0	t + 7	t + 15	t + 22	t + 28	t + 35	t + 43
0.0 L	-	-	-	-	-[a]	-	-
0.0 D	-	-	-	-	-	-	-
1.8 L	100	0	0	0	0	0	0
1.8 D	100[b]	0	0	0	0	0	0
18 L	100	104	10	30	75	125	45
18 D	100	131	11	32	75	131	40

*Approximate nominal concentration phenol (mg/l). # Values to 2 s.f.

L: Sample stored in light, D: Sample stored in dark. [a] Spurious replicate value, replicate ignored. [b] One replicate only.

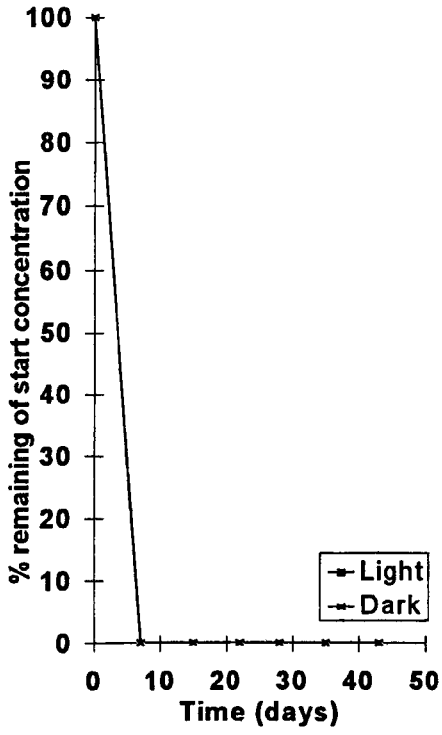
Fig. 13. Stability of phenol study 1: 6 week study, percent remaining of start concentration over time

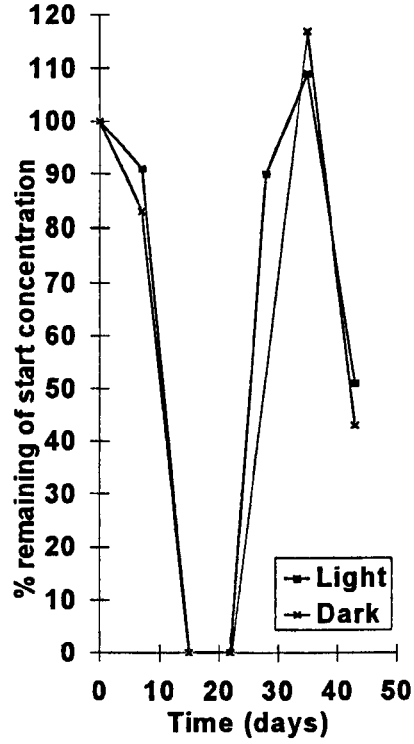
(1) 1.8 mg/l phenol, no formalin

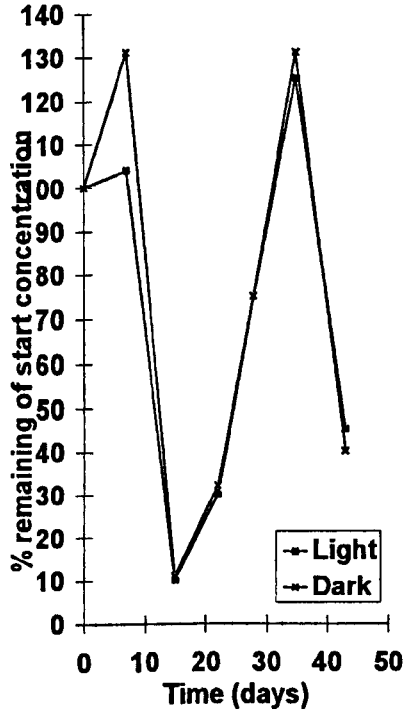
(2) 1.8 mg/l phenol, 3% formalin

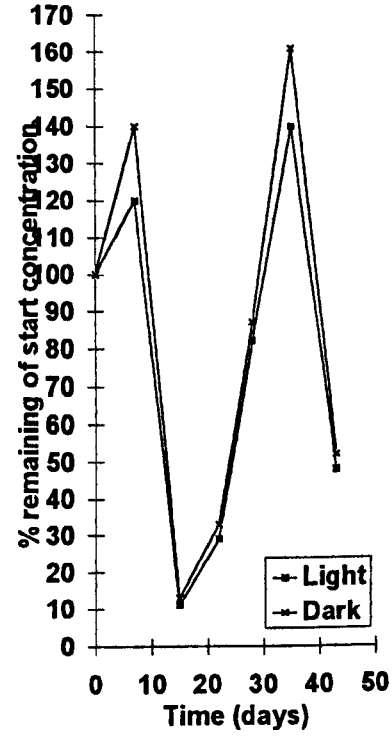
(3) 18 mg/l phenol, no formalin

(4) 18 mg/l phenol, 3% formalin


% remaining of start concentration
Time (days)
Light
Dark


% remaining of start concentration
Time (days)
Light
Dark


% remaining of start concentration
Time (days)
Light
Dark


% remaining of start concentration
Time (days)
Light
Dark

conclusively establish maintenance of phenol concentration, although due to the presence of measurable phenol concentrations at the end of the study, it is probable that there was phenol present in preserved samples at the end of the test period. It would also seem that low concentrations samples were subject to considerable loss of phenol concentration if unpreserved.

3.3.1.2 STUDY 2

Tables 16 - 19 show mean measured phenol concentrations over the eight day test period. All replicate data are presented in Tables 103 and 104, Appendix I. Following problems associated with Study 1, improvements had been made to the analysis technique. Four replicate values appeared spurious, including one of the 0.0mg/l phenol samples, and were ignored. Unpreserved samples showed considerable loss in phenol concentration over the test period, particularly in the lower concentrations. There was only a small degree of error in a given run of samples integral to the method of analysis shown by slight differences in replicate measurement values of samples stored with and without 3% formalin (Tables 105 and 106, Appendix I). Excluding the 0.0mg/l phenol samples and values where one or both replicates were ignored as a result of spurious measurements, of the seventy remaining mean measured values, approximately 61% of mean measured values exhibited maximum and minimum measured concentrations of ±0% of the mean. For a further 26% the maximum and minimum values were within 5.9% of the mean. Of the remainder, maximum and minimum values were within 26% of the mean with the exception of values where complete loss of phenol was observed for one replicate.

Tables 17 and 19 and Fig 14. shows mean % of start concentration over time for samples stored with and without formalin. Considerable difference was observed between preserved and unpreserved samples at the end of the test period although no appreciable difference was observed between samples stored in the light and dark. This would indicate no photolysis of phenol over the test period but significant loss if unpreserved with 3% formalin.

Table 16. Mean measured phenol concentrations stored with 3% formalin in the light and dark by GC-MS for 8 day phenol stability study 2

Sample*	Measured concentration of phenol (mg/l) at time t (days)#					
	t = 0	t + 1	t + 2	t + 3	t + 4	t + 8
0.0 L	0	0	0	0	0	0
0.0 D	0	0	0	0	0	0
1.8 L	1.4	1.3	1.1	1.3	1.1	1.1
1.8 D	1.3	1.3	1.2	1.3	1.1	1.1
5.6 L	4.0	3.9	3.7	4.0	3.6	3.3
5.6 D	4.0	3.9	3.7	4.0	3.6	3.2
18 L	13	14	12	14	12	11
18 D	13	14[a]	12	14	13	11

Table 17. Mean measured phenol concentrations stored with 3% formalin as a % of the mean measured start concentrations for stability study 2

Sample*	% remaining of start concentration at time t (days)					
	t = 0	t + 1	t + 2	t + 3	t + 4	t + 8
0.0 L	-	-	-	-	-	-
0.0 D	-	-	-	-	-	-
1.8 L	100	92	77	98	82	80
1.8 D	100	98	92	101	84	84
5.6 L	100	98	92	100	88	83
5.6 D	100	99	94	100	90	80
18 L	100	104	104	104	93	82
18 D	100	111[a]	107	107	102	84

Approximate nominal concentration phenol (mg/l), L Samples stored in light, D Samples stored in dark, # Values to 2 s.f., [a] Spurious replicate value, replicate ignored (Table #, Appendix I).

Table 18. Mean measured phenol concentrations stored with no added formalin in the light and dark by GC-MS for 8 day phenol preservation study 2

Sample*	Measured concentration of phenol (mg/l) at time t (days)#					
	t = 0	t + 1	t + 2	t + 3	t + 4**	t + 8
0.0 L	0.0	0.0	0.0	0.0	0.0	0.0[a]
0.0 D	0.0	0.0	0.0	0.0	0.0	0.0
1.8 L	1.3	1.2	0.99	0.92	0.73	0.36
1.8 D	1.3	1.3	0.80	-[b]	0.44	0.36
5.6 L	4.1	4.0	3.9	3.4	2.7	2.2
5.6 D	4.1	3.9	3.1	2.8	2.4	1.4
18 L	14	13	12	13	11	9.8
18 D	13	14	12	12	11	8.9

Table 19. Mean measured phenol concentrations stored with no formalin as a % of the mean measured start concentrations for stability study 2

Sample*	% remaining of start concentration at time t (days)					
	t = 0	t + 1	t + 2	t + 3	t + 4**	t + 8
0.0 L	-	-	-	-	-	-[a]
0.0 D	-	-	-	-	-	-
1.8 L	100	93	74	69	54	27
1.8 D	100	93	60	-[b]	33	27
5.6 L	100	98	85	84	67	53
5.6 D	100	96	76	69	58	33
18 L	100	102	90	96	85	74
18 D	100	107	95	94	86	69

* Approximate nominal concentration phenol (mg/l), # Values to 2 s.f., L: Sample stored in light, D: Sample stored in dark. ** Quantisation based on average instead of daily calibration, [a] Spurious replicate value, replicate ignored, [b] Spurious values for both replicates, both replicates ignored (Table 104, Appendix I).

Fig. 14. Stability of phenol study 1: 8 day study, percent remaining of start concentration over time

(1) 1.8 mg/l phenol, no formalin

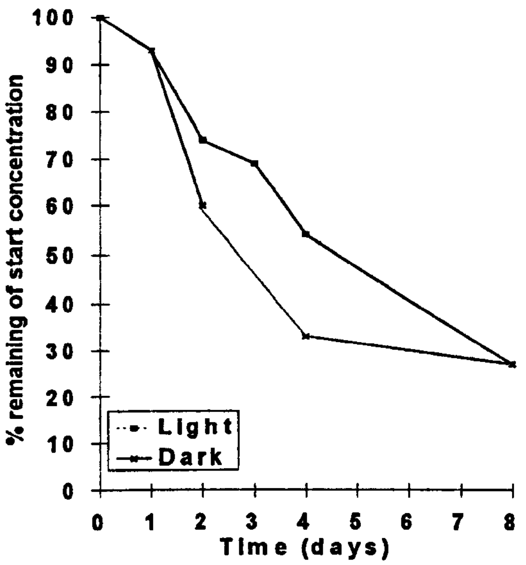
(2) 1.8 mg/l phenol, 3% formalin

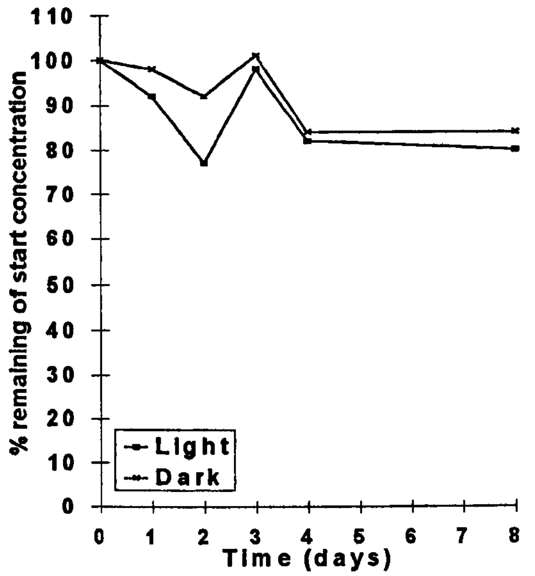
(3) 5.6 mg/l phenol, no formalin

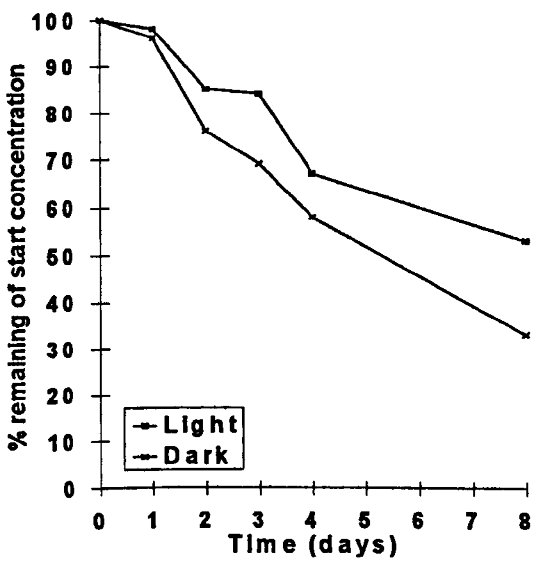
(4) 5.6 mg/l phenol, 3% formalin

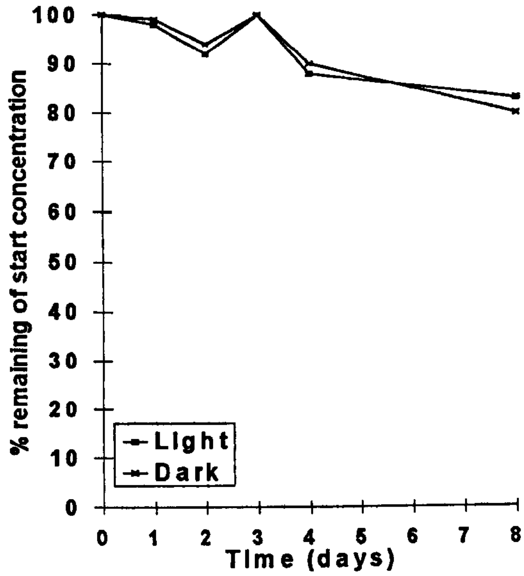
(5) 18 mg/l phenol, no formalin.

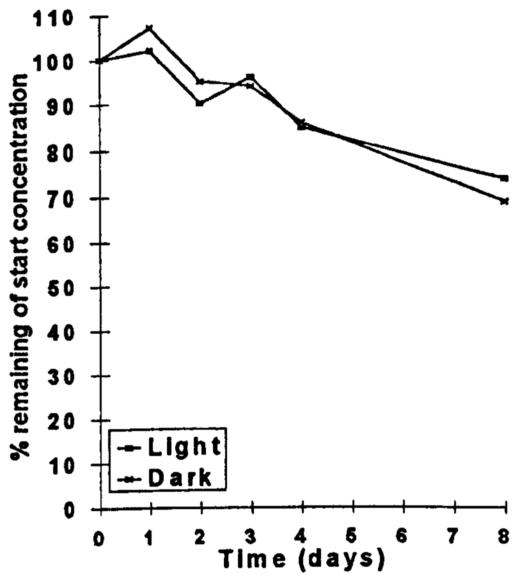
(6) 18 mg/l phenol, 3% formalin.

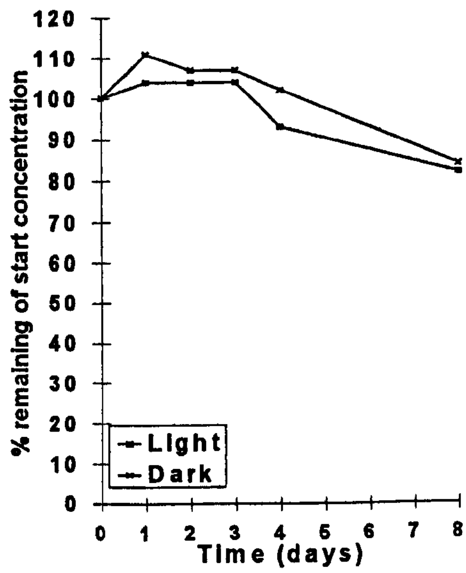

% remaining of start concentration
100
90
80
70
60
50
40
30
20
10
0
Light
Dark
0 1 2 3 4 5 6 7 8
Time (days)


% remaining of start concentration
110
100
90
80
70
60
50
40
30
20
10
0
Light
Dark
0 1 2 3 4 5 6 7 8
Time (days)


% remaining of start concentration
100
90
80
70
60
50
40
30
20
10
0
Light
Dark
0 1 2 3 4 5 6 7 8
Time (days)


% remaining of start concentration
100
90
80
70
60
50
40
30
20
10
0
Light
Dark
0 1 2 3 4 5 6 7 8
Time (days)

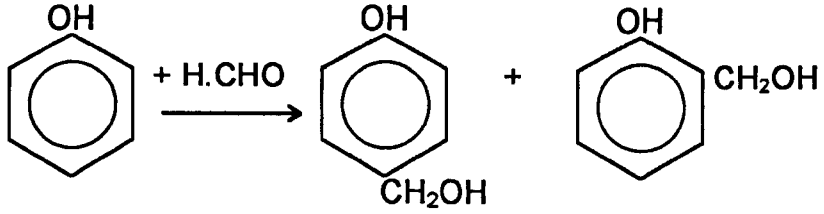

% remaining of start concentration
110
100
90
80
70
60
50
40
30
20
10
0
Light
Dark
0 1 2 3 4 5 6 7 8
Time (days)


% remaining of start concentration
120
110
100
90
80
70
60
50
40
30
20
10
0
Light
Dark
0 1 2 3 4 5 6 7 8
Time (days)

There is good evidence that the greatest cause of instability of phenol in aqueous samples is microbiological and not chemical (Carter and Huston, 1978). At concentrations of >0.5%, phenolic substances are good preservatives in themselves and are used to inhibit microorganism bioactivity as biocides and disinfectants (Carter and Huston, 1978; Fang *et al.*, 1996; Sharp, 1983; Zobell and Brown, 1944). At lower concentrations, however, they are readily biodegraded (Erikson, 1940) although concentrations of phenol as low as 0.05% have been shown to inhibit microorganism activity but not dependably (Zobell and Brown, 1944). Most reported preservation techniques of aqueous samples containing phenol recommend storage at 4°C in combination with the addition of chemical preservatives (Carter and Huston, 1978; Ettinger *et al.*, 1943; Zobell and Brown, 1944). Such chemical preservative techniques, involving acidification with concentrated sulphuric acid or addition of sulphamic or phosphoric acid in conjunction with copper sulphate, resulted in effective preservation for at least 28 days, in the case of the latter method, particularly at 4°C (Carter and Huston, 1978; Ettinger *et al.*, 1943). Storage of large numbers of samples in refrigerated conditions, however, was impractical in this thesis due to lack of space. In a study of the effects of various chemical preservatives on oxygen consumption as a directly proportional measure of bacterial activity in sea water samples, the use of 0.25% formaldehyde alone as a preservative at 22°C resulted in no observed oxygen consumption over the 10 day study period and was considered to be a dependable preservative of phenol samples (Zobell and Brown, 1944). This compares to 1.2% formaldehyde (equating to 3% formalin) used in samples stored at 20°C±2°C in this thesis. Results of the stability studies reported in this thesis suggest that, given possible error inherent to the analytical method, preservation of phenol samples with 3% formalin was sufficiently dependable for the given conditions for at least the duration of the study assuming microbiological activity to be the main source of instability.

Instability as a result of chemical reaction of phenol samples has been noted to be less under oxidising acidic rather than basic conditions (Carter and Huston, 1978). It is generally thought, however, that in practice basic preservation

techniques are not a main cause of instability of phenolic samples. Phenol reacting with the formaldehyde in solution by a similar mechanism as described by the Lederer-Manasse condensation reaction (1894) was thought possible but unlikely. At low temperature, in the presence of dilute acid or alkali the main product of this reaction is p-hydroxybenzyl alcohol (Finar, 1959; Cram and Hammond, 1959).



In the absence of water the addition reaction which parallels the above reaction has been performed at temperatures of between 68°C and 98°C resulting in slower rates of reaction at lower temperatures (Sprung, 1941). Even at 98°C, however, the condensation reaction is extremely slow compared to the addition reactions. The reported reactions were performed at equimolar concentrations. The effect of increasing the ratio of formaldehyde to phenol had the effect of decreasing the speed of reaction. The stored samples in this thesis contained concentrations of formaldehyde in vast excess to the phenol concentration at ratios of at least in the region of 4500 : 1 (mol/l), effectively negating any possible condensation reactions. Samples were also neutral, with no acid or alkali catalyst.

3.3.2 QUALITY OF TOXICITY DATA - REFERENCE SUBSTANCES

In order to establish accurate prediction of mode of action of one component within a two component mixture, it is essential to have confidence in the accuracy of the toxicity data for each of the components involved. Replication of toxicity tests helps provide some confidence in measured values; intuitively any mean value will be more impressive given a larger number of replicates, (n). Therefore, replication has been performed for most toxicity tests involving both individual and mixtures of substances. All toxicity tests involving individual

substances were performed at least twice. Phenol was tested four times (Table 20). All reference substance EC50 values reported in this chapter are mean values which were calculated based on nominal values and adjusted for mean differences between nominal and mean measured concentrations following GC-MS analysis where ≥20% mean difference between nominal and measured concentrations was revealed (Table 93, Appendix I). All test replicate EC50 values based on nominal concentrations are reported in Table 74, Appendix I with associated confidence limits. Water quality data are presented in Tables 114 and 115, Appendix II).

3.3.2.1 REFERENCE SUBSTANCES - POLAR NARCOTICS

It is difficult to compare the observed toxicity of phenol and 2-chlorophenol with other acute toxicity data for these substances, as reported values cover a wide range of species, duration of test and test conditions (Table 21). The pH is often not presented, making comparison of data difficult. Whilst the pK_a for phenol is sufficiently high to make the assumption that most acute toxicity tests involving aquatic species will be conducted at a pH below the pK_a, this may not be the case for other phenolic substances. Even reported acute toxicity data for single species can be seen to cover an order of magnitude, such as those reported by Walker (1988) for 48 hour toxicity data of phenol to *D. magna* (Table 22). The mean toxicity values of phenol and 2-chlorophenol reported in this thesis can be considered consistent with some reported 48 hour toxicity values to *D. magna*, at least in terms of order of magnitude, bearing in mind the lack of information regarding test conditions, condition of neonates used in tests etc. of reported data.

Mode of action of the phenols in question and the accuracy of measured response of such mode of action in the selected test system are central issues. The maximum variation about the mean was +14% for phenol indicating good repetition. Maximum and minimum EC50 values for 2-chlorophenol were within ±

Table 20. Observed EC50 values for reference substances used in mixture toxicity studies

Substance	MW	log P*[a]	Mean Observed EC50 (mg/l)#[b]	Predicted EC50 (mg/l)#[c] A	B
Phenol	94	1.46	7.4	34	-
2 -chlorophenol	128.5	2.15	2.3[e]	17	-
C11.8 LAS	341[f]	-[d]	5.7	-	-
1-Pentanol	88	1.41	400	-	390
1-Hexanol	102	1.95	130	-	150
1-Octanol	130	3.03	23	-	22
1-Nonanol	144	3.57	7.5	-	8.4
1-Decanol	158	4.11	3.5	-	3.1

A Calculated using 0.63 log P + 2.52 (equation 10)

B Calculated using 0.87 log P + 1.13 (equation 9)

* Calculated by Hansch and Leo (1979) method. No Roberts (1991) PDBF required in calculations as log P for LAS not calculated.

Values to 2 s.f.

[a] Values to 3 s.f.

[b] All test replicates and confidence intervals presented in Table 74, Appendix I.

[c] Values calculated in mol/l before conversion to mg/l.

[d] Exact isomer distribution unknown. Unable to calculate log P value, therefore, although this does not effect calculation of MW.

[e] EC50 value corrected for measured test solution concentrations (Table 93, Appendix I).

[f] Based on average chain length (Table 23).

Table 21. Toxicity data for phenols

Species	TD (hours)	pH	LC50 (mg/l)*#				Reference
			Phenol	2-CP	p-Cresol	2,4,6-TCP	
Crangon septemspinosa	96	8.0	7.5	5.3	-	-	McLeese *et al.*, 1979
Mya arenaria	96	$<pK_a$	-	-	-	3.9	McLeese *et al.*, 1979
C. auratus	2.5	7.0	85	93	-	4.5	Kishino and Kobayashi, 1996
P. reticulata	96	7.0	43	14	-	2.3	Saarkoski and Viluksela, 1982
P. reticulata	96	7.3	30	8.2	-	-	Könemann and Musch, 1981
P. reticulata	96	7.8	31	14	-	-	Könemann and Musch, 1981
P. reticulata	96	7.7	43	14	-	2.3	Shigeoka *et al.*, 1987
D. carinata	24	7.7	95	25	-	7.5	Shigeoka *et al.*, 1987
D. pulex	24	7.7	45	21	-	3.9	Shigeoka *et al.*, 1987
D.magna	24	7.7	32	9.0	-	1.7	Shigeoka *et al.*, 1987
D.magna	48	-	12	7.4	21	-	Kopperman *et al.*, 1974
D.magna	48	7.4-7.6	7.7[a]	-	-	-	Lewis, 1983
D.magna	7 day	-	-	3.7	-	-	LeBlanc *et al.*, 1988
D.magna	24	7.0	-	18	-	5.5	Devillers and Chambon, 1986

TD: Test duration. (T)CP: (Tri)chlorophenol. * Data converted to mg/l where not reported in these units. # Values to 2 s.f..

[a] Average LC50 for values measured at different loading densities.

Table 22. Phenol acute toxicity data for *D. magna*

*Average test temperature, S: Static, R: Renewal, N: Nominal Concentrations, M: Measured Concentrations, NR: Not reported.

28% of the mean (Table 85, Appendix I). This relatively poor repetition compared to that of phenol was a result of adjustment of one test replicate EC50 to account for measured test solution concentrations (Table 93, Appendix I) combined with the second replicate EC50 based on nominal test concentrations (Table 74, Appendix I).

GC-MS analysis of test solutions from selected tests involving phenol showed that measured concentrations over the 48 hour test period had <20% mean difference from nominal concentrations (Table 93, Appendix I). There was no appreciable drop in concentration over a 24 hour period indicating that there was no loss of test material due to degradation for the duration of the test. It can be assumed, therefore, that test organisms were being exposed to concentrations close to nominal and that observed EC50 values are an accurate description of the inherent toxicity of phenol to *D. magna*. No adjustment was required for EC50 values calculated for phenol based on nominal test concentrations.

GC-MS analysis of test solutions from the selected test involving 2-chlorophenol showed that measured concentrations over the 48 hour test period had only 44% mean agreement with nominal concentrations requiring adjustment of the EC50 value based on nominal test concentrations (Table 93, Appendix I). There was no appreciable drop in concentration, however, over a 24 hour period indicating that there was no loss of material due to degradation. The uniformity of disagreement between measured and nominal concentrations over all measured test concentrations indicates that it was not a result of loss of test substance during the storage period before analysis, as loss of phenol in unpreserved samples does not occur uniformly between samples (Tables 18 and 19). This suggests that the difference was due to loss of material either during preparation of stock or during dilution of prepared stock in the preparation of the test solutions. Again due to the uniformity of difference it would seem unlikely that the error was as a result of incorrect dilution when preparing test solutions. Hence it is likely that it was an incorrectly prepared stock solution which was at fault. The balance printout indicated correct amount of weighed test substance and so it

would seem that loss of test substance occurred at the stock preparation stage possibly as a result of volatilisation of test material, loss due to spillage or to adsorption on vessel surfaces.

The selected phenols can be considered to be polar narcotics as observed by Saarikoski and Viluksela (1982) and Veith and Broderius (1990). Measured EC50 values for phenol and 2-chlorophenol, however, were lower than predicted by the polar narcosis equation (equation 10)(Table 20). To investigate the mode of action of phenols further, p-cresol and 2,4,6-trichlorophenol were used as individual substances in acute toxicity studies to *D. magna*. These were tested twice (Table 24 and Table 75, Appendix I). Water quality data are presented in Tables 114 and 115, Appendix II. The maximum and minimum EC50 values for each of these substances were within ± 33% and ± 28% of the means respectively (Table 86, Appendix I). This is again a result of adjustment of one or both of the replicate EC50 values to account for measured test concentrations (Table 93, Appendix I). The EC50 values were lower to *D. magna* than other reported values for these substances (Table 21). 2,4,6-trichlorophenol in particular was not consistent with reported values. Reported data were generally obtained at a pH above the pK_a of this substance which, as a result, was mainly present in its dissociated form. As toxicity is mainly a result of the diffusion of the undissociated form across membranes (Kishino and Kobayashi, 1995), the EC50 value reported in this thesis based on either nominal or measured test solution concentrations was lower than other reported EC50 values and probably a better representation of the true inherent toxicity.

GC-MS analysis of test concentrations from the selected test involving p-cresol showed that measured concentrations over the 48 hour test period had only 67% mean agreement with nominal concentrations requiring adjustment of the EC50 value based on nominal test concentrations (Table 93, Appendix I). There was only one appreciable drop in concentration, however, for the highest concentration analysed over the first 24 hour period of the toxicity test. As the measured concentration for this concentration at 0 hours was inconsistent with the

Table 24. Observed EC50 values for p-cresol and 2,4,6-trichlorphenol

Substance	MW	log P*[a]	Observed	Predicted EC50[e]
			EC50 (mg/l)#[b]	(mg/l)#[c]
p-cresol	108	1.96	6.0[d]	19
2,4,6-trichlorophenol	197.5	4.03	0.35[d]	1.7

* Calculated by Hansch and Leo (1979) method. No Roberts (1991) PDBF required in calculations.

Values to 2 s.f.

• Values to 3 s.f.

[b] Mean values. All test replicates and confidence intervals presented in Table 75, Appendix I

[c] Values calculated in mol/l before conversion to mg/l.

[d] EC50 value corrected for measured test solution concentrations (Table 93, Appendix I).

• Calculated using 0.63 log P + 2.52 (equation 10).

subsequent measurements at 24 hours of the old and renewed test solutions and at 48 hours, it may be that this measured value was in error. However, without replication of measurements it was not possible to establish this fact with certainty and the value was assumed to be accurate for the purposes of establishing a mean measured concentration for the test. The remaining measured concentrations showed <20% drop over the 24 hour period indicating that there was acceptable loss of test substance over the 48 hour test period. Overall differences between nominal and mean measured concentrations indicated either loss of test substance during the storage period prior to analysis or error in stock preparation due to solubility problems. Whilst measured concentration values were spurious over the long term phenol stability study, there was no evidence to suggest that method of storage was insufficient for the given period for phenolic substances and thus stock preparation was likely to be at fault (Figs. 13 and 14).

GC-MS analysis of test solution concentrations of 2,4,6-trichlorophenol in replicates 1 and 2 exhibited only 76% and 52% mean agreement between nominal and measured concentrations (Table 93, Appendix I). Stock solution concentration was below the reported water solubility of 800 mg/l (Shigeoka *et al.*, 1988). In general the lowest measured concentrations were more consistent over the 48 hour toxicity test period than were the highest concentrations. There was no trend in loss of measured concentration over any 24 hour period and in some cases they appeared to increase over 24 hours by as much as 100%. Repeated measured values also showed discrepancy. In general, however, it can be seen that the measured values showed ≥20% difference from nominal values and the EC50 value based on nominal concentrations was adjusted according to mean % difference between nominal and mean measured values.

Whilst it is clear that there was some problem in measuring the concentration of 2,4,6-trichlorophenol and any measured values must be treated with caution, neither p-cresol nor 2,4,6-trichlorophenol was used in any mixture toxicity tests or for any other analysis in this thesis other than to provide an

overview of the QSAR of phenols to *D. magna*. When plotted with phenol and 2-chlorophenol as log (1/EC50) in mol/l versus log P (Fig. 15), calculated using the Hansch and Leo (1979) method, the data yielded the regression line :

$$\log (1/EC50) = 0.65 \log P + 3.16 \qquad \text{Equation 17.}$$

(n = 4, rsq = 0.959, se = 0.185)

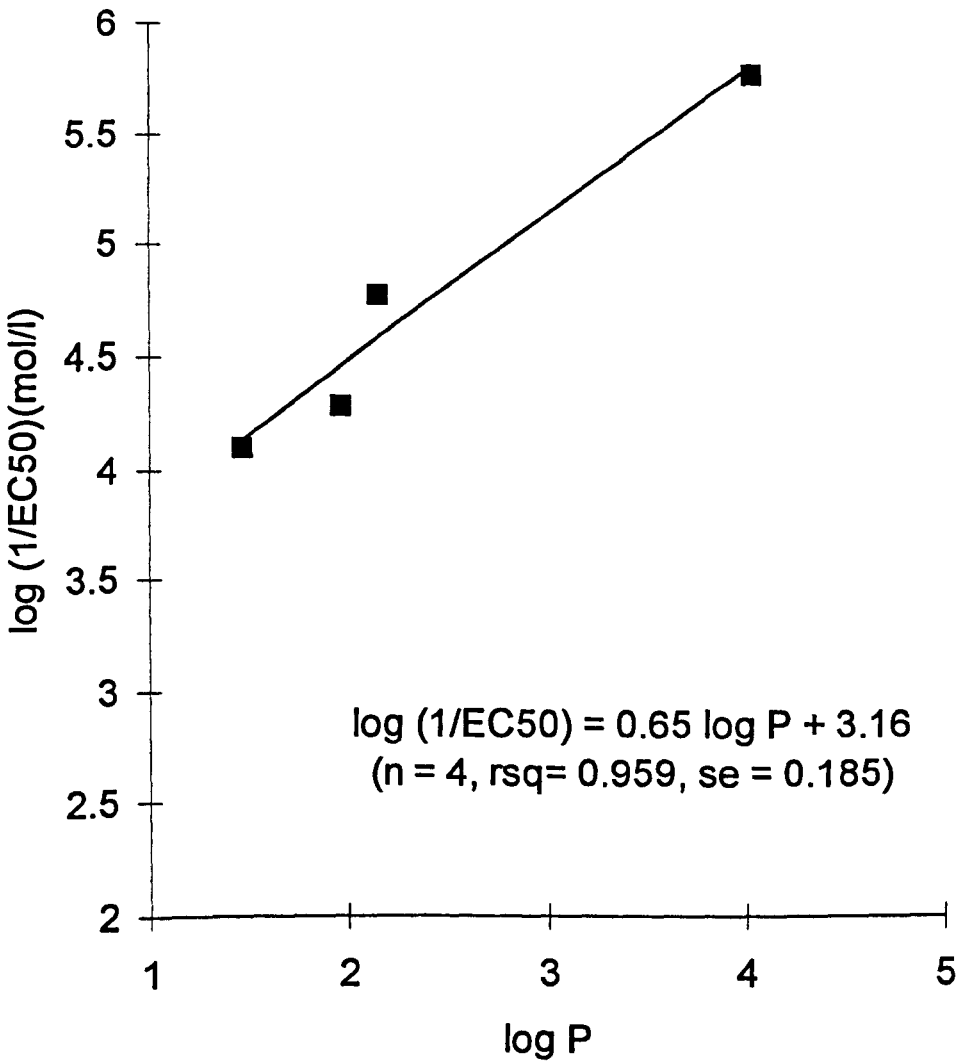
Thus a QSAR similar to that reported by Saarikoski and Viluksela (1982) (equation 10) would appear to be developing. The rsq value is good for equation 17, but it is not a convincing relationship based on only four values. To develop a reliable QSAR for phenols to *D. magna*, is beyond the scope of this thesis, but equation 17 does provide some indication that the toxicity and mode of action of phenols to *D. magna* is consistent with other reported polar narcosis QSARs (Table 2).

The toxicity of C11.8 LAS (Table 20) was fairly consistent with other reported toxicity data to both *D. magna* and other species (Table 1). The maximum and minimum EC50 values were within ±18% of the mean (Table 85, Appendix I).

MBAS analysis of test solutions from selected mixture toxicity tests showed that measured test concentrations over the 48 hour test period had <20% mean difference from nominal test concentrations (Table 95, Appendix I). There was no appreciable drop in concentration over a 24 hour period indicating that there was no loss of test material due to degradation. It can be assumed, therefore, that test organisms were exposed to concentrations close to nominal. No adjustment was required to the EC50 value based on nominal concentrations.

No reduction in the bioavailability would be expected as a result of the presence of micelles as the observed EC50 value for C11.8 LAS (5.6 mg/l, 0.017 mmol/l) occurs well below the CMC for C12 LAS as the nearest reported worst case (Table 4). The observed EC50 for C11.8 LAS can, therefore, be considered to be an accurate description of inherent toxicity of this substance to *D. magna*.

Fig. 15. Log P versus log (1/EC50)(mol/l) for phenols



3.3.2.2 REFERENCE SUBSTANCES - NON-POLAR NARCOTICS

EC50 values for alcohols used as reference substances are based only on nominal concentrations (Table 20). Time restrictions prevented analysis of all stored samples and preference was given to phenolic substances with which there were more potential errors, such as dissociation, degradation etc., and mixture studies. Reproducibilty of results was good with most maximum and minimum EC50 values being within ±3% of the mean with the exception of 1-octanol which showed ±9.8% such variation (Table 85, Appendix I). EC50 values are consistent with reported values for various species (Table 25). In addition all replicate EC50 values and hence mean measured EC50 values were well predicted by the general narcosis equation (equation 9)(Table 20). Plotted as log P, calculated using the Hansch and Leo (1979) method, versus log 1/EC50 (mol/l) (Fig. 16), produced the regression line:

$$\log(1/EC50) = 0.85 \log P + 1.18 \qquad \text{Equation 18.}$$
$$(n = 5, rsq = 0.998, se = 0.0538)$$

which is very similar to the baseline narcosis equation of Könemann (1981) (equation 9). Although only based on 5 values, the statistics are fairly good and it suggests a non-polar narcosis mode of action for alcohols as expected.

3.3.3 MIXTURE TOXICITY

Concentration addition would be expected for all substances which have the same mode of action, as previously discussed (Hermens *et al.*, 1985). Thus in all combinations of mixtures used in toxicity studies in this thesis (Table 9), one component of the binary mixture was selected to be either a polar narcotic or a non-polar narcotic as the reference substance. Mean EC50 values for phenols, LAS, alcohols and FAES substances are all good representations of their respective inherent individual toxicity. In terms of TUs the EC50 of an individual

Fig. 16. Log P versus log (1/EC50)(mol/l) for alcohols

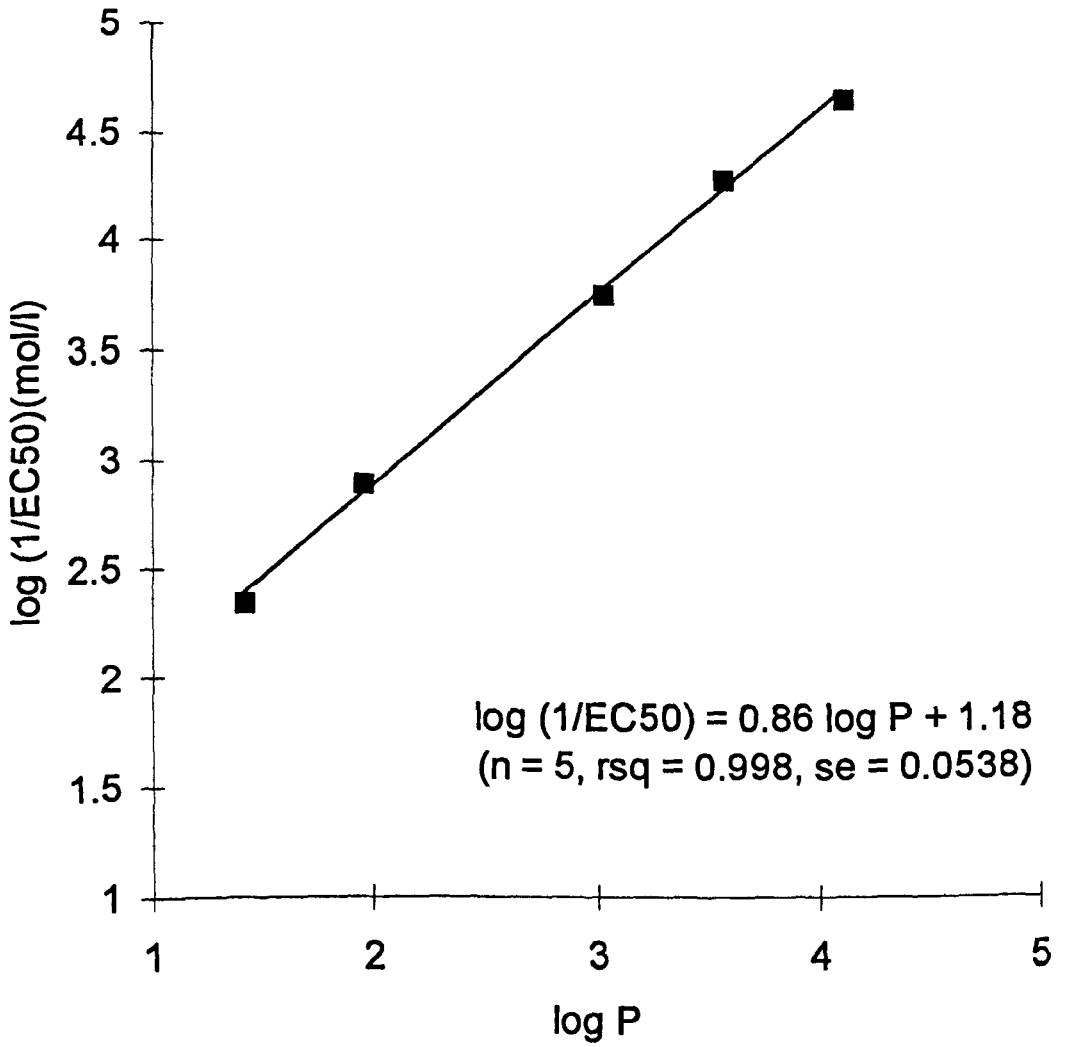


Table 25. Toxicity data for alcohols

Species	TD	LC50 (mg/l)*				Reference
		1-Pentanol	1-Hexanol	1-Octanol	1-Decanol	
D. pulex	24	340	150	27	3.2	Ikemoto *et al.*,1992
O. latipes	96	600	170	18	5.5	Ikemoto *et al.*,1992
P. promelas	96	600	130	14	5.5	Broderius and Kahl, 1985
Golden Orfe	24	-	-	20	-	Zhao and Wang, 1993
D. magna	48	400	130	23	3.5	Table 20[a]

TD: Test duration (hours)

* Values to 2 s.f.

[a] Values reported in this thesis.

compound is defined as 1 TU for that substance. In each of the mixtures the concentration of each component at the EC50 of the mixture was determined as a fraction of its individual EC50 value to provide the TU for each component (equation 14). These were summed to determine a total TU at the EC50 of the mixture. If FAES substances are acting by polar narcosis then concentration addition would be expected to be observed for all mixture studies where the second component was either a phenol or LAS. Thus a total TU = 1 would be observed, within the limits of experimental error, if strict concentration addition occurred. Response addition would be expected for mixtures of FAES and alcohols which would result in total TU = 2.

All mixtures reported in this thesis could be considered to behave by concentration addition, response addition or less than concentration addition. None of the mixtures was observed to exhibit synergistic behaviour.

3.3.3.1 INITIAL MIXTURE TOXICITY STUDIES

Where possible, mixture toxicity studies were performed twice. For each test replicate the TUs of each component at the EC50 were calculated for each component and hence total TU at the EC50 of the mixture as described. For replicates of a particular test mixture, mean total TU was calculated for that mixture. Table 26 shows mean total TU and mean observed EC50 values for each of the initial mixtures studies. Table 27 expresses EC50 values and component TU values for each test replicate. Associated confidence limits are reported in Table 76, Appendix I. The polar narcosis and non-polar narcosis reference substances in these mixtures were C11.8 LAS and 1-decanol respectively. All values in Table 26 and 27 are based on nominal concentrations. Water quality data are reported in Tables 116 and 117, Appendix II. MBAS analysis of FAES and LAS substances in chapter 2 in general indicated that measured concentrations do not differ greatly from nominal concentrations (Tables 91 and 92, Appendix I). The EC50 of 1-decanol is well within its limit of solubility and combined with the high rsq value from the regression analysis of alcohol toxicity,

Table 26. Mean observed EC50 values and total TU at the EC50 for mixtures with LAS and 1-decanol as the reference substances

Mixture		% # (A,B)	Mean EC50 of mixture (mg/l)*	Mean Total TU at EC50*
A	B			
LAS	C13 methyl	30,70	12	0.83
LAS	C13 methyl	20,80	21	1.2
LAS	C13 methyl	10,90	26	1.0
LAS	C12 methyl	20,80	37	1.5
LAS	C12 ethyl	30,70	20	1.1
LAS	C7 heptyl	30,70	20	1.2
LAS	C10 n-butyl	30,70	17	0.96
LAS	C10 n-butyl	20,80	30	1.2
LAS	C10 n-butyl	10,90	42	0.96
LAS	C8 hexyl	20,80	25	0.97
1-decanol	C13 methyl	20,80	14	0.88
1-decanol	C12 ethyl	20,80	14	1.0
1-decanol	C7 heptyl	20,80	15	0.93
1-decanol	C8 hexyl	20,80	14	0.86

A: Reference substance component, B: Test substance component (FAES).

* Values to 2 s.f..

nominal % component in mixture.

Table 27. Observed EC50 and TU values for each test replicate mixture with LAS and 1-decanol as the reference substances

Mixture		% # (A,B)	Test Replicate	EC50 (mg/l)*	TU of component at EC50 of mixture*		Total TU*
A	B				A	B	
LAS	C13 methyl	30,70	1	12	0.63	0.20	0.83
LAS	C13 methyl	20,80	1	25	0.88	0.49	1.4
			2	17	0.60	0.33	0.93
LAS	C13 methyl	10,90	1	26	0.46	0.57	1.0
LAS	C12 methyl	20,80	1	37	1.3	0.21	1.5
LAS	C12 ethyl	30,70	1	15	0.79	0.070	0.86
			2	20	1.3	0.12	1.4
LAS	C7 heptyl	30,70	1	18	0.95	0.090	1.0
			2	22	1.2	0.11	1.3
LAS	C10 n-butyl	30,70	1	17	0.89	0.070	0.96
LAS	C10 n-butyl	20,80	1	28	0.98	0.13	1.1
			2	32	1.1	0.15	1.3
LAS	C10 n-butyl	10,90	1	42	0.74	0.22	0.96
LAS	C8 hexyl	20,80	1	28	0.98	0.12	1.1
			2	21	0.74	0.099	0.84
1-decanol	C13 methyl	20,80	1	11	0.63	0.21	0.84
			2	16	0.91	0.31	1.2
1-decanol	C12 ethyl	20,80	1	15	0.86	0.080	0.94
			2	13	0.74	0.069	0.81
1-decanol	C7 heptyl	20,80	1	18	1.0	0.10	1.1
			2	12	0.69	0.069	0.76
1-decanol	C8 hexyl	20,80	1	14	0.80	0.062	0.86

A: Reference substance component. B: Test substance component (FAES).

nominal % component in mixture. * Values to 2 s.f..

there is no reason to expect large differences between measured and nominal concentrations for either substance. It should be noted that the relative proportions of components in these mixtures were not calculated according to equation 16. Relative % components of substances were chosen for these initial mixture studies from predictions calculated by :

$$(1/EC50)_{mixture} = (1/100)\ \Sigma\ [\%component_i / EC50_i] \qquad \text{Equation 19.}$$

Equation 19 is an extension of TU theory which can be used for prediction of toxicity of mixtures of smaller numbers of components which act by the same mode of action. Thus whilst components within a mixture may not be present at equitoxic concentrations, prediction of toxicity can in theory be calculated for any mixture of similarly acting substances where the % component is known and where one component is not considerably more toxic than the other. Predictions of EC50 values of the mixtures were calculated assuming concentration addition for 10%,90%, 20%,80% and 30%,70% mixtures and toxicity tests carried out accordingly.

Where replicates have been carried out most maximum and minimum total TU values are within ±20% of the mean (Table 87, Appendix I).

For the 1-decanol / FAES mixtures, there was an average ±0.14 TU deviation from strict additivity (Table 27) indicating concentration addition and non-polar narcosis mode of action for FAES substances, contrary to prediction. Further investigation revealed that response addition may occur although the FAES component was present in each of these mixtures at a concentration below the threshold which would be expected to induce any significant response.

At the observed EC50 for an equitoxic binary mixture, a total of 1TU would be observed, within experimental error, for components which behave by concentration addition and a total of 2 TU for those acting by response addition as previously discussed. For non-equitoxic binary mixtures, a total of 1 TU would still be observed at the EC50 for components which act by concentration addition by virtue of being able to exchange a proportion of one component with the other

without altering the observed response. For non-equitoxic binary mixtures of components which behave by response addition, however, the observed total TU will depend on the TUs of each component relative to each other, expressed as a proportion of the total TU for the mixture. As a result the observed EC50 for any non-equitoxic binary mixture of components which act by response addition will result largely when the proportionally greatest component TU = 1. It is, therefore, possible to predict toxicity of mixtures, in terms of TU, for non-equitoxic binary mixtures of components which are predicted to act by response addition. As an example, the predicted total TU value for replicate 1 for the 20%,80% mixture of 1-decanol / C12 ethyl FAES is calculated as follows (as for equation 16):

1) TU for 1-decanol as a proportion of total at EC50 of mixture :

$$\frac{(0.2 / \text{EC50 1-decanol})}{(0.2 / \text{EC50 1-decanol} + 0.8 / \text{EC50 C12 ethyl FAES})} = 0.91(91\%) \quad \text{Equation 20.}$$

2) TU C12 ethyl FAES as % of total at EC50 of mixture = 0.09 (9%)

Equation 21.

3) Predicted total TU of mixture : 1 + (1/0.91 x 0.09) = 1.1 (2 s.f.)

Equation 22.

Table 28 shows the largest component TU value for each replicate as a proportion of the total with predicted total TU values calculated by both the response addition and concentration addition models for the 1-decanol mixtures. The individual toxicity of the 1-decanol component was considerably greater than the FAES component and accounted for ≥91% of the total TU observed at the EC50 of the mixture with the exception of 1-decanol / C13 methyl FAES mixtures in which 1-decanol accounted for 75% of the total TU. The predicted total TU = 1.1 and 1.3 for all mixtures by the response addition model compared to total TU = 1 predicted by the concentration addition model. Whilst the FAES components were at a low percentage of the respective EC50, even at TUs of 0.02, 50% mortality has been observed in a mixture of 50 chemicals acting by concentration

Table 28. Observed and predicted total TU values for mixtures with 1-decanol as the reference substance

Mixture		% # (A,B)	TR	LCT*	Total TU at EC50*	Predicted total TU at EC50*	
A	B					C	D
1-decanol	C13 methyl	20,80	1	0.75	0.84	1.0	1.3
			2	0.75	1.2	1.0	1.3
1-decanol	C12 ethyl	20,80	1	0.91	0.94	1.0	1.1
			2	0.91	0.81	1.0	1.1
1-decanol	C7 heptyl	20,80	1	0.91	1.1	1.0	1.1
			2	0.91	0.76	1.0	1.1
1-decanol	C8 hexyl	20,80	1	0.93	0.86	1.0	1.1

A: Reference substance component.

B: Test substance component (FAES).

C: Calculated by concentration addition model.

D: Calculated by response addition model.

TR: Test replicate.

LCT: Largest component TU as proportion of total TU for mixture (equation 20).

* Values to 2 s.f..

Nominal % component in mixture.

addition (Hermens *et al.* 1985). The lowest such value for any FAES component in the 1-decanol / FAES mixtures in this thesis was 0.06 and would, therefore, be expected to contribute to the toxicity of the mixture if acting by concentration addition with the reference component. By application of hypothesis tests for the mean of the population of differences of observed total TU values and values predicted by both concentration and response addition models as paired samples, the observed total TU values favoured prediction calculated by the concentration addition model (calculated t < tabulated t) and not the response addition model (calculated t > tabulated t) at the 5% level (Tables 29 and 30)(Rees, 1989). However, when considering the inherent error in the system associated with a degree of subjective decision in immobility observation, nominal concentration based EC50 values etc., the close similarity in predictions from both models made it impossible to establish whether the FAES component was acting by concentration addition with 1-decanol as a non-polar narcotic or as a different mode of action but was present at a concentration below the threshold to exhibit any significant response.

At low environmental concentrations, all hydrophobic substances act on common secondary sites of action and are considered to act by non-polar narcosis (equation 9) (Könemann, 1981). This effect is often masked by other more toxic effects at concentrations in the region of the EC50 of the substance. As a result a substance at such a low concentration could still be considered to contribute to mixture toxicity with other substances of similar mode of action at low concentration. A third possibility for the observed total TU values is that concentration addition occurred between FAES substances and 1-decanol as a result of FAES acting by the secondary non-polar narcosis mechanism due to its presence at low concentrations.

For LAS and FAES mixtures which were expected to behave by concentration addition, with the exception of LAS / C12 methyl FAES, there was an approximate ±0.2 TU deviation from strict concentration addition (Table 26). This is consistent variability with reported mixture toxicity investigations of Broderius *et al.* (1995). This indicated polar narcosis mode of action for FAES

Table 29. Calculated and tabulated t values for mean of differences between observed total TU values at the EC50 of mixtures and predicted values calculated by the concentration addition model

	Reference substance of mixtures.					
	LAS[a]	LAS[b]	LAS[c]	1-decanol	Phenol[d]	Alcohols
H_0/H_1	A	A	A	A	A	A
d(mean)	0.099	0.11	0.096	0.070	0.12	0.77
sd	0.225	0.254	0.229	0.162	0.246	0.316
n	15	3	12	7	12	30
α	0.05	0.05	0.05	0.05	0.05	0.05
υ	14	2	11	6	11	29
Calc. t	1.70	0.751	1.45	1.14	1.66	13.3
Tab. t	1.761	2.920	1.796	1.943	1.796	1.699
Conc.	Accept H_0	Accept H_0	Accept H_0	Accept H_0	Accept H_0	Reject H_0

A: H_0: $\mu_d = 0$: predicted and observed values give same mean score.

A: H_1: $\mu_d > 0$: observed values give higher mean score than predicted values.

Conc.: Conclusion - accept or reject H_0.

[a] All LAS mixtures.

[b] LAS mixtures for which predicted total TU values calculated by the concentration and response addition models appear significantly different.

[c] LAS mixtures for which predicted total TU values calculated by the concentration and response addition models do not appear significantly different.

[d] Includes 2-chlorophenol mixtures.

Table 30. Calculated and tabulated t values for mean of differences between observed total TU values at the EC50 of mixtures and predicted values calculated by the response addition model

	Reference substance of mixtures.					
	LAS[a]	LAS[b]	LAS[c]	1-decanol	Phenol[d]	Alcohols
H_0/H_1	A	A	A	A	A	A
d(mean)	0.13	0.52	0.038	0.23	0.66	0.043
sd	0.333	0.303	0.270	0.155	0.366	0.354
n	15	3	12	7	12	30
α	0.05	0.05	0.05	0.05	0.05	0.05
υ	14	2	11	6	11	29
Calc. t	1.57	2.99	0.48	3.89	6.26	0.671
Tab. t	1.761	2.92	1.796	1.943	1.796	1.699
Conc.	Accept H_0	Reject H_0	Accept H_0	Reject H_0	Reject H_0	Accept H_0

A: H_0: $\mu_d = 0$: predicted and observed values give same mean test score.

A: H_1: $\mu_d > 0$: predicted values give higher mean score than observed values.

Conc.: Conclusion - accept or reject H_0.

[a] All LAS mixtures.

[b] LAS mixtures for which predicted total TU values calculated by the concentration and response addition models appear significantly different.

[c] LAS mixtures for which predicted total TU values calculated by the concentration and response addition models do not appear significantly different.

[d] Includes 2-chlorophenol mixtures.

substances. The toxicity of LAS was high relative to those of the FAES substances, resulting in higher TU and similar predictions made by both the response and concentration addition models for the majority of these mixtures. Hypothesis testing for the mean of differences revealed total TU values to be equally well predicted by both concentration and response addition models when applied to all mixtures with LAS as the reference component (Tables 29 and 30). The similarity between predictions calculated by both models made it difficult to establish mode of action for FAES substances from observed EC50 values. The exceptions to this were LAS / C13 methyl FAES as 20%,80% and 10%,90% mixtures, in which significantly larger total TUs were predicted by the response addition model than were observed, where predicted total TU at the EC50 were calculated using equations 20,21 and 22 (Table 31). When these values were separated from the other LAS mixtures and hypothesis tests reapplied, these three values were found now to be significantly different from predicted values calculated by the response addition model (Table 30) and similar to those calculated by the concentration addition model (Table 29). This indicated polar narcosis mode of action for the FAES substances in these studies (Table 31). The remaining mixtures, tested separately, could still not be statistically separated from either model. It would seem reasonable, therefore, to assume that the remaining LAS / FAES mixtures were also acting by concentration addition. No replicate was performed for the LAS / C12 methyl FAES mixture as the outlier due to lack of test substance.

These initial mixture toxicity studies proved inconclusive as to the mode of action of FAES substances although for the highlighted LAS / C13 methyl FAES mixtures, the observed concentration addition indicated polar narcosis.

Table 31. Observed and predicted total TU values for mixtures with LAS as the reference substance

Mixture		% # (A,B)	TR	LCT*	Observed TU at EC50*	Predicted total TU at EC50*	
A	B					C	D
LAS	C13 methyl	30,70	1	0.76	0.83	1.0	1.3
LAS	C13 methyl	20,80	1	0.63	1.4	1.0	1.6
			2	0.65	0.93	1.0	1.5
LAS	C13 methyl	10,90	1	0.55	1.0	1.0	1.8
LAS	C12 methyl	20,80	1	0.87	1.5	1.0	1.1
LAS	C12 ethyl	30,70	1	0.92	0.86	1.0	1.1
			2	0.93	1.4	1.0	1.1
LAS	C7 heptyl	30,70	1	0.91	1.0	1.0	1.1
			2	0.92	1.3	1.0	1.1
LAS	C10 n-butyl	30,70	1	0.93	0.96	1.0	1.1
LAS	C10 n-butyl	20,80	1	0.88	1.1	1.0	1.1
			2	0.88	1.3	1.0	1.1
LAS	C10 n-butyl	10,90	1	0.77	0.96	1.0	1.3
LAS	C8 hexyl	20,80	1	0.89	1.1	1.0	1.1
			2	0.88	0.84	1.0	1.1

A: Reference substance component. B: Test substance component (FAES).

C: Calculated by concentration addition model.

D: Calculated by response addition model.

TR: Test replicate.

LCT: Largest component TU as proportion of total TU for mixture.

* Values to 2 s.f..

Nominal % component in mixture.

3.3.3.2 MIXTURE STUDIES WITH PHENOLS - CONCENTRATION ADDITION PREDICTED

Most mixture studies in this section were repeated twice. The concentration of each component at the EC50 was expressed in terms of TUs and summed to produce a total TU for each study as for previous studies. Table 32 shows mean total TU and mean observed EC50 values for each of the mixtures in which concentration addition was predicted. Table 33 expresses EC50 values and component TU values for each of these test replicates. Associated confidence limits are presented in Table 77, Appendix I. Water quality data are presented in Tables 118 and 119, Appendix II. TU and EC50 values were initially calculated based on nominal values and then adjusted for mean differences between nominal and measured concentrations following MBAS / GC-MS analysis of the individual components (Table 94 and 95, Appendix I). Table 34 shows mean EC50 and TU values for each mixture following adjustment for test solution concentration analysis. Previously adjustments were made to observed EC50 values where ≥20% mean difference occurred between nominal and measured concentrations. Due to the subtle differences being observed in mixture toxicity, however, adjustments were made to the TUs of each component even when the difference represented a change of only a few percent (Tables 94 and 95, Appendix I).

Results based on nominal concentrations initially indicated less than additive joint toxicity, contrary to prediction (Table 32). Following MBAS analysis of FAES and LAS components in mixtures with phenol, ≤20% mean difference between measured test solution concentrations and nominal concentrations was found, with the majority much less (Table 95, Appendix I). Adjustments were made to the TU of the FAES component as previously mentioned (Table 35). A mean of 0.18 mg/l contamination of methylene blue active substance was observed in 24 hour old, 24 hour new and 48 hour control samples of the phenol / C14 methyl FAES mixture. This was most probably C14 methyl FAES. Contamination of glassware, both test and storage vessels, is a possible explanation although

Table 32. Mean observed EC50 values and total TU values at the EC50 for mixtures with phenols as the reference substances (no adjustments for test solution concentration analysis).

Mixture		% # (A,B)	Mean EC50 of mixture (mg/l)*[a]	Mean Total TU at EC50*[a]
A	B			
Phenol	C14 methyl	46,54	8.6	1.1
Phenol	C12 amyl	51,49	13	1.8
Phenol	C14 ethyl	48,52	12	1.6
Phenol	C12 n-butyl	32,68	17	1.5
Phenol	C11.8 LAS	56,44	6.4	0.97
2-CP	C14 n-butyl	47,53	4.0	1.4

A: Reference substance component. B: Test substance component (FAES, LAS).

* Values to 2 s.f..

Nominal % component to give equitoxic concentrations in mixture.

[a] Values calculated from replicate values based on nominal concentrations (no adjustments for test solution concentration analysis)(See Table 33 for replicate values).

Table 33. Observed EC50 and TU values for each test replicate mixture with Phenols as the reference substances (no adjustments for test solution concentration analysis)

Mixture		% # (A,B)	TR	EC50 (mg/l)*[a]	TU of component at EC50 of mixture*[b]		Total TU*
A	B				A	B	
Phenol	C14 methyl	46,54	1	10	0.62	0.64	1.3
			2	7.1	0.44	0.45	0.89
Phenol	C12 amyl	51,49	1	12	0.83	0.82	1.7
			2	14	0.96	0.95	1.9
Phenol	C14 ethyl	48,52	1	13	0.84	0.85	1.7
			2	11	0.71	0.72	1.4
Phenol	C12 n-butyl	32,68	1	16	0.69	0.68	1.4
			2	18	0.78	0.77	1.6
Phenol	C11.8 LAS	56,44	1	6.4	0.48	0.49	0.97
			2	6.3	0.48	0.49	0.97
2-CP	C14 n-butyl	47,53	1	4.7	0.96[c]	0.67[c]	1.6
			2	3.2	0.65[c]	0.46[c]	1.1

A: Reference substance component. B: Test substance component (FAES, LAS).

TR: Test Replicate. 2-CP: 2-chlorophenol.

Nominal % component to give equitoxic concentrations in mixture.

* Values to 2 s.f.,.

[a] EC50 value of mixture.

[b] Values based on nominal concentrations.

[c] Components not at equitoxic concentrations since tests performed before GC-MS analysis of test solution concentrations of 2-CP required adjustment of EC50 of individual reference substance.

Table 34. Mean observed EC50 values and total TU values at the EC50, adjusted for measured component concentrations by MBAS and GC-MS, for mixtures with phenol as the reference substance

Mixture		% # (A,B)	Mean EC50 of mixture (mg/l)*[a]	Mean Total TU at EC50*[a]
A	B			
Phenol	C14 methyl	46,54	7.4	0.92
Phenol	C12 amyl	51,49	9.8	1.3
Phenol	C14 ethyl	48,52	9.5	1.2
Phenol	C12 n-butyl	32,68	16	1.4
Phenol	C11.8 LAS	56,44	6.1	0.94
2-CP	C14 n-butyl	47,53	2.7	0.99

A: Reference substance component. B: Test substance component (FAES, LAS).

* Values to 2 s.f.. 2-CP: 2-chlorophenol.

Nominal % component to give equitoxic concentrations in mixture.

[a] Values calculated from replicate values based on measured concentrations (See Table 35 for replicate values and Tables 94 and 95, Appendix II for analysis results).

Table 35. Observed EC50 and TU values, adjusted for measured component concentrations by MBAS and GC-MS, for each test replicate mixture with phenol as the reference substance

Mixture		% # (A,B)	TR	EC50 (mg/l)*[a]	TU of component at EC50 of mixture*[b]		Total TU*
A	B				A	B	
Phenol	C14 methyl	46,54	1	8.2	0.43	0.59	1.0
			2	6.6	0.38	0.45[c]	0.83
Phenol	C12 amyl	51,49	1	7.6	0.37	0.67	1.0
			2	12	0.71	0.93	1.6
Phenol	C14 ethyl	48,52	1	11	0.46	0.94	1.4
			2	7.9	0.29	0.72	1.0
Phenol	C12 n-butyl	32,68	1	15	0.76	0.56	1.3
			2	17	0.71	0.76	1.5
Phenol	C11.8 LAS	56,44	1	5.9	0.44	0.46	0.90
			2	6.3	0.48[c]	0.49[c]	0.97
2-CP	C14 n-butyl	47,53	1	2.5	0.49	0.54	1.0
			2	2.9	0.51	0.46[c]	0.97

A: Reference substance component, B: Test substance component (FAES, LAS).
TR: Test Replicate. 2-CP: 2-chlorophenol.
nominal % component to give equitoxic concentrations in mixture.
* Values to 2 s.f..
[a] Adjusted EC50 value of mixture - calculated from adjusted TU and individual EC50 values of each component.
[b] Values based on measured concentrations - adjusted from those presented in Table 33 according to measured concentrations as mean % of nominal (Tables 94 and 95, Appendix I).
[c] No test solution concentration analysis performed - no adjustment made.

prewashing usually prevented such contamination. No contamination was observed in the 0 hour sample. The rarity of observed contamination in the control samples suggests contamination at the analysis stage and not in test solutions during the test period. No observable effect was noted on control organisms and due to the low concentration involved the contamination was considered insignificant. No other control contamination was observed in the other studies analysed.

GC-MS analysis of phenol and 2-chlorophenol components revealed mean agreement between measured and nominal concentrations to be highly variable, ranging from 41% to 110% mean agreement between measured and nominal concentrations between studies (Table 94, Appendix I). Only five of the thirteen studies measured showed mean difference of <20%. The initial explanation for these low measured concentrations compared to nominal was loss of test substance during the storage period before analysis, due to either microbial or chemical activity. There was no correlation, however, between loss of test substance and the storage period. Table 36 shows storage time of studies before GC-MS analysis of phenols. These are maximum storage times since there was a 48 hour lag between samples preserved at 0 hour and those at 48 hour and analyses of samples from a particular study were performed over several days in some instances. Stability studies performed to investigate this phenomenon indicated that there was no evidence for phenol degradation in samples stored with the addition of 3% formalin over the storage period (see 3.3.1 Stability of phenol studies for results and discussion).

For the toxicity studies with a large 95% confidence interval for mean % agreement of measured and nominal concentrations, this was indicative of considerable variability of measured concentrations as a % of nominal about the mean (Table 94, Appendix I). Clearly as the error term is inversely proportional to n, increasing the number of measured values for each study would decrease the error term. However, where variability was observed to be large, this was mostly due to loss of phenol over a 24 hour period, in some cases as a result of complete loss of substance. In such studies, partial or complete loss of phenol was

Table 36. Storage times and measured concentrations as mean percent of nominal for phenol samples

	Mixture	Storage Time (days)	Measured Concentration Phenol / 2-CP (mean % of nominal)*
Phenol	C12 n butyl FAES	16	110
Phenol	C14 ethyl FAES	20	41
Phenol	C11.8 LAS	22	92
Phenol	C12 amyl FAES	36	46
Phenol	C12 amyl FAES	42	76
Phenol	C14 ethyl FAES	43	55
Phenol	C12 n butyl FAES	44	91
Phenol	Nonanol	49	89
2-CP	C14 butyl FAES	52	51
Phenol	Nonanol	57	66
Phenol	C14 methyl FAES	58	86
Phenol	C14 methyl FAES	60	70
2-CP	C14 butyl FAES	64	78

* Values to 2 s.f.

2-CP: 2-chlorophenol.

observed for both measured concentrations and over both 0-24 hour and 24-48 hour periods, although not necessarily at a constant rate. This would indicate that the difference between measured and nominal concentration was, at least in part, due to degradation or adsorption of phenol over the test period.

Result of phenol stability studies (Tables 12, 14, 16 and 18) show 73%±1.78% agreement of measured concentrations with nominal concentrations at time t = 0 for all concentrations and replicates with and without formalin preservative. GC-MS analysis was performed immediately on these samples resulting in no storage time. The low variability in measurements indicated reproducibility in test solution preparation and in the GC-MS measurement technique although there is always some inherent error. Loss of material may have been due to stock preparation technique. Alternatively due to the hygroscopicity of the sample and consequent absorption of water during storage, the assumed activity of the phenol sample during weighing was incorrect with resulting disagreement in measured and nominal concentrations.

No satisfactory explanation could be found for the difference between measured and nominal concentrations of phenol. There was no evidence for loss as a result of storage (section 3.3.1) and as a result total TUs were calculated for mixtures based on measured concentrations (Tables 34 and 35). For four of the six mixtures, the maximum and minimum total TU values were within ±10% of the mean based on measured concentrations where applicable (Table 88, Appendix I). For the remaining two studies, phenol / C14 ethyl FAES and phenol / C12 amyl FAES, maximum and minimum total TU values were within ±17% and ±23% of the mean respectively. Four of the six mixtures with mean total TUs were within ±0.2TU of strict additivity indicating these mixtures to be exhibiting strict additivity (Table 34). The two remaining mixtures were only within 0.4TU of strict additivity. Adjustments made for measured concentrations resulted in slight non-equitoxic concentrations of each component, due in part to the similarity in toxicity of the individual components. These were not the large imbalances observed for the initial 1-decanol and LAS mixture studies. Table 37 shows total TU values predicted by the response addition model (equations 20, 21 and 22), accounting

Table 37. Observed and predicted total TU values for mixtures with phenols as the reference substance

Mixture		% # (A,B)	TR	LCT*	Observed TU at EC50*	Predicted total TU at EC50*	
A	B					C	D
Phenol	C14 methyl	46,54	1	0.59	1.0	1.0	1.7
			2	0.54	0.83	1.0	1.9
Phenol	C12 amyl	51,49	1	0.67	1.0	1.0	1.5
			2	0.58	1.6	1.0	1.7
Phenol	C14 ethyl	48,52	1	0.67	1.4	1.0	1.5
			2	0.72	1.0	1.0	1.4
Phenol	C12 n-butyl	32,68	1	0.55	1.3	1.0	1.8
			2	0.47	1.5	1.0	2.1
Phenol	C11.8 LAS	56,44	1	0.51	0.90	1.0	2.0
			2	0.51	0.97	1.0	2.0
2-CP	C14 n-butyl	47,53	1	0.58	1.0	1.0	1.7
			2	0.47	0.97	1.0	2.1

A: Reference substance component.

B: Test substance component (FAES, LAS).

C: Calculated by concentration addition model.

D: Calculated by response addition model.

TR: Test replicate. 2-CP: 2-chlorophenol.

LCT: Largest component TU as proportion of total TU for mixture (equation 20).

* Values to 2 s.f..

Nominal % component in mixture.

for changes in relative concentrations of each component and by the concentration addition model which were unaffected by adjustments due to measured concentrations. Intuitively, most replicate values favoured the concentration addition model which was also strongly favoured statistically at the 5% level (Tables 29 and 30), despite some replicates behaving as less than additive. Concentration addition shown by phenol / LAS indicates that LAS does behave by a similar mode of action to polar narcosis, and is, therefore, an acceptable polar narcosis reference substance. Results are also strongly indicative of FAES behaving by a similar mode of action to polar narcosis.

3.3.3.3 MIXTURE STUDIES WITH ALCOHOLS - RESPONSE ADDITION PREDICTED

Most mixture studies in this section were repeated twice. The concentration of each component at the EC50 was expressed in terms of TUs and summed to produce a total TU for each study as for previous studies. Table 38 shows mean total TU and mean observed EC50 values for each of the equitoxic mixtures in which response addition was predicted. Table 39 expresses EC50 values and component TU values for each of these test replicates. Associated confidence limits are presented in Table 78, Appendix I. Water quality data are presented in Tables 120 and 121, Appendix II. All TUs were based on nominal values with the exception of 1-nonanol / phenol studies. For most of the mixtures, the maximum and minimum total TU values were within ±10% of the mean. The remaining mixtures of the nine for which the calculation applied replicates were within ±20% of the mean (Table 88, Appendix I). All equitoxic mixtures were within a mean of ±0.4TU of predictions calculated by response addition. Predicted total TU values calculated by the response and concentration addition models are presented in Table 40. Whilst predictions calculated by response addition are mostly TU = 2 for equitoxic concentrations, slight deviations from equitoxic concentrations in two mixtures resulted in slight lowering of the predicted total TU value (equations 20, 21 and 22). Adjustments for measured concentrations in 1-nonanol / phenol

Table 38. Mean observed EC50 values and total TU values at the EC50 for nominally equitoxic mixtures with alcohols as the reference substances

Mixture		% # (A,B)	Mean EC50 of mixture (mg/l)*[a]	Mean Total TU at EC50*[a]
A	B			
Pentanol	C8 sec hexyl	50,50	630	1.6
Hexanol	C12 ethyl	50,50	280	2.0
Hexanol	C10 butyl	43,57	230	1.6
Hexanol	C8 hexyl	41,59	270	1.7
Hexanol	C7 heptyl	50,50	250	1.9
Octanol	C12 butyl	60,40	37	1.9
Octanol	C13 methyl	36,64	55	1.7
Nonanol	C12 amyl	51,49	15	2.1
Nonanol	C14 methyl	47,53	19	2.3
Nonanol	C11.8 LAS	57,43	12	1.8
Nonanol	Phenol	51,49	[12] 16[b]	[1.7] 2.2[b]

A: Reference substance component, B: Test substance component (FAES, LAS).

* Values to 2 s.f..

Nominal % component to give equitoxic concentrations in mixture.

[a] Values calculated from replicate values (Table 39).

[b] Values adjusted according to measured concentrations as mean % of nominal (Table 94, Appendix I) - values in [ ] are based on measured concentrations.

Table 39. Observed EC50 and TU values for each test replicate mixture (nominally equitoxic) with alcohols as the reference substances

Mixture		% # (A,B)	TR	EC50 (mg/l) *[a]	TU of component at EC50 of mixture*[a]		Total TU*
A	B				A	B	
Pentanol	C8 sec hexyl	50,50	1	570	0.71	0.71	1.4
			2	690	0.86	0.86	1.7
Hexanol	C12 ethyl	50,50	1	290	1.1	0.97	2.1
			2	270	1.0	0.90	1.9
Hexanol	C10 butyl	43,57	1	250	0.83	0.84	1.7
			2	210	0.69	0.70	1.4
Hexanol	C8 hexyl	41,59	1	270	0.85	0.89	1.7
			2	270	0.85	0.89	1.7
Hexanol	C7 heptyl	50,50	1	200	0.77	0.71	1.5
			2	300	1.2	1.1	2.3
Octanol	C12 butyl	60,40	1	35	0.91	0.88	1.8
			2	39	1.0	0.98	2.0
Octanol	C13 methyl	36,64	1	58	0.91	0.91	1.8
			2	51	0.80	0.80	1.6
Nonanol	C12 amyl	51,49	1	16	1.1	1.1	2.2
			2	14	0.95	0.95	1.9
Nonanol	C14 methyl	47,53	1	18	1.1	1.1	2.2
			2	19	1.2	1.2	2.4
Nonanol	C11.8 LAS	57,43	1	12	0.91	0.91	1.8
			2	11	0.84	0.83	1.7
Nonanol	Phenol	51,49	1	18	[0.56]1.2	[1.1]1.2	[1.7]2.4
			2	14	0.95	[0.61]0.95	[1.6]1.9

A:Reference substance component. B:Test substance component (FAES, LAS). TR: Test Replicate. # Nominal % component to give equitoxic concentrations in mixture. * Values to 2 s.f.. [a] Values in [ ] based on measured concentrations, otherwise on nominal (Table 94, Appendix I analysis results).

Table 40. TU values for nominally equitoxic alcohol mixtures

Mixture		% # (A,B)	TR	LCT*	Total TU*	Predicted total TU	
A	B					C	D
Pentanol	C8 sec hexyl	50,50	1	0.50	1.4	1	2
			2	0.50	1.7	1	2
Hexanol	C12 ethyl	50,50	1	0.53	2.1	1	1.9
			2	0.55	1.9	1	1.8
Hexanol	C10 butyl	43,57	1	0.50	1.7	1	2
			2	0.50	1.4	1	2
Hexanol	C8 hexyl	41,59	1	0.51	1.7	1	2
			2	0.51	1.7	1	2
Hexanol	C7 heptyl	50,50	1	0.52	1.5	1	1.9
			2	0.52	2.3	1	1.9
Octanol	C12 butyl	60,40	1	0.51	1.8	1	2
			2	0.51	2.0	1	2
Octanol	C13 methyl	36,64	1	0.50	1.8	1	2
			2	0.50	1.6	1	2
Nonanol	C12 amyl	51,49	1	0.50	2.2	1	2
			2	0.50	1.9	1	2
Nonanol	C14 methyl	47,53	1	0.50	2.2	1	2
			2	0.50	2.4	1	2
Nonanol	C11.8 LAS	57,43	1	0.50	1.8	1	2
			2	0.50	1.7	1	2
Nonanol	Phenol	51,49	1	0.65	1.7	1	1.5
			2	0.59	1.6	1	1.7

A: Reference substance component. B: Test substance component (FAES, LAS).

C: Calculated by concentration addition. D: Calculated by response addition .

TR: Test replicate. LCT: Largest component TU as proportion of total TU for mixture (equation 20).

* Values to 2 s.f.. # Nominal % component in mixture.

mixtures (Table 94, Appendix I) resulted in considerable reduction in predicted total TU values for these replicates, in good agreement with observed total TUs.

Table 41 shows mean results of the non-equitoxic mixtures of alcohol and FAES substances. Replicate values are presented in Table 42. Associated confidence limits are presented in Table 79. Water quality data are presented in Tables 122 and 123, Appendix II. Maximum and minimum values were within ±15% of the mean for the 1-hexanol / C7 heptyl FAES (23%,77%) mixtures and within ±7.0% for the remaining mixtures (Table 88, Appendix I). Table 43 shows predicted total TU values calculated by the response (equations 20, 21 and 22) and concentration addition models. Observed total TU values at the EC50 of the mixtures were well predicted by the response addition model. No explanation can be given for 1-octanol / C13 methyl FAES (16%,84%) mixtures which exhibited considerably less toxicity than predicted. All these non-equitoxic mixtures exhibited significantly higher total TU values at the EC50 than predicted by concentration addition.

The observed total TU values for both equitoxic and non-equitoxic mixtures were consistent with values predicted by the response addition model, which proved highly significant at the 5% level (Table 30). Comparison of observed total TU values with values predicted by the concentration addition model proved highly insignificant at the 5% level (Table 29). This is strongly indicative of FAES behaving by a different mode of action to non-polar narcosis.

3.3.3.4 MIXTURE STUDIES - GENERAL COMMENTS

If the limits of classification of mixtures as strict concentration addition are to be defined by 1TU±0.2TU as denoted by Broderius *et al.* (1995), then it is reasonable to define the limits of strict response addition as the values predicted for equitoxic and non-equitoxic concentrations ±0.2TU in the same way. It follows that those mixtures which exhibited total TU exceeding such limits for either model, despite being strongly favoured by the respective model, could not be strictly classed into either of these categories. However, it must be recognised

Table 41. Mean observed EC50 values and total TU values at the EC50 for non-equitoxic mixtures with alcohols as the reference substances

Mixture		% # (A,B)	Mean EC50 of mixture (mg/l)*[e]	Mean Total TU at EC50*[e]
A	B			
Hexanol	C7 heptyl	73,27[a]	180	1.4
Hexanol	C7 heptyl	23,77[b]	180	1.3
Octanol	C12 butyl	50,50[c]	32	1.7
Octanol	C13 methyl	16,84[b]	78	2.2
Octanol	C13 methyl	50,50[d]	43	1.5

A: Reference substance component. B: Test substance component (FAES).

* Values to 2 s.f..

Nominal % component in mixture.

[a] % component to give 1 : 3 relative toxic concentrations of components A : B.

[b] % component to give 3 : 1 relative toxic concentrations of components A : B.

[c] % component to give 1 : 1.4 relative toxic concentrations of components A : B.

[d] % component to give 1.8 : 1 relative toxic concentrations of components A : B.

[e] See Table 42 for replicate values.

Table 42. Observed EC50 values and TU values at the EC50 for replicate non-equitoxic mixtures with alcohols as the reference substances

Mixture		% # (A,B)	TR	EC50 (mg/l)*[a]	TU of component at EC50 of mixture*[b]		Total TU*
A	B				A	B	
Hexanol	C7 heptyl	73,27[a]	1	180	1.0	0.35	1.4
Hexanol	C7 heptyl	23,77[b]	1	150	0.27	0.83	1.1
			2	200	0.35	1.1	1.5
Octanol	C12 butyl	50,50[c]	1	32	0.70	1.0	1.7
Octanol	C13 methyl	16,84[b]	1	82	0.57	1.7	2.3
			2	73	0.51	1.5	2.0
Octanol	C13 methyl	50,50[d]	1	41	0.89	0.50	1.4
			2	44	0.96	0.54	1.5

A: Reference substance component. B: Test substance component (FAES).

TR: Test replicate.

* Values to 2 s.f..

Nominal % component in mixture.

[a] % component to give 1 : 3 relative toxic concentrations of components A : B.

[b] % component to give 3 : 1 relative toxic concentrations of components A : B.

[c] % component to give 1 : 1.4 relative toxic concentrations of components A : B.

[d] % component to give 1.8 : 1 relative toxic concentrations of components A : B.

Table 43. Observed and predicted total TU values at the EC50 for replicate non-equitoxic mixtures with alcohols as the reference substances

Mixture		% # (A,B)	TR	LCT*	Total TU*	Predicted total TU	
A	B					C	D
Hexanol	C7 heptyl	73,27[a]	1	0.74	1.4	1	1.4
Hexanol	C7 heptyl	23,77[b]	1	0.75	1.1	1	1.3
			2	0.73	1.5	1	1.4
Octanol	C12 butyl	50,50[c]	1	0.59	1.7	1	1.7
Octanol	C13 methyl	16,84[b]	1	0.75	2.3	1	1.3
			2	0.75	2.0	1	1.3
Octanol	C13 methyl	50,50[d]	1	0.64	1.4	1	1.6
			2	0.64	1.5	1	1.6

A: Reference substance component.

B: Test substance component (FAES).

C: Calculated by concentration addition model.

D: Calculated by response addition model.

TR: Test replicate.

LCT: Largest component TU as proportion of total TU for mixture (equation 20).

* Values to 2 s.f..

Nominal % component in mixture.

[a] % component to give 1 : 3 relative toxic concentrations of components A : B.

[b] % component to give 3 : 1 relative toxic concentrations of components A : B.

[c] % component to give 1 : 1.4 relative toxic concentrations of components A : B.

[d] % component to give 1.8 : 1 relative toxic concentrations of components A : B.

that there will always be some error inherent in both observed EC50 values and any analytical procedures. Whilst this may be only a few percent this can in some instances be the difference between being categorised as strictly concentration / response addition or as less than additive if applying strict limits of ±0.2TU. Some total TU values reported in this thesis which were >±0.2TU of the predicted value may indeed be the result of differences between nominal and true concentrations. However, phenol mixtures with measured concentrations of both components exhibited less than additive response in some cases.

Broderius *et al.*, (1995) reported results which suggested that outside the 1TU±0.2TU limits of concentration addition all mixtures resulted in less than additive joint toxicity ~~with no fixed pattern, which~~ This only sometimes resulted in strict response addition, although most showed consistently greater toxicity than predicted by response addition. It was considered by Broderius *et al.* that completely independent action was not the most likely mode of action for diverse compounds when mortality was the endpoint. Analysis of test solutions appeared to have been performed for these studies but no information was given as to the extent. Clearly it is possible that some of the responses, where concentrations of components were less than nominal, were classed as less than additive when in fact they were behaving by strict response addition. It must also be considered that these mixtures covered a diverse range of substances. Substances researched in this thesis comprise homologous series, each expected to behave in a similar way to others described by the same QSAR. In addition, results of 1-nonanol / phenol mixtures were observed with a mean total TU of 1.7(to 2 s.f.), within ±0.15 TU(to 2 s.f.) of the mean value predicted by response addition (Table 40). Considering that mode of action for both these substances is already established, this observation is a good indication that substances which act by different modes of action will behave by response addition if applied together as a binary mixture. As a result, where differences occur between predicted and observed total TU values for mixtures involving non-reference substances from the same series, these are likely to be as a result of inherent errors in the test system. It seems unlikely that as a result of inherent variability, particularly when

considering the confidence interval associated with each EC50 (Table 77, 78 and 79 , Appendix I), binary mixtures will necessarily fall within such defined limits as ±0.2TU of the respective model. It would perhaps be more appropriate to consider mixture toxicity as a sliding scale as considered by Plackett and Hewlett (1967) of which concentration addition and response addition are the two extremes. As a result it would probably not be applicable clearly to define limits for either strict non-additivity or strict additivity although results from this thesis indicate that it is likely that mixtures acting by different or similar modes of action will exhibit total TU values which will tend to aggregate around strict concentration and response addition respectively.

If all results are considered as part of such a sliding scale all equitoxic or near equitoxic phenol / FAES, 2-chlorophenol / FAES, phenol / LAS and LAS / C13 methyl FAES mixture results, in which concentration addition was predicted, appear at or near the concentration addition end of the scale and are significantly favoured by the concentration addition model over the response model. All equitoxic and non-equitoxic alcohol / FAES, alcohol / LAS and alcohol / phenol mixture results, in which response addition was predicted, appear at or near the response addition end of the scale and are significantly favoured by the response addition model over the concentration addition model. On weight of evidence it would thus seem appropriate to conclude that 1) phenol acts by a different mode of action from that of alcohols and can be assumed to act as a polar narcotic. 2) LAS acts by a similar mode of action to that of phenol, but by a different mode of action from that of alcohols, and can be considered to act as a polar narcotic and thus be suitable as a reference substance. 3) FAES substances act by a similar mode of action to that of phenols and LAS and a different mode of action to alcohols and can, therefore, be considered to act by polar narcosis.

3.3.4 CONCLUSION

Results of mixture toxicity studies would suggest that FAES substances act by a similar mode of action to that of LAS and phenols and by a different mode of action from that of alcohols suggesting that they behave by polar narcosis. It would seem, therefore, that differences in observed regression lines for LAS and FAES substances (Fig. 11) are probably not a result of FAES substances behaving by a different mode of action but may be a result of a need to modify the log P calculation of Hansch and Leo (1979). As the slopes are highly similar, the log P values associated with any pair of LAS and FAES substances of given equal response are distinguishable by a constant factor. As a result any possible modification to its calculation will not be associated with chain length or water sharing factor as this would vary according to each structure. It seemed probable, therefore, that modification of the proximity factor as the constant for each FAES substance log P calculation, was required.

CHAPTER 4

THE DEVELOPMENT OF THE PROXIMITY FACTOR MODIFICATION

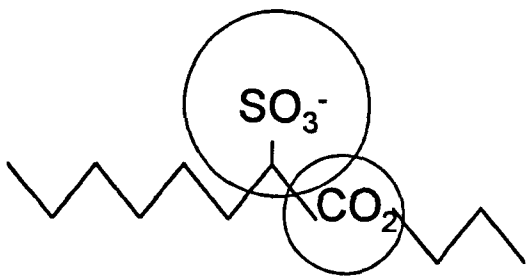
4.1 INTRODUCTION

4.1.1 PROXIMITY FACTORS DERIVED BY HANSCH AND LEO

Full calculation of log P using the method derived by Hansch and Leo (1979) has been discussed in sections 1.4 and 2.2.9. The method considers a variety of interactions which can occur between fragments within a molecule such as halogen fragment with halogen fragment, halogen fragment with polar fragment and polar fragment. It is the latter of these with application to non-aromatic systems which is of concern to substances tested in this thesis.

Polar fragments possess a negative fragment value due to the stronger interaction of the fragment with water which surrounds them in what is termed the Hydration Sheath. When two polar groups are proximal, electron attracting effects reduce the dipole of each fragment which, combined with loss of accessible surface area, has the effect of reducing the overall negative contribution of the two fragments to log P (Fig. 17).

Fig. 17. Schematic representation of Hydration Sheaths in FAES



In addition, water sharing between overlapping sheaths reduces the overall reduction in free energy. The method conforms to the idea that polar fragments

are capable of interaction only when positioned within a molecule by a maximum of three carbons separation. The effect is most important when fragments are geminal or separated by two carbons, but where four carbons intervene the two fragments are given their full constant. The proximity effects are, therefore, proportional to the amount of intrinsic hydrophilic character of the two fragments. The value of each fragment is reduced by approximately 40% and 25% when separated by one and two carbons respectively.

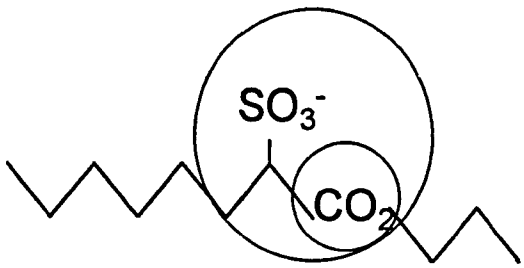
When applied to aliphatic rings, the proximity effect is lower than for chains by approximately 76%, which reflects the relative inflexibility of the former.

The system becomes more complex when dealing with molecules in which there is opportunity for interactions between various polar groups. This is not helped by the problems associated with measuring partition coefficients for very hydrophilic substances.

Calculation of the proximity effect is, as described in section 2.2.9, by application of the factor to a simple summation of the two polar fragments. This considers only total contribution of the two fragments to log P and effects of separation. It does not consider whether the calculated total contribution to log P results from two polar fragments of equal size (where size taken as the scope of influence on free energy of the water surrounding any polar fragment and is considered as a spherical volume), or from two different sized fragments where one may be considerably larger than the other.

The proximity factors of Hansch and Leo (1979) are empirically derived values, calculated on the basis of measured partition coefficients. It is unlikely that substances containing such different polar fragments such as seen in FAES molecules were considered at the time of their derivation. It would seem likely that where one polar fragment is considerably larger than its proximate neighbour, then this larger fragment will have more influence on the overall contribution of polar fragments to log P, possibly to the complete exclusion of the smaller fragment (Fig. 18). Thus it would seem reasonable to employ a proximity factor which accounts not only for separation, but also for relative size (compared to each other) of polar fragments within a molecule.

Fig. 18. Schematic representation of the influence of the largest polar fragment in FAES as an extreme case



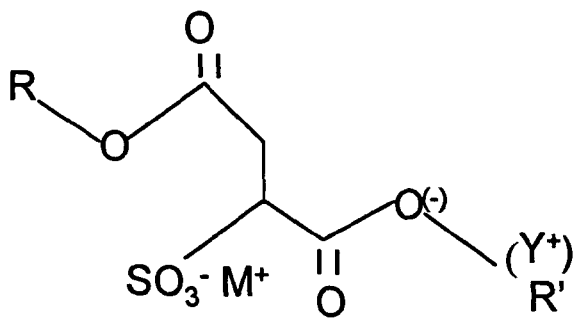
To examine the possibility of the application of such a proximity factor to the Hansch and Leo method of log P calculation, a theoretical model based on scope of influence of the hydration sheath considered as a spherical volume in addition to polar group separation was calculated and applied to the conventional log P calculation of FAES and other anionic compounds in this chapter. EC50 values for *D. magna* were observed for these other substances in order to plot log (1/EC50) as mol/l versus log P calculated with and without the new proximity factor. If the new proximity factor was applicable, prediction of EC50 values for these substances would be possible using the new log P value with an equation similar to that of LAS (equation 40) assuming mode of action could be established to be similar to LAS. Mode of action was established through testing in mixtures, with phenol or 1-hexanol as reference substances, to suggest concentration or response addition in the same way as for chapter 3. The resulting modified log P versus log (1/EC50) plots for these substances were compared to those for the reference LAS substances as authentication of the validity of the proximity factor modification.

The anionic compounds used in this chapter (excluding LAS and FAES) were either supplied or synthesised to contain different proximate polar group fragments and to induce a measurable toxic response to *D. magna*. These included sulphosuccinates (Fig. 19) and sulphonated amides, both being employed commercially.

4.1.2 SULPHOSUCCINATES

Sulphosuccinates are surface active metal (usually sodium) salts of either monoesters or diesters of sulphosuccinic acid (Domsch and Irrgang, 1996) and are based on reactions of maleic acid anhydride with hydroxyl group carrying molecules followed by sulphation with sodium sulphite for monoesters or with sodium sulphite, sodium bisulphite or sodium metabisulphite for diesters. The monoesters are prepared from a wide variety of raw materials such as fatty acids, fatty alcohols, ethoxylated fatty alcohols and others, the majority being of non-petrochemical origin. The diesters, on the other had, are derived principally from branched and unbranched alcohols in the presence of strong acid (Linfield, 1976).

Fig. 19. General structure of sulphosuccinates



Where R = alkyl, alkylamide etc.,
R' = alkyl,
$M^+ = Y^+$ = alkaline metal ion.
( ) = monoester form.

Historically, sulphosuccinates have been described in their diester form from the mid-1930s. Due to the very high price of maleic acid anhydride they were used only in a few formulations until the mid-1960s when, with the advent of major plastics industries, the price of maleic acid anhydride decreased dramatically and large scale production of sulphosuccinates became possible. Sulphosuccinates

are used in many cosmetic and toiletry products (Falbé, 1987). Those based on lauryl alcohol ethers, for example, are applied in many mild shampoos. Their good wetting properties make them extremely useful in products such as glass cleaners and floor cleaners, and diesters are applied as emulsifiers and have widespread use in the pharmaceutical industry (Domsch and Irrgang, 1996).

The monoester sulphosuccinate used in this thesis was disodium lauryl sulphosuccinate (DLAS) in which R = C12 chain, M^+ and Y^+ = Na^+. The large variety of educts used in the production of monoester sulphosuccinates leads to a large number of different physical properties associated with these molecules, although they are mostly associated with the lipophilic part of the molecule. Hard water resistance is generally very good and lime soap formation low (Domsch and Irrgang, 1996). Unfortunately DLAS exhibits only average resistance to hard water. In hard water DLAS clear solutions can be obtained only up to 0.1% w/v. However, whilst the ester group is sensitive to strong acid and alkali, leading to hydrolysis, all these substances, including the diesters, can be considered stable at pH 6-8 (Domsch and Irrgang, 1996).

Sulphosuccinates generally have good biodegradability and show >80% complete degradation although there are exceptions, such as dicyclohexyl sulphosuccinate, which show no degradation after 28 days.

There is little published information regarding the acute toxicity of sulphosuccinates to aquatic organisms. The LC50 for diisooctyl sodium sulphosuccinate (DOSS), the most widely used of the diesters, has been determined as 28mg/l (Goodrich *et al.*, 1991).

4.1.3 SULPHONATED AMIDES

Sulphonated amides are sulphoalkyl amides of fatty acids of general structure:

$$R\text{-}CON(R')CH_2CH_2SO_3^-Na^+$$

They are commonly prepared commercially by the Schotten-Baumann addition reaction of acyl chlorides with an amine, commonly taurine ($N_2HCH_2CH_2SO_3H$), under alkaline conditions. They are used commonly in industry as scale inhibitors, for flotation of minerals, and as wettable powders in agricultural formulations. They have also found wide application in washing liquids, shampoos and in bar soap, and in their widest application in the textile industry as wetting agents.

Sulphonated amides are insensitive to hard water, have good detergency and wetting ability and are hydrolytically stable in acid conditions (Linfield, 1976).

4.1.4 WATER HARDNESS

A number of the substances used in this chapter proved to be of low solubility. In order to establish an EC50 for the substances of lower solubility it was necessary to obtain an EC50 value at low hardness in order to achieve solutions of test substance at concentrations sufficiently high to induce an EC50 value to *D. magna*.

Hardness is a property conferred on water by the presence of alkaline earth salts and expressed as mg equivalent of $CaCO_3/l$. It is attributable mainly to the salts of Mg, Ca, Sr and Ba, although in practice the total hardness of a medium is overwhelmingly attributable to the contribution of Ca and Mg salts. The measurement of total hardness (TH) using titration methods is not sufficiently accurate for any slight differences in hardness to be observed as a result of changes in the trace amounts Sr^{2+} and Ba^{2+} ions compared to those of Ca^{2+} and Mg^{2+} ions. This is particularly the case when considering accepted variation in composition and associated physical properties of a medium expected when preparing large volumes.

The effects of alteration of water hardness on the physical properties of the substances dissolved in it can be pronounced, particularly for anionic surfactants. Counterions associated with an ionic amphiphile have great influence on micellar properties (Attwood and Florence, 1983). A change in counterion to one of greater valency leads to a decrease in the CMC of the substance. For example, for lauryl

sulphate a change in counterion from Na^+ to K^+ results in a decrease in the CMC from 8.32 to 7.17mmol/l at 25°C (Attwood and Florence, 1983). Clearly also the presence of increased $[Ca^{2+}]$ and $[Mg^{2+}]$ ions in a saturated solution of anionic surfactant will alter the equilbrium of the solution. When a saturated solution of the electrolyte is in contact with non-dissolved electrolyte then the following equilibria can be considered:

$$AB_{solid} \rightleftharpoons AB_{dissolved} \rightleftharpoons A^- + B^+$$

with the equilibrium constant as:

$$K = \frac{[A^-][B^+]}{[AB]_{dissolved}}$$

As AB_{solid} and undissociated $Ab_{dissolved}$ are in equilibrium, the latter of these can be considered to remain constant and the solubility product (K_{sp}) of the electrolyte can be assumed to be:

$$K_{sp} = [A^-][B^+]$$

As a result where $[B^+]$ is increased the K_{sp} is exceeded and the salt is precipitated to restore equilibrium. In solutions of surfactant substances of low solubility, increased precipitation is also induced by an increase in $[Ca^{2+}]$ which acts competitively as an alternative counterion to the electrolyte. Solubility of less soluble anionic surfactants can be improved, therefore, by a reduction in the hardness of the solvent.

Reduction in hardness of a solvent, however, introduces further complications when attempting to establish acute toxicity of a substance using daphnids cultured in hard medium (*ca.* 240mg/l as $CaCO_3$). Firstly it potentially causes additional stress to the test organism. It is certainly the case that maximum productivity of *D. magna* can be observed in harder medium (350mg/l as $CaCO_3$)(Lewis and Maki, 1981). Numbers of neonates and brood size

increased with hardness (range :50-350mg/l as $CaCO_3$) although no significant difference in adult mortality was observed. Published acute toxicity data of *D. magna* obtained at hardness as low as 15mg/l as $CaCO_3$ report no observed detrimental effects to the test organisms as a result of low hardness tested (Hokanson and Smith, 1971; Lewis and Perry, 1981). Thus by allowing sufficient acclimation time of test organisms to low hardness, no increased toxicity arising from the significant addition physiological stress, associated with sudden change in hardness, should result (Maki and Bishop, 1979).

Secondly it is well established that the toxicity of anionic surfactants varies with the hardness of the medium; (Maki and Bishop, 1979; Persoone *et al.*, 1989) (section 1.3.5). The evidence for any systematic increase or decrease in toxicity is variable. LAS and other anionic surfactants such as alkyl sulphates have been found to have increased toxicity to bluegill and other species in hard water (Gafa, 1974; Holman and Macek, 1980; Hokanson and Smith, 1971). Results were noted, however, to be dependent on whether commercial products or individual components were tested (Hokanson and Smith, 1971) and the toxicity of LAS to *D. magna* was observed to decrease with hardness (Lewis and Perry, 1981).

Due to differences in EC50 values which would be expected when testing at lower hardness it was necessary to also establish the EC50 values of the other more soluble substances used in this chapter at an equivalent hardness. It was also essential to establish the change in EC50 values of the LAS and selected FAES substances due to the lowering of medium hardness, to obtain a second reference log P based QSAR for the toxicity of LAS to *D. magna* at low hardness. A range of 48 hour EC50 studies were carried out to this end.

A number of preliminary studies were also required in order to establish solubility of the substances of lower solubility and any detrimental effects on the test organisms.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

In addition to the twenty-one FAES and six LAS (chain length C9 - C14) substances used in previous chapters, various other anionic surfactants were either synthesised or obtained.

Lauryl sulphosuccinate (DLAS) and sodium lauroyl diethoxy isethionate (SLDI) (Fig. 20) were obtained internally at Unilever Research and were 100% and 96% active respectively.

Geropon T77 (sodium methyl oleyl taurate) (Fig. 20) was supplied by Rhône-Poulenc and was 67 - 76% active.

Sodium sulphomethyl myristylamide (SMMA) (Fig. 20), sodium sulphoethyl myristylamide (SEMA)(Fig 20) and octadecyl 1,2-disulphate disodium salt (DSDS) (Fig.20) were synthesised using the following detailed methods.

Where mixture toxicity studies have been performed, previously detailed phenol and 1-hexanol were used as reference substances.

Fig. 20. Anionic surfactant structures synthesised / supplied for chapter 4

SEMA : CH_3-$(CH_2)_{12}$-CONH-CH_2-CH_2-$SO_3^-Na^+$

SMMA : CH_3-$(CH_2)_{12}$-CONH-CH_2-$SO_3^-Na^+$

Geropon: CH_3-$(CH_2)_7$-CH=CH-$(CH_2)_7$-CON(CH_3)-CH_2-CH_2-$SO_3^-Na^+$

DLAS: CH_3-$(CH_2)_{11}$-CO_2-CH_2-CH$(SO_3^-Na^+)$-$CO_2^-Na^+$

DSDS: CH_3-$(CH_2)_{15}$-CH$(OSO_3^-Na^+)CH_2$-$OSO_3^-Na^+$

SLDI: CH_3-$(CH_2)_{10}$-CO_2-CH_2-CH_2-(O-CH_2-CH_2)-(O-CH_2-CH_2)-$SO_3^-Na^+$

4.2.2 SYNTHESIS OF SMMA

Sulphomethyl amides can be cheaply synthesised by the reaction of fatty amides with formaldehyde-bisulphite:

$$R\text{-}CONH_2 + HOCH_2SO_3^- \xrightarrow{180\text{-}190^\circ C} R\text{-}CONHCH_2SO_3^- + H_2O$$

This is a satisfactory and cheap method for synthesis of this surfactant but the forcing conditions necessary for the reaction to occur result in a number of side products. In addition the large volumes of solvent required for recrystallising steps can result in handling difficulties.

A second common method for their preparation requires much milder conditions. This route involves the Schotten-Baumann reaction of acyl chloride with aminomethanesulphonic acid in the presence of alkali:

$$R\text{-}COCl \xrightarrow{H_2NCH_2SO_3H} R\text{-}CONHCH_2SO_3^- + HCl$$

This method was used for the preparation of SMMA. Myristoyl chloride and aminomethanesulphonic acid were supplied by Lancaster Synthesis and were 98% and 97% pure respectively.

To a stirred slurry of 80g (0.72 mol) aminomethane sulphonic acid in 600 ml deionised water, aqueous NaOH (20%w/v) was added dropwise. Approximately 150ml was required to raise the pH of the slurry from approximately 3.5 to 8 - 9. To the resulting clear solution, 98ml (approximately 0.36mol) myristoyl chloride and aqueous NaOH were added dropwise simultaneously in order to maintain pH 8 - 9. The solution was stirred continuously although the stirrer speed required initial adjustment to account for the resulting precipitate which reduced homogeneity and caused pH to rise slightly. The temperature rose to approximately 30-35°C over this period.

The mixture was stirred for a further 2.5 hours at room temperature and then cooled to *ca.* 0°C. The white crystalline precipitate was filtered and washed with approximately 200-300ml iced water.

Approximately 60g of the washed precipitate was mixed with 250ml hot ethanol and dissolved by the addition of small volumes of deionised water. The filtered hot solution was then allowed to cool and recrystallise before filtering and drying the resulting white crystals. Peaks near the region of 1725-1700cm^{-1} on the IR spectrum respectively indicated the presence of fatty acid contaminant (Fig. 55, Appendix III) in addition to the expected sulphonated amide product.

Removal of the fatty acid was attempted by three Soxhlet extractions, each run for a period of approximately 14 hours, using diethyl ether, acetone and hexane as consecutive solvents. NMR and IR traces of the product following each extraction revealed the contaminant peak to remain. Fatty acid is usually removed by such techniques and it was considered that presence of unreacted myristoyl chloride may have produced a soap by-product in reaction with NaOH:

$$C_{13}H_{27}\text{-}COCl + NaOH \longrightarrow C_{13}H_{27}\text{-}COO^{-}Na^{+} + HCl$$

4.2.2.1 SOAP AND INORGANICS EXTRACTION

The remaining 12g of product after all extractions was dissolved in 300ml deionised water and heated on a steam bath to approximately 50°C. HCl (20%v/v) was added dropwise to adjust solution to approximately pH 3. Resulting hydrolysis caused the formation of fatty acid:

$$C_{13}H_{27}\text{-}COO^{-}Na^{+} + NaOH \xrightarrow{\text{Dilute acid}} C_{13}H_{27}\text{-}COOH + NaCl$$

The fatty acid was removed with repeated extractions using warmed hexane. The aqueous layer was cooled overnight to induce precipitation of the purified SMMA which was filtered, washed with acetone and dried. NMR traces

indicated no further organic contaminants. Extracted contaminant was identified as fatty acid from its IR spectrum following evaporation of the solvent Recrystallisation in deionised water with a final washing with acetone and isopropyl alcohol (IPA), failed to remove remaining inorganic contaminants.

Final analysis by NMR identified 83.5% purity of the active SMMA product (7.5g) , calculated by weight percent product (section 2.2.3), (Fig. 56, Appendix III).

4.2.3 SYNTHESIS OF SEMA

Synthesis of SEMA was performed, again using the Schotten-Baumann reaction of myristoyl chloride with taurine (2-aminoethanesulphonic acid) in the presence of alkali. Taurine was supplied by Aldrich Chemical Co., U.K. and was of 99% purity.

To a stirred slurry of 35.5g (0.3 mol) taurine in 300 ml deionised water, aqueous NaOH (20%w/v) was added dropwise. Approximately 30ml was required to raise the pH of the slurry to 8 - 9. Warming to approximately 27°C was required to produce a clear solution to which 41ml (0.15mol) myristoyl chloride and aqueous NaOH were added dropwise simultaneously in order to maintain pH 8 - 9. The solution was stirred continuously for a further 2.5 hours at room temperature over which time the pH dropped slowly requiring continuous correction with aqueous NaOH. A further 100ml deionised water was added as the precipitation increased to aid mobility of the mixture. At the end of this period the pH ceased to decrease indicating that the reaction was complete.

The mixture was cooled to *ca.* 0°C and the white crystalline precipitate was filtered and washed with approximately 200-250ml iced water.

Approximately 50g of the crude product was recrystallised from 220ml ethanol. The IR spectrum of the product identified fatty acid contaminant. Following continuous extraction of 14.4g recrystallised product (sufficient to fill Soxhlet thimble) with ether for approximately 26 hours, the NMR trace identified

the presence of a contaminant in the fatty acid region of the spectrum. Fatty acid would have been expected to have been removed by continuous extraction and following experience with SMMA, the contaminant was assumed to be soap.

4.2.3.1 SOAP AND INORGANICS EXTRACTION

The remaining product following extraction (13.6g) was dissolved in 400ml hot deionised water and acidified to pH3 with dilute HCl (20%v/v) added dropwise. The hydrolysed material was removed with repeated extractions using warm hexane as for SMMA. Following cooling of the mother liquor and resultant precipitation of purified SEMA product, NMR analysis revealed no further organic contaminant although only 75% active product. Extracted contaminant was identified as fatty acid from its IR spectrum.

The remaining purified product was dissolved in 90ml hot deionised water and allowed to cool. The precipitated purifed product was filtered and dried.

Final analysis by NMR identified 81% purity of the active SEMA product (3.5g) , calculated by weight percent product (section 2.2.3), (Fig. 57, Appendix III).

4.2.4 SYNTHESIS OF DSDS

DSDS was synthesised following the reactions:

$$C_{16}H_{33}\text{-}CH(OH)CH_2(OH) \xrightarrow{Cl_3SO_3H} C_{16}H_{33}\text{-}CH(OSO_3H)CH_2(OSO_3H) + HCl$$

$$C_{16}H_{33}\text{-}CH(OSO_3H)CH_2(OSO_3H) \xrightarrow{NaOH} C_{16}H_{33}\text{-}CH(OSO_3^-Na^+)CH_2(OSO_3^-Na^+) + H_2O$$

1,2-octadecanediol and chlorosulphonic acid were supplied by Lancaster Synthesis and were of >99% and 98% respectively.

To a stirred slurry of 30g (0.105mol) 1,2-octadecanediol in approximately 250ml ether, 15.5ml (0.23mol) chlorosulphonic acid was added dropwise over a period of 1.5 hours. The reaction mixture was chilled to approximately 5°C over the reaction period which was characterised by a gradual change from the starting ethereal slurry to a clear, yellow solution.

The mother liquor was added to 200ml of excess aqueous NaOH (9.9%w/v), producing a white precipitate. Excess ether was evaporated by standing overnight and completed over a steam bath.

The remaining aqueous mixture was neutralised with dilute HCl (2M) added dropwise. Care was taken during this process not to allow pH to drop to 3-4 at any stage and vigorous stirring of the slurry was required to avoid any such localised effects. Low pH of this nature would have induced hydrolysis of the intermediate. Following neutralisation, the slurry was freeze dried for a period of seven days.

4.2.4.1 RECRYSTALLISATION

30g of the crude product was recrystallised from 200ml IPA. Precipitated material was filtered and dried. Extracted substance after evaporation of the IPA solvent was identified as 1,2-octadecanediol from its NMR spectrum.

The purified product was recrystallised a further time, from 600ml ethanol although solubility of the product was achieved only by the addition of 20% deionised water. Extracted pink crystalline material after evaporation of ethanol was identified by IR as largely inorganic with trace amounts of parent diol and disulphated material (Fig. 58, Appendix III). Filtered and dried recrystallised precipitate was identified by NMR as probably disulphated product. No further organic contaminants were identified although the NMR trace indicated only 67 - 68% purity. In addition the relatively large volume of deionised water required to dissolve product for recrystallisation, indicated large amounts of inorganic

impurities. Two further recrystallisations from ethanol required 10% and 5% deionised water respectively to dissolve product.

Mass spectrometry identified a final product of MW 490 corresponding to octadecyl 1,2-disulphate disodium salt (DSDS) with trace amounts (<<1%) of monosulphated material (Fig. 59, Appendix III). Final NMR analysis indicated 90% purity of active product, calculated by weight percent product (section 2.2.3), with no further organic contaminant (Fig. 60, Appendix III).

4.2.5 TEST SUBSTANCE SOLUBILITY STUDIES

The EC50 values of LAS and FAES substances and for phenol and 1-hexanol have already been established to be below the limit of solubility for all chain lengths tested. Small scale solubility studies were performed for SMMA, SEMA and DSDS in order to establish approximate solubility of each of these substances prior to testing. For each study approximately 0.01g of the substance was accurately weighed into a vial. Initially 10ml of standard Elendt M7 medium was added at room temperature to the vial to establish solubility at 1000mg/l. Following failure to dissolve, heat (≤60°C) and sonication at full power were applied to the sample, allowing the sample to cool or stand overnight respectively between application of each technique. Following further failure to dissolve, additional volumes of Elendt M7 medium of 7.9ml, 13.4ml, 24.3ml and 44.4ml were added to establish solubility at 560mg/l, 320mg/l, 180mg/l and 100mg/l with warming and sonication if required. The process was repeated using distilled water where the substance failed to dissolve in Elendt M7 medium.

Studies were also performed using a series of solvents to attempt dispersion of the test substance. 1ml methanol, ethanol, dimethyl sulphoxide (DMSO) and ethyl acetate were applied to a 0.01g sample and heated to dissolve the test substance. Whilst hot, the solution was pipetted into 8ml distilled water under sonication. Sonication was continued for a further 30 minutes to disperse the solvent and test substance. At the end of this period the volume was made up to 10ml with distilled water and cooled. The process was repeated with 80ml

distilled water, making up to 100ml, where precipitation occurred in the 10ml water study.

Further solvents of 1-propanol and acetone were applied at 0.1ml in 10ml hot distilled water with sonication at full power for 30 minutes where no satisfactory dispersion had previously been achieved.

Where no suitable solubility or dispersion of the test substance could be achieved by heating, sonication or use of solvents a number of studies were performed to establish the possibility of performing studies at low water hardness to eliminate or reduce precipitation of the test material. Preliminary work required some investigation into survivability of *D.magna* neonates at low hardness in addition to improved solubility of test substances at low water hardness.

4.2.6 PREPARATION OF TOTAL HARDNESS STOCK FOR REDUCED HARDNESS STUDIES

Alterations to the TH of Elendt medium were achieved by replacement of the $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$ stock solutions (Table 107, Appendix II) used in general preparation of the medium, with a new stock combining these two salts at the correct ratio (Stock A), added separately at volumes calculated to attain any required TH as follows:

MW: Ca: 40.08, Mg: 24.3, $CaCO_3$: 100.08,
$CaCl_2.2H_2O$: 147.08, $MgSO_4.7H_2O$: 246.6

mg equivalent $CaCO_3$ /l = 100.08 / 40.08 [Ca^{2+}(mg/l)] + 100.08 / 24.3 [Mg^{2+} (mg/l)].
= 2.497 [Ca^{2+}(mg/l)] + 4.119 [Mg^{2+} (mg/l)].

4.2.6.1 NOMINAL TOTAL HARDNESS OF STANDARD ELENDT M7 MEDIUM

In any 50l batch preparation, Ca^{2+} and Mg^{2+} ions were present as $CaCl_2.2H_2O$ (293.8g/l) and $MgSO_4.7H_2O$ (246.6g/l) stocks respectively for general Elendt M7 medium preparation. These were added at volumes of 50ml and 25ml respectively. Expressed as [Ca^{2+}(mg/l)] and [Mg^{2+} (mg/l)] to estimate nominal TH of the medium:

50l Elendt contains ((50/1000) x 293.8) / 147.08 = 0.100 mol $CaCl_2.2H_2O$.
50l Elendt contains 0.100 x 40.08 = 4.01g Ca^{2+}
[Ca^{2+}] = 80mg/l.

50l Elendt contains ((25/1000) x 246.6) / 246.6 = 0.025 mol $MgSO_4.7H_2O$.
50l Elendt contains 0.025 x 24.3 = 0.608g Mg^{2+}.
[Mg^{2+}] = 12mg/l.

Nominal TH of standard Elendt M7 medium:
mg equivalent $CaCO_3$ /l = (2.497x 80) + (4.119 x 12) = 249.

Ratio of $CaCl_2.2H_2O$: $MgSO_4.7H_2O$ required in Stock A
= 14.7 / 6.17 : 1 = 2.4 : 1.

Stock A was prepared as 1.0g $MgSO_4.7H_2O$ and 2.4g $CaCl_2.2H_2O$ dissolved in 1000ml distilled water.

4.2.6.2 CALCULATION OF VOLUMES OF $CaCl_2.2H_2O$ / $MgSO_4.7H_2O$ STOCK REQUIRED FOR A SPECIFIED TOTAL HARDNESS

Volumes of $CaCl_2.2H_2O$ / $MgSO_4.7H_2O$ stock required to attain a specified TH in a given volume of medium / test solution were calculated as a proportion of

the [Mg^{2+}(mg/l)] required to produce a TH of 249mg/l $CaCO_3$. For example to calculate the volume of Stock A required in 500ml medium to attain TH of 13mg/l $CaCO_3$ would be calculated as follows:

TH of 249 mg/l of $CaCO_3$ ≡ 12mg/l Mg^{2+},

TH of 240 mg/l of $CaCO_3$ ≡ (13 / 249) x 12 = 0.627mg/l ($2.58x10^{-5}$ mol/l) Mg^{2+}.

≡ $2.58x10^{-5}$ x 246.6 = $6.36x10^{-3}$g/l $MgSO_4.7H_2O$.

Equation 23.

Concentration of $MgSO_4.7H_2O$ in Stock A = 1.0g/l. Stock A already contains the correct ratio of [Ca^{2+}(mg/l)] to [Mg^{2+} (mg/l)] and, therefore, the volume Stock A required to be added to 500ml medium to attain nominally 240mg/l equivalent of $CaCO_3$ is a simple calculation based on [Mg^{2+}] alone as (6.36 / 1000) x 500 = 3.2ml. Equation 24.

The calculation would be equally valid based on [Ca^{2+}(mg/l)].

4.2.7 SOLUBILITY OF SMMA IN THE PRESENCE OF Ca^{2+} AND Mg^{2+} IONS

Two studies were performed to establish a saturated soluble fraction of SMMA and SEMA at a water hardness which would 1) not impair neonate survival 2) allow sufficient test substance to remain in solution to induce a response below the limit of solubility.

4.2.7.1 SOLUBILITY STUDY 1: SOLUBILITY OF SMMA IN THE PRESENCE OF A SERIES OF [Ca^{2+}] AND [Mg^{2+}] IONS

A stock solution of SMMA (100mg/l) was prepared by dissolving and warming the test material in Elendt M7 medium, prepared without the addition of $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$. The solution was allowed to cool slightly to a temperature where no precipitation occurred.

Two series of test solutions were prepared in 250ml conical flasks, the first as 100mg/l samples (100ml) in the presence of a geometric series of Ca^{2+} and Mg^{2+} ions on the logarithmic scale nominally equivalent to a water hardness of 0.0, 13, 24, 42, 75, 130, 240mg/l as $CaCO_3$, by the addition of the required volume of Stock A as calculated in section 4.2.6 (equations 23 and 24)(Table 44). Both the hardness and nominal test solution concentration were decreased slightly as Stock A was added to test solutions which were already at nominal concentration. The second series was prepared identically to the first to the exclusion of the SMMA test substance.

Test solutions were stirred continuously for a period of 5 hours. At the end of this period solutions were transferred to sealed centrifuge tubes and spun at 20,000 rpm for 15 minutes. Samples of the soluble fraction (SF) supernatant were then taken to determine TH of each test solution. The remaining supernatant was pipetted into vials and preserved with approximately 3% formalin for analysis by MBAS (Table 57).

Samples were taken from the second series of solutions at time t = 0 for determination of TH of the SF at the start of the test (Table 57). The assumption was made that these values would be synonymous with TH values for the first series since it was impossible to measure TH of the first series due to almost immediate precipitation of test substance and consequent drop in TH on contact of Stock A with SMMA test substance. Test solution concentration at time t = 0 was established through MBAS analysis of the test solution of a hardness of 0.0mg/l as $CaCO_3$ solution (Table 57).

4.2.7.2 SOLUBILITY STUDY 2: SOLUBILITY OF A SERIES OF CONCENTRATIONS OF SMMA IN THE PRESENCE OF $[Ca^{2+}]$ AND $[Mg^{2+}]$ IONS

A stock solution of SMMA (320mg/l) was prepared by dissolving and warming the test material in Elendt M7 medium prepared without the addition of $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$. The solution was allowed to cool slightly to a

Table 44. Volumes of Stock A addition required in preparation of 500ml Elendt M7 medium , prepared without the addition of conventional hardness stocks, to achieve given medium hardness

Required mg/l equivalent of $CaCO_3$ of medium	Conc. of $MgSO_4.7H_2O$ (equation 23)(g/l)	Volume of Stock A required (equation 24)(ml)*
7.5	3.7×10^{-3}	1.83
13	6.36×10^{-3}	3.15
24	0.0117	5.85
42	0.021	10.5
75	0.0365	18.3
130	0.063	31.5
240	0.117	58.5

*Values to 3 s.f.

temperature where no precipitation occurred before preparation of the test solutions.

Test solutions (500ml) were prepared in conical flasks on the logarithmic scale 320, 180, 100, 56, 32, 0.0mg/l by serial dilution of the stock solution with Elendt M7 medium prepared without the addition of $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$. To each test solution was added 3.2ml Stock A in order to attain 13mg/l equivalent of $CaCO_3$. The solutions were made up to volume, flasks sealed and the solutions stirred continuously for 24 hours.

At time t-10 (before the addition of Stock A), t = 0 (addition of Stock A), t+30 minutes, t+60, t+180, t+360, t+540 and t+1440, samples (20ml) were pipetted from each test solution into centrifuge tubes and spun at 20,000 rpm for 15 minutes. The soluble fraction (SF) supernatant was pipetted into vials and preserved with 3% formalin for analysis of test solution concentration by MBAS (Table 58). Further samples to include both soluble and precipitated material as a total fraction (TF) were also taken and pipetted directly from test solution vessels into vials for preservation and subsequent MBAS analysis of test solution concentration (Table 58).

Samples were taken from the control at t-10 and from all test solution concentrations at t+1440 for determination of TH of the SF (Table 58).

4.2.7.3 SOLUBILITY STUDY 3: SOLUBILITY OF A SERIES OF CONCENTRATIONS OF SEMA IN THE PRESENCE OF $[Ca^{2+}]$ AND $[Mg^{2+}]$ IONS

This study was performed by the same method as solubility study 2 (section 4.2.7.2) except that SEMA was used instead of SMMA as the test substance. Due to time restriction, however, measured concentrations were obtained for SF and TF only for the 100mg/l test solution concentration (Table 59).

4.2.8 EXPOSURE OF *D. MAGNA* TO LOW TOTAL HARDNESS

Three studies were performed in which <24 hour old *D. magna* neonates, cultured at standard water hardness, were exposed to a geometric series of water hardness concentrations based on a logarithmic scale for a period of 48 hours. The first two of these were prepared as 0.0, 13, 24, 42, 75, 130, 240mg/l equivalent of $CaCO_3$, the third as 0.0, 1.3, 2.4, 4.2, 7.5, 13mg/l equivalent of $CaCO_3$. The studies were performed as acute studies according to the 48 hour acute toxicity test procedure outlined in section 2.2.6, in which $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$ were treated as test substances with no further addition of any test material. Test solution were prepared as Elendt M7 medium which had been prepared without the addition of $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$, with the addition of the required volumes of Stock A as calculated in section 4.2.6 (Table 44). Neonates were cultured at standard water hardness and transferred to isolation medium as Elendt M7 prepared at 13mg/l as $CaCO_3$, prior to transfer to test solutions. Samples were removed at 0, 24 and 48 hours from old and new test solutions to determine TH of all test solution concentrations and pH, dissolved oxygen and temperature of selected concentrations at time of neonate addition / transfer. The aim of the studies was, therefore, not to establish EC50 values but to apply ANOVA using Dunnetts test to the study data, to observe differences in treatments on immobility of neonates.

4.2.9 TOXICITY TESTING

All acute toxicity data were determined by exposing less than 24 hour old *Daphnia magna* neonates to a series of concentrations of each test substance on a logarithmic scale according to the standard 48 hour acute toxicity test procedure as outlined in section 2.2.6.

With the requirement to prepare test solutions of SMMA and SEMA at low water hardness in order to achieve a soluble fraction, a number of studies were performed at 13mg/l equivalent of $CaCO_3$. This hardness was chosen to a) be on

a logarithmic scale, b) be similar to values used in other data for LAS substances unreported in this thesis, c) allow sufficient test substance to remain in solution as the soluble fraction to obtain an observable response. Substances previously tested for EC50 values at standard water hardness were now required to be tested at low water hardness to establish changes in observed EC50 values due to reduced hardness. In addition to SMMA and SEMA, such studies were performed for C9 - C14 LAS substances, selected FAES substances, DSDS, Geropon T77, lauryl sulphosuccinate and SLDI, conforming to the standard 48 hour acute toxicity test procedure outlined in section 2.2.6 in all other aspects.

The highest test solution concentration prepared for the SMMA and SEMA studies were equivalent to the stock solution concentration. The nominal concentration of these test solutions dropped very slightly, therefore, on addition of Stock A. This was acceptable, however, as all concentrations were measured using MBAS.

Samples at 0, 24 and 48 hours were taken from old and new solutions to provide water quality analysis (section 2.2.6). In addition to the standard water quality analysis, samples were taken from each test solution concentration at 0, 24 and 48 hours for SMMA and SEMA studies to determine TH of the SF.

In addition to the standard samples taken from the control, lowest and highest test solution concentrations of selected studies for preservation with 3% formalin and MBAS analysis, samples of the soluble fraction were preserved at 0, 24 and 48 hours for each old and new concentration of the SMMA and SEMA studies.

Mixture toxicity studies were conducted in order to establish mode of action of the test substance. Concentration ranges were determined by assuming concentration addition for those substances expected to behave by a similar mode of action and by assuming response addition of those substances expected to behave by different mode of action.

4.2.10 PREPARATION OF TEST SUBSTANCE STOCK / TEST SOLUTIONS TO BE TESTED AT STANDARD WATER HARDNESS

Stock solutions of the individual test substances to be tested at standard water hardness were prepared as outlined in section 2.2.5, by dissolving the test material in Elendt M7 medium as the preferred solvent although distilled water was an acceptable alternative solvent where the stock solution was present at ≤10% of any final test solution.

Test solutions were prepared by serial dilution of the stock solution with Elendt M7 medium as outlined in section 2.2.5.

4.2.11 PREPARATION OF TEST SUBSTANCE STOCK / TEST SOLUTIONS TO BE TESTED AS BINARY MIXTURES

Mixture toxicity stock solutions were prepared as binary equitoxic mixtures of the test substance with either phenol or 1-hexanol as the reference substance in order to establish mode of action of the test substance. Stock solutions with phenol were prepared for lauryl sulphosuccinate, DSDS and Geropon T77 at pH 7 as described in section 3.2.3.2.. Stock solution with 1-hexanol was prepared for SLDI as described in section 3.2.3.2..

Test solutions were prepared by serial dilution of stock solutions with Elendt M7 medium (pH 7 adjusted where required) as described in section 3.2.3.3..

4.2.12 PREPARATION OF STOCK / TEST SOLUTIONS TO BE TESTED AT LOW WATER HARDNESS

Stock solutions of each test material to be tested at 13mg/l equivalent of $CaCO_3$ were prepared by dissolving the test material Elendt M7 medium which had been prepared without the inclusion of $CaCl_2.2H_2O$ or $MgSO_4.7H_2O$. For SMMA and SEMA, these required warming. These were again typically of

1000mg/l and 100mg/l depending on the range of test solution concentrations. For SMMA and SEMA stock solutions were prepared to be analogous to the previous precipitation studies involving these two materials (section 4.2.7), such that stock concentration for both test substances was 320mg/l.

Test solutions were prepared by serial dilution of the stock solutions with Elendt M7 medium prepared without Ca^{2+} or Mg^{2+} ions, typically on the progressive scale ...1.0, 0.56, 0.32, 0.18, 0.1mg/l.... To each test solution was added the required volume (3.2ml) of hardness Stock A to attain a TH of 13mg/l equivalent of $CaCO_3$ as calculated in section 4.2.6.2. (Table 44) before making up to volume.

Test solutions of SMMA and SEMA were prepared in a similar way to those in precipitation studies for these substances (section 4.2.7), by serial dilution of warm stock solution solutions with Elendt M7 medium prepared without Ca^{2+} or Mg^{2+} ions. On addition of 3.2ml hardness Stock A to each test solution, prepared in conical flasks, and making up to volume, the conical flasks were sealed and the solutions stirred for a period of 5 hours in order to achieve saturation equilibrium. At the end of this period each test solution was transferred to sealed centrifuge tubes and spun for 15 minutes at 20,000 rpm to remove suspended precipitate. The soluble fraction (SF) supernatant was removed by pipette into the test vessels.

Solutions were prepared at the start of the test and renewed at 24 hours.

4.2.13 CULTURING CONDITIONS OF *DAPHNIA MAGNA*

Culturing conditions were as described in section 2.2.4.. In addition to the standard cultures, three further cultures were established using neonates from the standard cultures and maintained at 13mg/l equivalent of $CaCO_3$. In all other respects these cultures were maintained identically to the standard cultures.

4.2.14 STATISTICAL ANALYSIS OF MORTALITY DATA

The mortality of neonate at 48 hours was analysed using the computer program BMPDIN as described in section 2.2.7. Where measured test solution concentrations were available for all concentrations, these values were used instead of nominal values. Where only partial analysis was performed, 48 hour EC50 values were corrected for mean disagreement between nominal and measured values where applicable.

4.2.15 ANALYSIS OF TEST SOLUTION CONCENTRATIONS

Analysis of anionic surfactant test solution concentrations in both individual and mixture toxicity studies was carried out by MBAS as described in section 2.2.8.

Analysis of phenol and 1-hexanol test solution concentrations was carried out by GC-MS by the analytical support units at Unilever Research.

4.2.16 LOG P CALCULATION

All conventional log P calculation was carried out according to the Hansch and Leo (1979) method with the application of PDBF where appropriate, as described in section 2.2.9.

4.2.16.1 CALCULATION OF LOG P BY HANSCH AND LEO METHOD

SEMA : CH_3-$(CH_2)_{12}$-CONH-CH_2-CH_2-$SO_3^-Na^+$

$\log P = f_{CH3} + 14f_{CH2} + f_{CONH} + f_{SO3^-} + 16\text{-}1(Fb) + Fp\text{-}2$

$= 0.89 + 14(0.66) + (-2.71) + (-5.87) + 15(-0.12) + (-0.26(-5.87 + (-2.71)))$

$= 1.98$

SMMA : $CH_3-(CH_2)_{12}-CONH-CH_2-SO_3^-Na^+$

log P = $f_{CH3} + 13f_{CH2} + f_{CONH} + f_{SO3-} + 15\text{-}1(Fb) + Fp\text{-}2$

= $0.89 + 13(0.66) + (-2.71) + (-5.87) + 14(-0.12) + (-0.26(-5.87 + (-2.71)))$

= 2.81

Geropon T77: $CH_3-(CH_2)_7-CH=CH-(CH_2)_7-CON(CH_3)-CH_2-CH_2-SO_3^-Na^+$

log P for structures containing double bonds are calculated treating as saturated molecules and applying the bond factor (Fdou). Triple bonds are treated in the same way.

log P = $2f_{CH3} + 18f_{CH2} + f_{CON} + f_{SO3-} + 21\text{-}1(Fb) + Fp\text{-}2 + PDBF + Fdou$

= $2(0.89) + 18(0.66) + (-3.04) + (-5.87) + 20(-0.12) + (-0.26(-5.87 + (-3.04))) + (-1.44\log(1+1)) + (-0.55)$

= 3.69

DLAS: $CH_3-(CH_2)_{11}-CO_2-CH_2-CH(SO_3^-Na^+)-CO_2^-Na^+$

log P = $f_{CH3} + 12(f_{CH2}) + f_{CH} + f_{SO3-} + f_{CO2} + f_{CO2-} + 16\text{-}1(Fb) + FgBr + Fp\text{-}1 + 2(Fp\text{-}2)$

= $0.89 + 12(0.66) + 0.43 + (-5.87) + (-1.49) + (-5.19) + 15(-0.12) + (-0.22) + (-0.42(-5.87 + (-5.19))) + (-0.26(-5.87 + (-1.49))) + (-0.26(-5.19 + (-1.49)))$

= 2.97.

DSDS: CH_3-$(CH_2)_{15}$- $CH(OSO_3^-Na^+)$-CH_2-$OSO_3^-Na^+$

log P = f_{CH3} +16f_{CH2} + f_{CH} + 2f_{SO4-} + 19-1(Fb) + Fp-2 + FgBr

= 0.89 + 16(0.66) + 0.43 + 2(-5.23) + 18(-0.12) + (-0.26(-5.23 + (-5.23))) + (-0.22)

= 1.76

SLDI: CH_3-$(CH_2)_{10}$-CO_2-CH_2-CH_2-(O-CH_2-CH_2)-(O-CH_2-CH_2)-$SO_3^-Na^+$

The Hansch and Leo method for the calculation of the contribution to log P of the EO unit (O-CH_2-CH_2) in ethoxylated non-ionic surfactants of general structure R-$(EO)_n$-OH has appeared deficient in the past (Roberts, 1991; Roberts and Marshall, 1995). The method calculates the contribution of each EO unit as slightly positive, whereas in reality the water solubility of these substances increases with additional EO units. An empirically derived log P increment of -0.10 for each EO group additional to the first unit has been found to be applicable (Roberts, 1991; Yoshimura, 1986). Accurate prediction of EC50 values can be dervied from log P values arising from such modification in combination with the general narcosis equation (equation 9). EO units within the SLDI structure can be dealt with in the same way such that only the first EO unit is considered by the conventional log P calculation with the application of the -0.1 increment for every additional EO unit:

log P = f_{CH3} + 14f_{CH2} + f_{SO3-} + f_{CO2} + f_O + 17-1(Fb) +2(Fp-2) + (-0.1)*

= 0.89 + 14(0.66) + (-5.87) + (-1.49) + (-1.82) + 16(-0.12) + (-0.26(-5.87 + (-1.82))) + (-0.26(-1.49 + (-1.82))) + (-0.1)*

= 1.79

* empirically derived value for ethoxylate group.

4.3 NEW PROXIMITY FACTOR CALCULATION

The proximity factor derived by Hansch and Leo (1979) was based on observations of measured log P fragments. As previously mentioned, it is unlikely that those observed contained such widely different fragment values as found in FAES and some other anionic surfactants. However, it is reasonable to assume that the their proximity factor is applicable to those values from which it was originally derived. This should be accounted for when deriving any modified factor. Hence two conditions made by the original proximity factor were incorporated into the modified proximity factor.

1) Polar fragments are capable of interaction only when positioned within a molecule by a maximum of three carbons separation.
2) The value of two proximal equal sized fragments is reduced by 42%, 26% and 10% when separated by one, two and three carbons respectively (where size is taken to mean the scope of influence on free energy of the water surrounding any polar fragment e.g. $f_{SO3^-} = -5.87$ is large compared to $f_{CO2} = -1.49$).

Where the two polar fragments are of equal size, therefore, the scale of the effect of water sharing between overlapping sheaths and the effects of proximity on the reduction of overall negative contribution of the two fragments, can be considered equivalent to that calculated by the original proximity factor. As the difference in size between polar fragments increases, the influence of the larger fragment on free energy of the surrounding water increases relative to the smaller fragment. This results in complete overlap of the hydration sheaths where the size difference of the fragments is sufficiently large and the fragment separation is sufficiently small. It is necessary, therefore, to calculate the extent of overlap of the hydration sheaths of any two proximate polar fragments in order to establish loss of negative log P contribution of each fragment.

The zone of influence, or hydration sheath, of any polar fragment on surrounding water molecules cannot in actuality be considered to have a 'cut-off' point. However, as the influence reduces with increasing distance from the centre

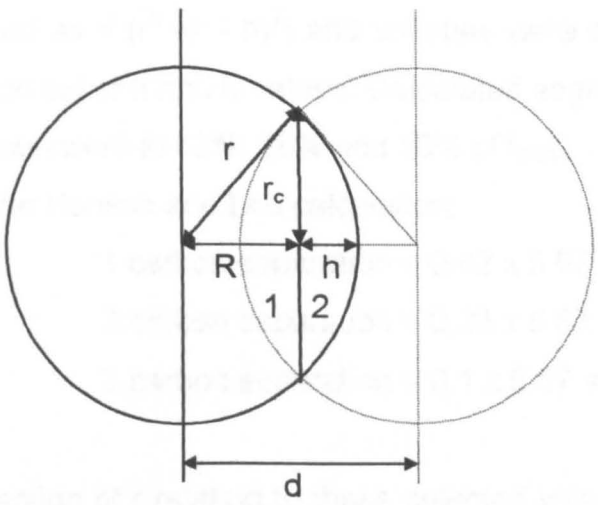
of the fragment, it can be considered to reach a point where its influence is negligibly small. The fragment value is a measure of the extent of influence within this zone. As all surrounding water is influenced equally by an individual fragment it is reasonable to consider the hydration sheath as a spherical volume (f) of radius (r), where f is now considered as a positive value:

e.g. volume ($4/3\pi\ r^3$) f_{SO3}^- = 5.87 , r = $\sqrt[3]{(3f)/(4\pi)}$ = 1.119. Equation 25.

Units conceptually are in log P units which will be assumed for all remaining calculations unless otherwise stated.

Thus the overlap of hydration sheaths can be considered as the overlapping volume of two spheres (Fig. 21).

Fig. 21. Schematic overlap of hydration sheaths of two equal sized polar fragments '1' and '2'



where R = distance from centre of sphere to centre of overlap.

h = distance from centre of overlap to edge of overlap.

r = radius.

r_c = distance from point of overlap at surface to centre of overlap.

d = distance between centres of spheres.

Fig. 21. indicates R > h. Considering that for equal sized fragments at one carbon separation the 42% of each volume is overlapped it was probable that for such equal sized volumes h > R.

The overlapping volume can thus be considered as the sum of the volume of segments 1 and 2, each calculated (James, 1992) by :

$$1/6\pi h\,(3r_c^2 + h^2) \qquad \text{Equation 26.}$$

As r_c and h, and consequently volume, of the segments are dependent on the distance between the centres of each sphere it was necessary to establish centre of sphere separation. Overlapping volume for any two equal sized fragments is identical for each fragment and is consistent with 42%, 26% and 10% reduction of the fragment value. Arbitrarily selecting the fragment value for SO_3^-, as one of the larger fragments, a series of h values were calculated as fraction of r. Fractions were chosen in the range 0 - 1 such that a value of 0 indicated no overlap and a value of 1 indicated complete overlap. r_c values for each h value were calculated as $\sqrt{(r^2 - (r - h)^2)}$ and volumes were calculated for the segments relating to each set of h and r_c values. Calculated segment volumes were selected which were equivalent to 42%, 26% and 10% of f_{SO3}^-.

From the Hansch and Leo calculation:

f_{SO3}^- = 5.87 at: 1 carbon separation = 0.42 x 5.87 = 2.47 (2.465 to 4 s.f.).

2 carbon separation = 0.26 x 5.87 = 1.53 (1.526 to 4 s.f.).

3 carbon separation = 0.1 x 5.87 = 0.587 (0.5870 to 4 s.f.).

The fraction of r relating to these selected volumes could then be used for any given value of r to calculate h for fragments at 1 carbon, 2 carbon and 3 carbon separation respectively (Tables 45 and 46).

Table 46 indicates that h is related to r at:

1 carbon separation by: h = 0.893 r.

2 carbon separation by: h = 0.668 r.

3 carbon separation by: h = 0.392 r.

Table 45. Values of h, rc and segment volume (vol) calculated as fractions of r for fragment (f_{SO3}^-)of value 5.87, where f_{SO3}^- assumed to be volume of hydration sheath (all values conceptually are in log P units)

r[a]	Fraction	h#	r_c#	vol#
1.119	0	0	0	0
	0.1	0.111905	0.487783	0.042558
	0.15	0.167858	0.589497	0.094103
	0.2	0.22381	0.671431	0.16436
	0.25	0.279763	0.740183	0.252227
	0.3	0.335715	0.799162	0.356603
	0.35	**0.391668**	**0.850405**	**0.476387*** **
	0.4	**0.44762**	**0.895241**	**0.61048*** **
	0.45	0.503573	0.934592	0.75778
	0.5	0.559525	0.969126	0.917188
	0.55	0.615478	0.999344	1.087601
	0.6	0.671431	1.025627	1.26792
	0.65	**0.727383**	**1.048271**	**1.457044** **
	0.7	**0.783336**	**1.067506**	**1.653873** **
	0.75	0.839288	1.083516	1.857305
	0.8	0.895241	1.096441	2.06624
	0.85	**0.951193**	**1.10639**	**2.279578***
	0.9	**1.007146**	**1.113442**	**2.496218***
	0.95	1.063098	1.117651	2.715058
	1	1.119051	1.119051	2.935

* Approximate limits surrounding segment volume when equal sized fragments at 1 carbon separation (0.42 x 5.87 = 2.47).

** Approximate limits surrounding segment volume when equal sized fragments at 2 carbon separation (0.26 x 5.87 = 1.53).

*** Approximate limits surrounding segment volume when equal sized fragments at 3 carbon separation (0.1 x 5.87 = 0.587).(see Table 46 for exact values).

[a] Value to 4 s.f. # Value calculated by spreadsheet formula.

Table 46. Values of h, rc and segment volume (vol) calculated as fractions of r for fragment (f_{SO3}^-)of value 5.87 at closer defined fraction values, where f_{SO3}^- assumed to be volume of hydration sheath (all values conceptually are in log P units)

r[a]	Fraction	h#	r_c#	vol#
1.119	0.892	0.998193	1.112505	2.461379
	0.893*	**0.999312**	**1.112626**	**2.46573**
	0.894	1.000431	1.112746	2.470083
	0.667	0.746407	1.055183	1.523156
	0.668**	**0.747526**	**1.055578**	**1.527072**
	0.669	0.748645	1.055971	1.530991
	0.391	0.437549	0.887598	0.585337
	0.392***	**0.438668**	**0.888456**	**0.588109**
	0.393	0.439787	0.889312	0.590887

* Fraction required to calculate segment volume equivalent to that calculated by Hansch and Leo (1979) at 1 carbon separation (0.42 X 5.87 = 2.47).

** Fraction required to calculate segment volume equivalent to that calculated by Hansch and Leo (1979) at 2 carbon separation (0.26 X 5.87 = 1.53).

*** Fraction required to calculate segment volume equivalent to that calculated by Hansch and Leo (1979) at 3 carbon separation (0.1 X 5.87 = 0.587).

[a] Value to 4 s.f.

This can be applied to all fragment values.

Values of R in terms of r can now be calculated as r - h. This will hold whether R > h or h > R, since both fragments are of equal size. Since h is related to r as above, for any given value of r at 1,2 or 3 carbon separation, R will be related to r by (1-0.893), (1-0.668) and (1-0.392) respectively at :

1 carbon separation by: R = 0.107 r. Equation 27.

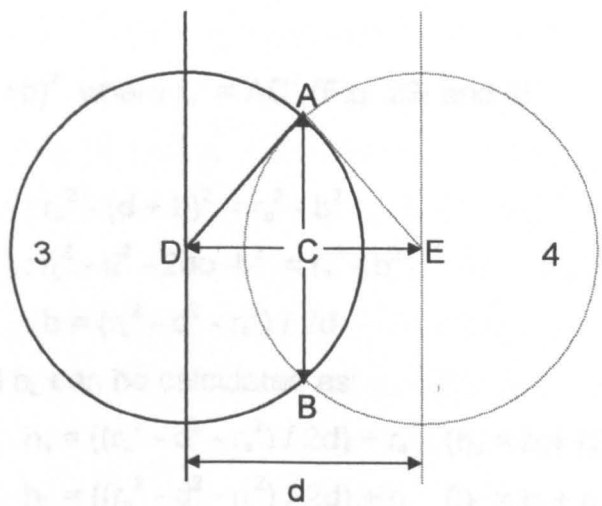
2 carbon separation by: R = 0.332 r. Equation 28.

3 carbon separation by: R = 0.608 r. Equation 29.

A value equivalent to separation of centres of spheres (d) can be calculated at 1,2 and 3 carbon separation for any two fragments by the summation of the R value of each fragment.

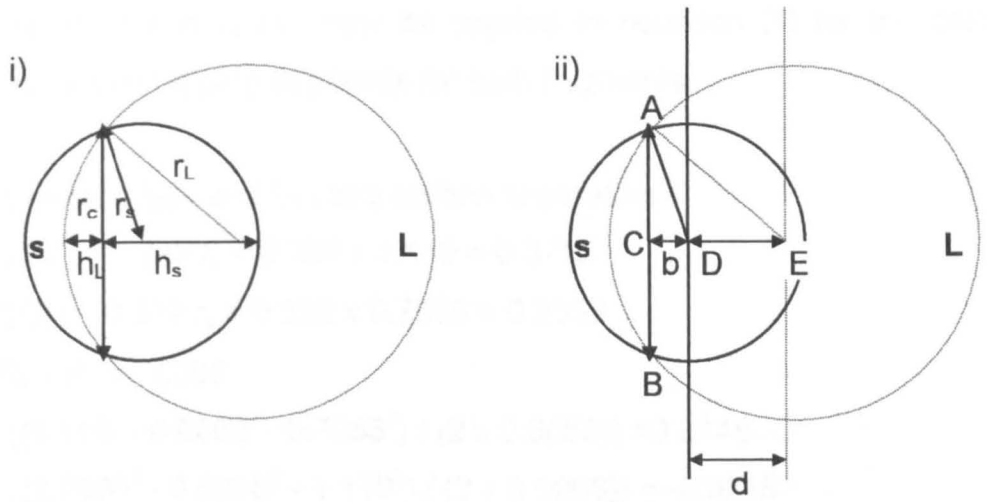
The calculation of a value for h is complicated, however, by the issue of difference in size of spheres. Where spheres are of equal size the intersection C of the line AB (Fig. 22) connecting the points of contact of spheres with line DE (Fig. 22) connecting sphere centres always be positioned at a distance d/2 from points D and E.

Fig. 22. Schematic diagram of overlapping fragments '3' and '4' showing points of intersection



As the size differential increases, however, CD decreases as CE increases as R_3 and R_4 change with r_3 and r_4 relative to each other. As the size differential continues to increase such that r_3 is small relative to r_4, then point C will no longer appear between points D and E (Fig. 23ii).

Fig. 23. Schematic diagram of overlapping fragments 's' and 'L' of different sizes. Diagrams i) and ii) represent the same pair of fragments



where fragments '3' and '4' are now classed relatively as 's' (small) and 'L' (large).

For this situation, calculation of R and consequently d can be calculated as before using equation 27, 28, and 29. However, calculation of h_s and h_L now requires an additional value 'b', calculated as:

$r_c^2 = r_L^2 - (d+b)^2$ where $r_c^2 = AC^2$ (Fig. 23i and ii)

$r_c^2 = r_s^2 - b^2$

therefore : $r_L^2 - (d + b)^2 = r_s^2 - b^2$

: $r_L^2 - d^2 - 2db - b^2 = r_s^2 - b^2$

: $b = (r_L^2 - d^2 - r_s^2) / 2d$

Thus h_s and h_L can be calculated as:

$h_s = ((r_L^2 - d^2 - r_s^2) / 2d) + r_s$ $(h_s = b_s + r_s)$. Equation 30.

$h_L = ((r_s^2 - d^2 - r_L^2) / 2d) + r_L$ $(h_L = b_L + r_L)$. Equation 31.

The calculation returns a negative value for b_L which is equal to a negative value of b_s + d (see example calculation below). This equation can be used universally for all fragment sizes and removes problems which would be associated with addition or subtraction of b_s where point C falls between D and E such as in Fig. 22.

$r_c{}^2$can be calculated as $r_L{}^2 - (d + b_s)^2$. Equation 32.

$d = R_s + R_L$. Equation 33.

Values for h and r_c can now be applied in equation 26 for the calculation of volume of overlapping segments for both fragments.

For example: f_{SO3}^- and f_{CO2} at 2 carbon separation:

$R_L (SO_3^-) = 0.332\ r_L = 0.332 \times 1.119 = 0.3715$

$R_s (CO_2) = 0.332\ r_s = 0.332 \times 0.7085 = 0.2352$

$d = R_L + R_s = 0.6068$

$b_s = ((1.119^2 - 0.6068^2 - 0.7085^2) / (2 \times 0.6068)) = 0.3148.$

$b_L = ((0.7085^2 - 0.6068^2 - 1.119^2) / (2 \times 0.6068)) = -0.9216.$

$h_s = 0.3148 + 0.7085 = 1.023.$

$h_L = -0.9216 + 1.119 = 0.1974.$

$r_c^2 = 1.1192 - (0.6068 + 0.3148)^2 = 0.4029$

volume segment s = $1/6\pi \times 1.023\ (3 \times 0.4029 + 1.023^2) = 1.208.$

volume segment L = $1/6\pi \times 0.1974\ (3 \times 0.4029 + 0.1974^2) = 0.1289.$

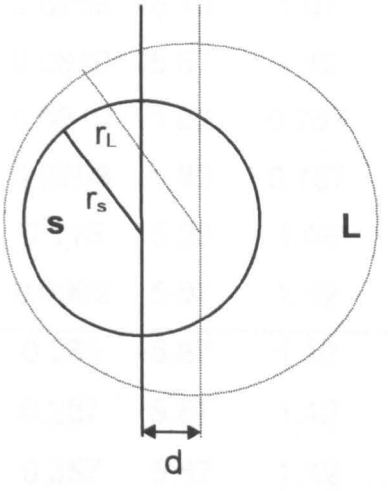
Total volume of overlapping hydration sheaths = 1.208 + 0.1289 = 1.337 (1.34).

The value of 1.34 accounts for the reduction in the negative contribution to log P by proximal polar fragments of SO_3^- and CO_2 at 2 carbon separation and should be used as a replacement for the Hansch and Leo proximity factor including the polar group branch factor (FgBr) such that for the above example total log P contribution of the polar fragments would be:

-5.87 + -1.49 + 1.34 = -6.02 at 2 carbon separation.

Further problems arise where the size differential is so large and d is sufficiently small that the two hydration sheaths completely overlap (Fig. 24). This effectively removes the negative contribution to log P of the smaller fragment.

Fig. 24. Schematic diagram of complete overlap of two fragment values where f_L is significantly larger than f_s



No calculation of overlap is necessary where complete overlap occurs. Log P contribution of the smaller fragment is considered as 0 and no further proximity factor is required.

Complete overlap can be assumed where$(r_L - d - r_s) > 0$.

Table 47 contains calculated values for r, R, d and $(r_L - d - r_s)$ for a number of common fragment combinations found in structures used in this chapter at 1, 2 and 3 carbon separation. At 1 carbon separation $(r_L - d - r_s) > 0$ for proximal pairs of fragments of f_{CO2} (1.49) / f_{SO3^-} (5.87), f_{CONH} (2.71) / f_{SO3^-} (5.87), f_{CO2} (1.49) / f_{COO^-} (5.19), f_O (1.82) / f_{SO3^-} (5.87) and f_{CON} (3.04) / f_{SO3^-} (5.87). No further proximity value need be calculated for these proximal pairs. Contribution of polar fragments to the log P calculation will be as a result of the largest fragment of the pair only. At 2 and 3 carbon separation $(r_L - d - r_s) < 0$ for all pairs selected.

Table 48 contains values for r, h and overlapping volumes for the above fragment pairs. It should be noted, as previously mentioned, that overlapping

Table 47. Testing for complete overlap (r_L - d - r_s) of hydration sheaths of pairs of polar fragments at 1,2 and 3 carbon separation

CS	f_s	r_s	R_s	f_L	r_L	R_L	d	r_L-d-r_s
1	1.49	0.709	0.0758	5.87	1.12	0.120	0.196	0.215
	2.71	0.865	0.0925	5.87	1.12	0.120	0.212	0.0419
	5.19	1.07	0.11	5.87	1.12	0.120	0.235	-0.190
	1.49	0.709	0.0758	5.19	1.07	0.115	0.191	0.175
	1.82	0.757	0.0810	5.87	1.12	0.120	0.201	0.161
	1.82	0.757	0.0810	1.82	0.757	0.0810	0.162	-0.162
	1.49	0.709	0.0758	1.82	0.757	0.0810	0.157	-0.108
	5.23	1.08	0.115	5.23	1.08	0.115	0.230	-0.230
	3.04	0.899	0.0962	5.87	1.12	0.120	0.216	0.00450
2	1.49	0.709	0.235	5.87	1.12	0.372	0.607	-0.196
	2.71	0.865	0.287	5.87	1.12	0.372	0.659	-0.405
	5.19	1.07	0.357	5.87	1.12	0.372	0.728	-0.683
	1.49	0.709	0.235	5.19	1.07	0.357	0.592	-0.226
	1.82	0.757	0.251	5.87	1.12	0.372	0.623	-0.261
	1.82	0.757	0.251	1.82	0.757	0.251	0.503	-0.503
	1.49	0.709	0.235	1.82	0.757	0.251	0.487	-0.438
	5.23	1.08	0.357	5.23	1.08	0.357	0.715	-0.715
	3.04	0.899	0.298	5.87	1.12	0.372	0.670	-0.449
3	1.49	0.709	0.431	5.87	1.12	0.680	1.11	-0.701
	2.71	0.865	0.526	5.87	1.12	0.680	1.21	-0.952
	5.19	1.07	0.653	5.87	1.12	0.680	1.33	-1.29
	1.49	0.709	0.431	5.19	1.07	0.653	1.08	-0.718
	1.82	0.757	0.461	5.87	1.12	0.680	1.14	-0.779
	1.82	0.757	0.461	1.82	0.76	0.461	0.921	-0.921
	1.49	0.709	0.431	1.82	0.76	0.461	0.891	-0.842
	5.23	1.08	0.655	5.23	1.08	0.655	1.31	-1.31
	3.04	0.899	0.546	5.87	1.12	0.680	1.23	-1.01

Values to 3 s.f..
CS: Carbon separation.

Table 48. Segment and total hydration sheath overlap of pairs of fragments at 1,2 and 3 carbon separation

CS	f_s	r_s	h_s	f_L	r_L	h_L	Vol_s	Vol_L	Voltot
1	5.19	1.07	1.17	5.87	1.12	0.791	2.93	1.68	4.61
	1.82	0.757	0.676	1.82	0.757	0.676	0.765	0.765	1.53
	1.49	0.709	0.858	1.82	0.757	0.451	0.978	0.387	1.37
	5.23	1.08	0.962	5.23	1.08	0.962	2.20	2.20	4.39
2	1.49	0.709	1.02	5.87	1.12	0.197	1.21	0.129	1.34
	2.71	0.865	0.918	5.87	1.12	0.407	1.48	0.512	1.99
	5.19	1.07	0.778	5.87	1.12	0.687	1.55	1.32	2.87
	1.49	0.709	0.963	5.19	1.07	0.228	1.13	0.163	1.29
	1.82	0.757	0.991	5.87	1.12	0.263	1.32	0.224	1.54
	1.82	0.757	0.506	1.82	0.757	0.506	0.473	0.473	0.947
	1.49	0.709	0.539	1.82	0.757	0.440	0.482	0.372	0.855
	5.23	1.08	0.719	5.23	1.08	0.719	1.36	1.36	2.72
	3.04	0.899	0.896	5.87	1.12	0.452	1.51	0.622	2.13
3	1.49	0.709	0.491	5.87	1.12	0.226	0.412	0.167	0.579
	2.71	0.865	0.471	5.87	1.12	0.307	0.493	0.301	0.794
	5.19	1.07	0.444	5.87	1.12	0.415	0.574	0.531	1.11
	1.49	0.709	0.467	5.19	1.07	0.232	0.379	0.168	0.547
	1.82	0.757	0.484	5.87	1.12	0.251	0.439	0.205	0.644
	1.82	0.757	0.297	1.82	0.757	0.297	0.182	0.182	0.365
	1.49	0.709	0.303	1.82	0.757	0.272	0.175	0.155	0.330
	5.23	1.08	0.422	5.23	1.08	0.422	0.524	0.524	1.05
	3.04	0.899	0.467	5.87	1.12	0.324	0.508	0.334	0.842

Values to 3 s.f.. CS: Carbon separation.

Vol_s: Volume of segment overlap of smaller fragment.

Vol_L: Volume of segment overlap of larger fragment.

Voltot : Total volume of overlap of both fragments.

No volumes calculated for pairs of fragments where $(r_L - d - r_s) > 0$ (Table 47).

volume values for equal sized fragments, for example 5.23 / 5.23 fragment combination, are consistent with values calculated using the Hansch and Leo proximity factor method at 1, 2 and 3 carbon separation e.g. 5.23 / 5.23 at 1 carbon separation = 4.39 = 0.42(5.23 + 5.23). Values calculated by the new method become progressively dissimilar to those of Hansch and Leo as the size differential of the pairs of fragments increases.

The assessment of volume overlap, however, requires a number of separate calculations making it time consuming. In order to reduce the number of calculations and thus simplify the equations for both r_L - d - r_s and overlapping volume calculations were derived in terms of f_L / f_s ratios expressed as fractions.

4.3.1 REFINEMENT OF (r_L - d - r_s) VALUE CALCULATION

The qualification for deciding whether two fragment hydration sheaths completely overlap is where (r_L - d - r_s) $\geq$ 0. A positive value indicates complete overlap, a negative value incomplete overlap requiring further calculations. A series of polar fragment pairs were arbitrarily chosen and values of (r_L - d - r_s) and ratios of the chosen pairs (f_L / f_s) calculated for each pair at 1 ,2 and 3 carbon separation (Table 49). Values less than the ratio at each carbon separation relating to the point at which (r_L - d - r_s) < 0 can be considered not to overlap completely. Table 49 indicates these values to be as follows:

At 1 carbon separation (f_L / f_s) $\leq$ 1.904 = incomplete overlap.
At 2 carbon separation (f_L / f_s) $\leq$ 7.928 = incomplete overlap.
At 3 carbon separation (f_L / f_s) $\leq$ 69.02 = incomplete overlap.

These values are considered of suitable accuracy when considering that fragment values are reported to a maximum of 3 s.f. and in situations where (r_L - d - r_s) approaches 0, differences in log P contribution become minimal.

Table 49. Values of pairs of fragment values expressed as (f_L / f_s) at point where (r_L - d - r_s) < 0.

CS	f_s#	f_L#	r_s#	r_L#	d#	f_s / f_L*	r_L-d-r_s
1	3.0814	5.8700	0.90272	1.1191	0.21633	1.905	4.98×10^{-6}
	3.0830	5.8700	0.90287	1.1191	0.21635	**1.904**	$\mathbf{-1.7 \times 10^{-4}}$
	3.0846	5.8700	0.90303	1.1191	0.21636	1.903	-3.4×10^{-4}
2	0.74032	5.8700	0.56119	1.1191	0.55784	7.929	2.02×10^{-5}
	0.74041	5.8700	0.56121	1.1191	0.55785	**7.928**	$\mathbf{-1.1 \times 10^{-5}}$
	0.74051	5.8700	0.56124	1.1191	0.55786	7.927	-4.3×10^{-5}
3	0.085023	5.8700	0.27278	1.1191	0.84624	69.04	3.4×10^{-5}
	0.085035	5.8700	0.27280	1.1191	0.84624	69.03	1.3×10^{-5}
	0.085048	5.8700	0.27281	1.1191	0.84625	**69.02**	$\mathbf{-8.4 \times 10^{-6}}$

CS: Carbon separation.

* Values to 4 s.f.

Values to 5 s.f.

Values in bold indicate values of (f_L / f_s) for which (r_L - d - r_s) < 0 (4 s.f.).

4.3.2 REFINEMENT OF VOLUME (HYDRATION SHEATH) OVERLAP EQUATIONS

A series of pairs of fragment values were chosen such that (f_L / f_s) ratios at 1, 2 and 3 carbon separation were within the range for which incomplete overlap of the fragments within each pair could be assumed to occur (Table 50, 51 and 52). In addition there are very few polar fragment values which would result in (f_L / f_s) ratios over approximately 25 : 1 (Hansch and Leo, 1979). Whilst the potential for these combinations exists, the vast majority result in values within the range 1 -10. For all pairs of polar fragments studied in this thesis, this is certainly the case. (f_L / f_s) ratios were chosen within the ranges 1 - 1.9, 1 - 7.9 and 1 - 7.9 for fragment s at 1, 2 and 3 carbon separation respectively. Thus for fragments at 2 and 3 carbon separation, the ratios chosen were the same. At 1 carbon separation these were changed to account for the effects of complete overlap on ratios.

Overlapping volumes were calculated for each of these pairs using the above methods and plotted as log (f_L / f_s) versus log(overlap volume / (f_s + f_L)) for 1, 2 and 3 carbon separation (Tables 50, 51 and 52, Figs. 25, 26 and 27).

The resulting relationships were generated as 3rd order polynomial functions with rsq values of 1.0 in each case (Figs. 25, 26 and 27). Overlapping volume equations as the new proximity functions can be expressed as:

For l carbon separation : Volume overlap =

$$\mathbf{(10E\,[-0.448(\log(f_L / f_s))^3 - 0.962(\log(f_L / f_s))^2 - 0.0042\log(f_L / f_s) - 0.377])x(f_s + f_L)}$$

Equation 34.

For 2 carbon separation : Volume overlap =

$$\mathbf{(10E\,[-0.059\,(\log(f_L / f_s))^3 - 0.385(\log(f_L / f_s))^2 - 0.0111\log(f_L / f_s) - 0.585])x(f_s + f_L)}$$

Equation 35.

For 3 carbon separation : Volume overlap =

$$\mathbf{(10E\,[0.0587(\log(f_L / f_s))^3 - 0.335(\log(f_L / f_s))^2 + 0.0027\log(f_L / f_s) - 0.999])x(f_s + f_L)}$$

Equation 36.

Table 50. Log (f_L / f_s) and log ((overlap volume) / (f_s + f_L)) for a series of fragment pairs at 1 carbon separation

f_s	f_L	log(f_L/f_s)*	log V/F*	f_s	f_L	log(f_L/f_s)*	log V/F*
4.01	5.87	0.165	-0.406	4	6	0.176	-0.410
3.47	5.87	0.228	-0.433	5	5	0	-0.377
5.19	5.87	0.0535	-0.380	1.29	2	0.190	-0.415
4.35	5.19	0.0767	-0.383	3.75	6	0.204	-0.421
4.52	5.87	0.114	-0.390	4	5	0.0969	-0.387
1.82	1.82	0	-0.377	1	1	0	-0.377
1.49	1.82	0.0869	-0.385	3.64	6	0.217	-0.427
5.23	5.23	0	-0.377	3	5	0.222	-0.430
4.7	5.87	0.0965	-0.386	4	4	0	-0.377
4	6	0.176	-0.410	3.53	6	0.230	-0.434
4.2	5	0.0757	-0.383	3.43	6	0.243	-0.441
3	4	0.125	-0.393	3.33	6	0.256	-0.448
4.35	5	0.0605	-0.381	3.24	6	0.268	-0.455
3.64	4	0.0410	-0.378	3.16	6	0.278	-0.462
3	3	0	-0.377	3.95	6	0.182	-0.412
6	6	0	-0.377	3.69	6	0.211	-0.425
3.81	4	0.0211	-0.377	3.28	6	0.262	-0.452
2	3	0.176	-0.410	4.9	6	0.0880	-0.385
5	6	0.0792	-0.383	5.33	6	0.0514	-0.379
2.4	3	0.0969	-0.387	5.87	5.87	0	-0.377
2	2	0	-0.377				

log (V/F) : log ((overlap volume of hydration sheaths) / (f_s + f_L))

* Values to 3 s.f.

Table 51. Log (f_L / f_s) and log ((overlap volume) / (f_s + f_L)) for a series of fragment pairs at 2 carbon separation

f_s	f_L	log(f_L/f_s)*	log V/F*	f_s	f_L	log(f_L/f_s)*	log V/F*
1.49	5.87	0.595	-0.740	4	6	0.176	-0.598
2.71	5.87	0.336	-0.634	5	5	0	-0.585
5.19	5.87	0.0535	-0.586	1	2	0.301	-0.625
1.49	5.19	0.542	-0.714	3	6	0.301	-0.625
1.82	5.87	0.509	-0.698	4	5	0.0969	-0.589
1.82	1.82	0	-0.585	1	1	0	-0.585
1.49	1.82	0.0869	-0.588	2	6	0.477	-0.685
5.23	5.23	0	-0.585	3	5	0.222	-0.606
3.04	5.87	0.286	-0.621	4	4	0	-0.585
1	6	0.778	-0.854	0.78	6	0.886	-0.939
2	5	0.398	-0.654	0.8	6	0.875	-0.930
3	4	0.125	-0.592	1.1	6	0.737	-0.825
1	5	0.699	-0.800	0.9	6	0.824	-0.888
2	4	0.301	-0.625	0.95	6	0.800	-0.870
3	3	0	-0.585	0.97	6	0.791	-0.864
6	6	0	-0.585	1.2	6	0.699	-0.800
1	4	0.602	-0.744	0.92	6	0.814	-0.881
2	3	0.176	-0.598	1.3	6	0.664	-0.779
5	6	0.0792	-0.588	0.86	6	0.844	-0.904
1	3	0.477	-0.685	5.87	5.87	0	-0.585
2	2	0	-0.585				

log (V/F) : log ((overlap volume of hydration sheaths) / (f_s + f_L))

* Values to 3 s.f.

Table 52. Log (f_L / f_s) and log ((overlap volume) / (f_s + f_L)) for a series of fragment pairs at 3 carbon separation

f_s	f_L	log(f_L/f_s)*	log V/F*	f_s	f_L	log(f_L/f_s)*	log V/F*
1.49	5.87	0.595	-1.10	4	6	0.176	-1.01
2.71	5.87	0.336	-1.03	5	5	0	-0.999
5.19	5.87	0.0535	-1.00	1	2	0.301	-1.03
1.49	5.19	0.542	-1.09	3	6	0.301	-1.023
1.82	5.87	0.509	-1.08	4	5	0.0969	-1.00
1.82	1.82	0	-0.999	1	1	0	-0.999
1.49	1.82	0.0869	-1.00	2	6	0.477	-1.07
5.23	5.23	0	-0.999	3	5	0.222	-1.01
3.04	5.87	0.286	-1.02	4	4	0	-0.999
1	6	0.778	-1.17	0.78	6	0.886	-1.22
2	5	0.398	-1.05	0.8	6	0.875	-1.21
3	4	0.125	-1.00	1.1	6	0.737	-1.16
1	5	0.699	-1.14	0.9	6	0.824	-1.19
2	4	0.301	-1.03	0.95	6	0.800	-1.18
3	3	0	-0.999	0.97	6	0.791	-1.18
6	6	0	-0.999	1.2	6	0.699	-1.14
1	4	0.602	-1.11	0.92	6	0.814	-1.19
2	3	0.176	-1.01	1.3	6	0.664	-1.13
5	6	0.0792	-1.00	0.86	6	0.844	-1.20
1	3	0.477	-1.07	5.87	5.87	0	-0.999
2	2	0	-0.999				

log (V/F) : log ((overlap volume of hydration sheaths) / (f_s + f_L))

* Values to 3 s.f.

Fig. 25. Log (fL / fs) versus log (Overlap volume / (fs + fL)) for fragment pairs at 1 carbon separation

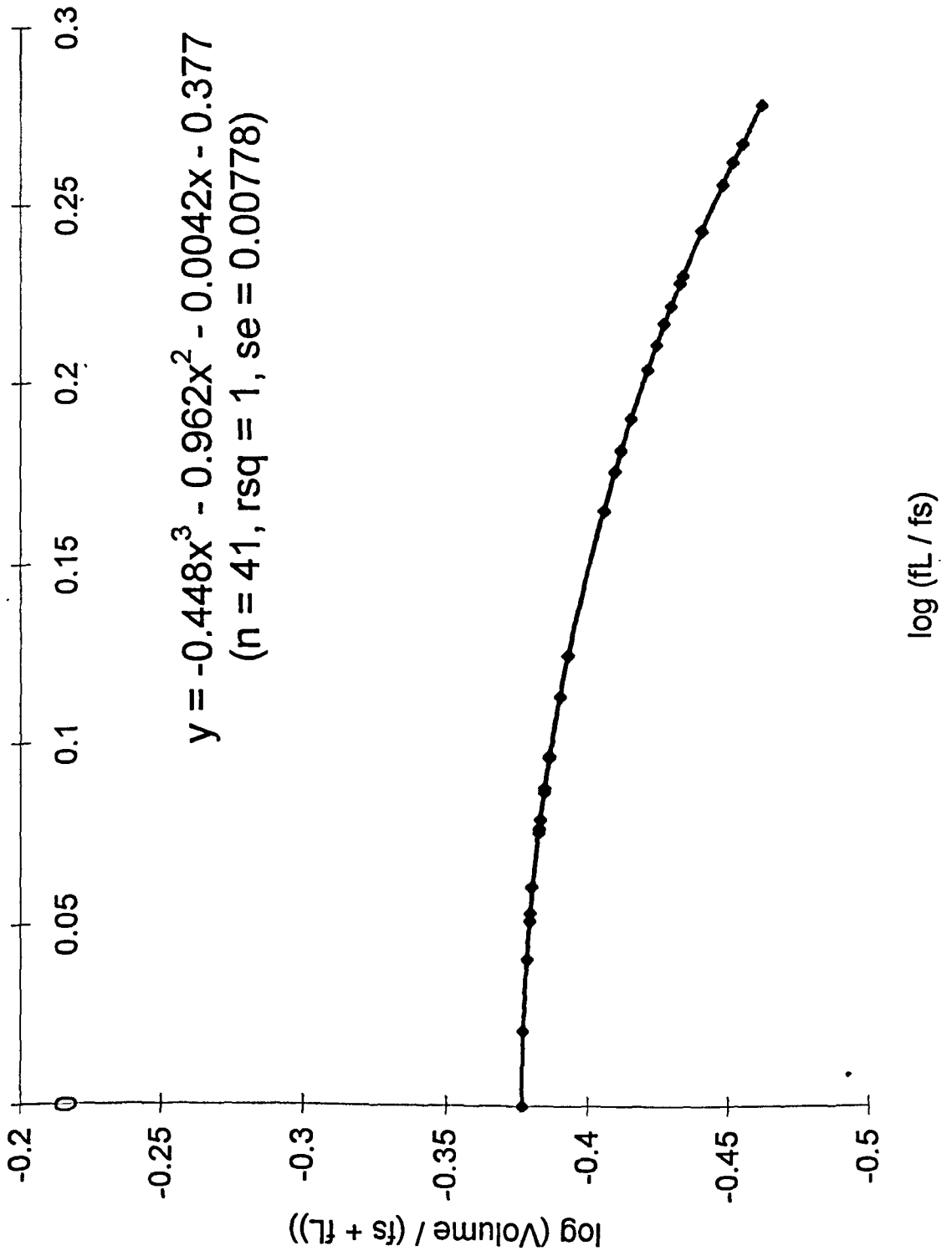


Fig. 26. Log (fL / fs) versus log (Overlap volume / (fs + fL)) for fragment pairs at 2 carbon separation

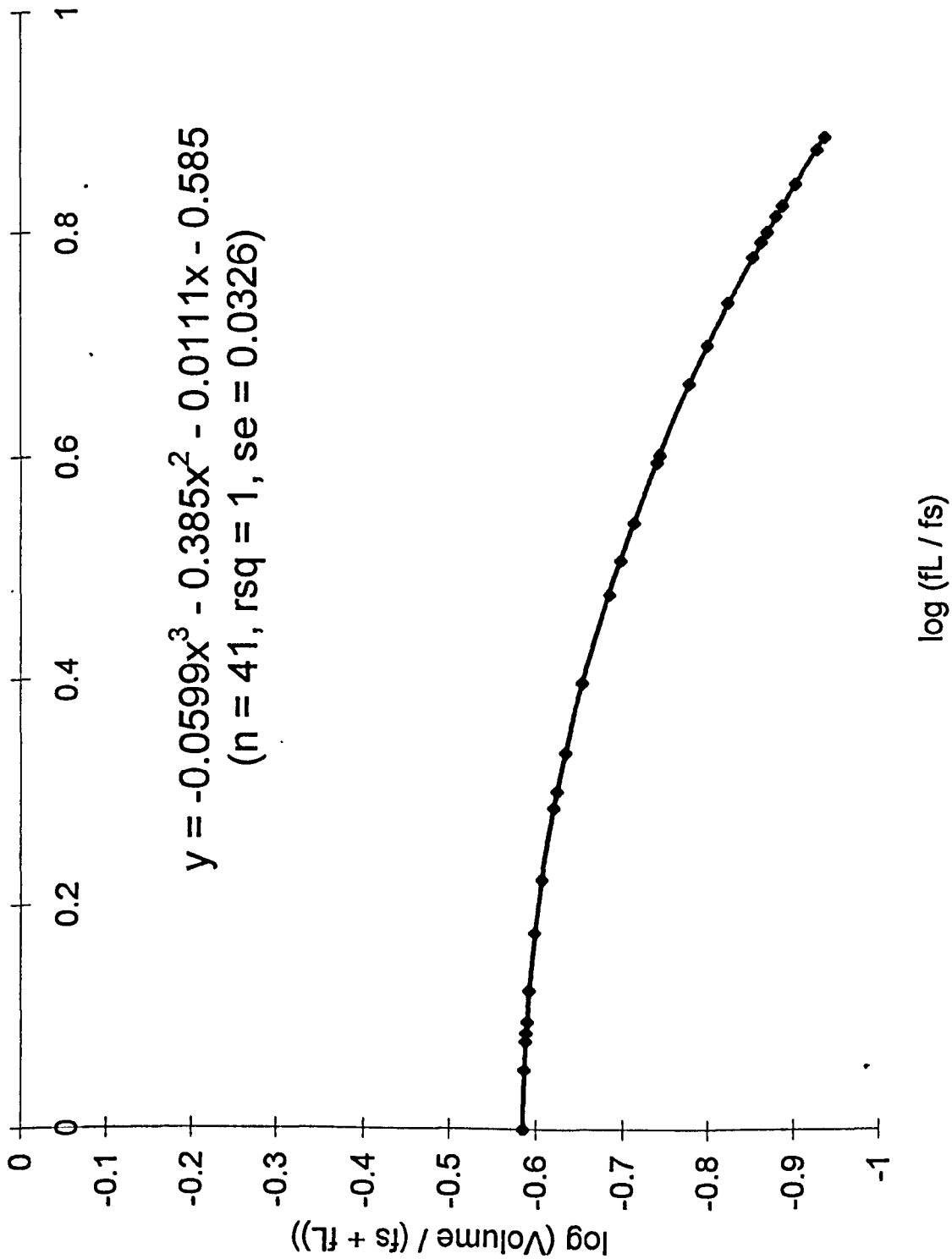
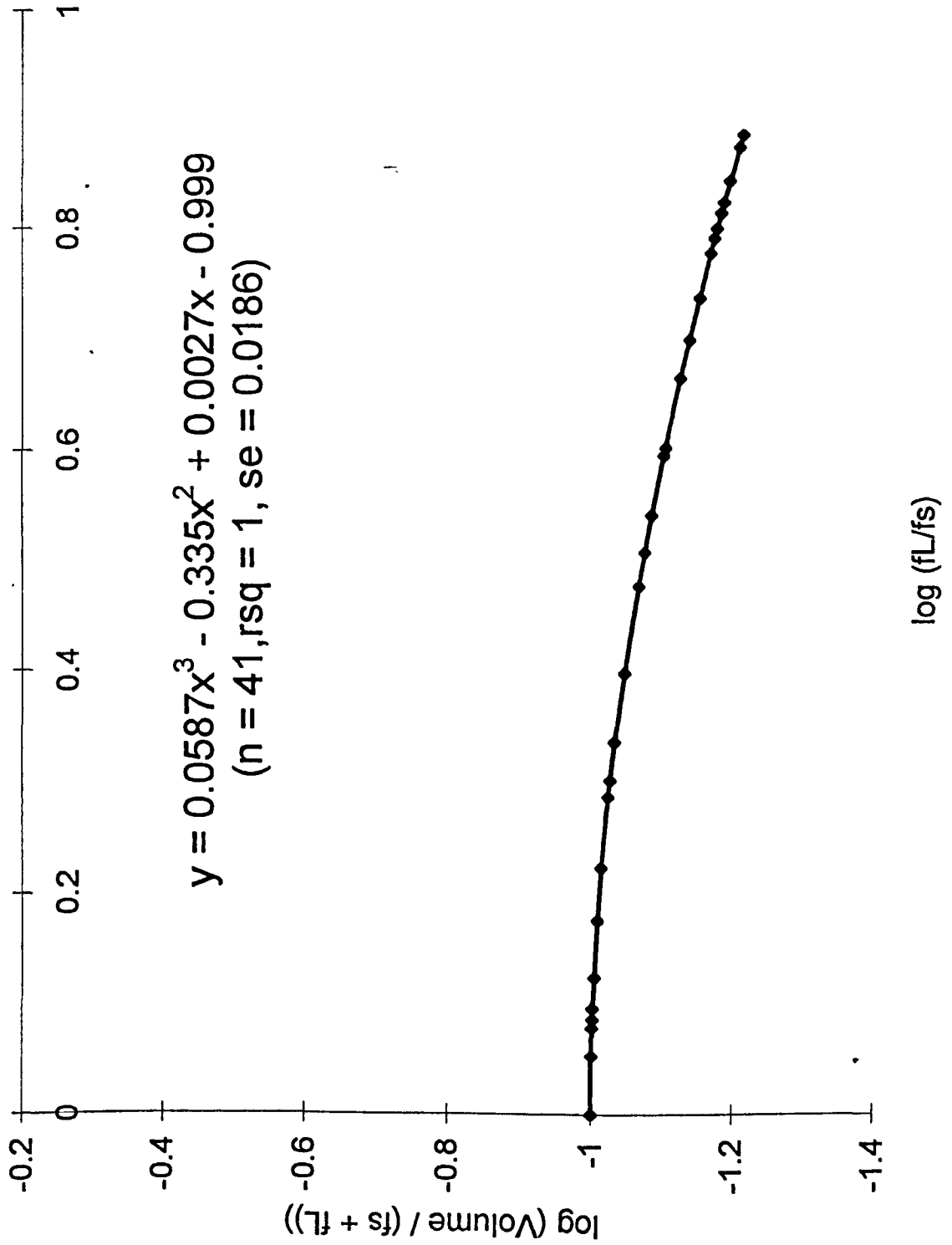


Fig. 27. Log (fL / fs) versus log (Overlap volume / (fs + fL)) for fragment pairs at 3 carbon separation



Overlapping volume calculated using equations 34, 35 and 36 provide values highly consistent with values calculated using equations 25 - 33. Tables 53, 54 and 55 show overlapping volume values calculated for a range of pairs of actual fragment values and artificial values of similar size to actual fragments at 1, 2 and 3 carbon separation within the range of (f_L / f_s) values required to produce incomplete overlap. These were calculated using the 'long hand' method using equations 25 - 33 and also with equations 34, 35 and 36. Figs. 28, 29 and 30 show plots of values calculated using equations 25 - 33 versus values calculated using the respective simplified equations 34, 35 and 36. Slopes of 1 in each case with very low standard error provide sufficient evidence for use of the simplified equations (34, 35 and 36) for calculation of overlapping hydration sheaths over the individual equations used in their derivation.

Whilst proximity factors defined by equations 34, 35 and 36 still involve greater complexity than those of Hansch and Leo, they provide a relatively easy method for calculation of overlapping hydration sheaths in terms of measured log P fragment values.

Table 53. Volume of overlapping hydration sheaths (as log P units) for a series of fragment pairs at 1 carbon separation calculated by the longhand method and by equation 34

f_s	f_L	longhand method*	Predicted using equation*	f_s	f_L	longhand method*	Predicted using equation*
4.01	5.87	3.88	3.88	4	6	3.89	3.89
3.47	5.87	3.45	3.44	5	5	4.20	4.20
5.19	5.87	4.61	4.61	1.29	2	1.26	1.26
4.35	5.19	3.95	3.95	3.75	6	3.69	3.69
4.52	5.87	4.23	4.23	4	5	3.70	3.69
1.82	1.82	1.53	1.53	1	1	0.84	0.84
1.49	1.82	1.37	1.36	3.64	6	3.60	3.60
5.23	5.23	4.39	4.39	3	5	2.97	2.97
4.7	5.87	4.34	4.34	4	4	3.36	3.36
4	6	3.89	3.89	3.53	6	3.51	3.50
4.2	5	3.81	3.81	3.43	6	3.42	3.41
3	4	2.83	2.83	3.33	6	3.32	3.32
4.35	5	3.89	3.89	3.24	6	3.24	3.24
3.64	4	3.20	3.20	3.16	6	3.16	3.16
3	3	2.52	2.52	3.95	6	3.85	3.85
6	6	5.04	5.04	3.69	6	3.64	3.64
3.81	4	3.28	3.27	3.28	6	3.28	3.27
2	3	1.95	1.95	4.9	6	4.49	4.49
5	6	4.55	4.55	5.33	6	4.73	4.73
2.4	3	2.22	2.22	5.87	5.87	4.93	4.93
2	2	1.68	1.68				

Longhand method uses equations 25 - 33.

* Values to2 d.p. (consistent with fragment values of Hansch and Leo, 1979).

Table 54. Volume of overlapping hydration sheaths (as log P units) for a series of fragment pairs at 2 carbon separation calculated by the longhand method and by equation 35

f_s	f_L	longhand method*	Predicted using equation*	f_s	f_L	longhand method*	Predicted using equation*
1.49	5.87	1.34	1.34	4	6	2.52	2.52
2.71	5.87	1.99	1.99	5	5	2.60	2.60
5.19	5.87	2.87	2.86	1	2	0.71	0.71
1.49	5.19	1.29	1.29	3	6	2.14	2.13
1.82	5.87	1.54	1.54	4	5	2.32	2.31
1.82	1.82	0.95	0.95	1	1	0.52	0.52
1.49	1.82	0.85	0.85	2	6	1.65	1.65
5.23	5.23	2.72	2.72	3	5	1.98	1.98
3.04	5.87	2.13	2.13	4	4	2.08	2.08
1	6	0.98	0.98	0.78	6	0.78	0.78
2	5	1.55	1.55	0.8	6	0.80	0.80
3	4	1.79	1.79	1.1	6	1.06	1.06
1	5	0.95	0.95	0.9	6	0.89	0.89
2	4	1.42	1.42	0.95	6	0.94	0.93
3	3	1.56	1.56	0.97	6	0.95	0.95
6	6	3.12	3.12	1.2	6	1.14	1.14
1	4	0.90	0.90	0.92	6	0.91	0.91
2	3	1.26	1.26	1.3	6	1.21	1.21
5	6	2.84	2.84	0.86	6	0.86	0.86
1	3	0.83	0.83	5.87	5.87	3.05	3.05
2	2	1.04	1.04				

Longhand method uses equations 25 - 33.

* Values to2 d.p. (consistent with fragment values of Hansch and Leo, 1979).

Table 55. Volume of overlapping hydration sheaths (as log P units) for a series of fragment pairs at 3 carbon separation calculated by the longhand method and by equation 36

f_s	f_L	longhand method*	Predicted using equation*	f_s	f_L	longhand method*	Predicted using equation*
1.49	5.87	0.58	0.58	4	6	0.98	0.98
2.71	5.87	0.79	0.79	5	5	1.00	1.00
5.19	5.87	1.11	1.11	1	2	0.28	0.28
1.49	5.19	0.55	0.55	3	6	0.85	0.85
1.82	5.87	0.64	0.64	4	5	0.90	0.90
1.82	1.82	0.36	0.36	1	1	0.20	0.20
1.49	1.82	0.33	0.33	2	6	0.68	0.68
5.23	5.23	1.05	1.05	3	5	0.77	0.77
3.04	5.87	0.84	0.84	4	4	0.80	0.80
1	6	0.47	0.47	0.78	6	0.41	0.41
2	5	0.63	0.63	0.8	6	0.42	0.42
3	4	0.69	0.69	1.1	6	0.50	0.50
1	5	0.43	0.43	0.9	6	0.44	0.44
2	4	0.56	0.56	0.95	6	0.46	0.46
3	3	0.60	0.60	0.97	6	0.46	0.46
6	6	1.20	1.20	1.2	6	0.52	0.52
1	4	0.39	0.39	0.92	6	0.45	0.45
2	3	0.49	0.49	1.3	6	0.54	0.54
5	6	1.10	1.10	0.86	6	0.43	0.43
1	3	0.34	0.34	5.87	5.87	1.18	1.18
2	2	0.40	0.40				

Longhand method uses equations 25 - 33.

* Values to2 d.p. (consistent with fragment values of Hansch and Leo, 1979).

Fig. 28. Plot of proximity factor calculated by equation 34 versus factor calculated by long hand method (equations 25 -33), for fragments at 1 carbon separation

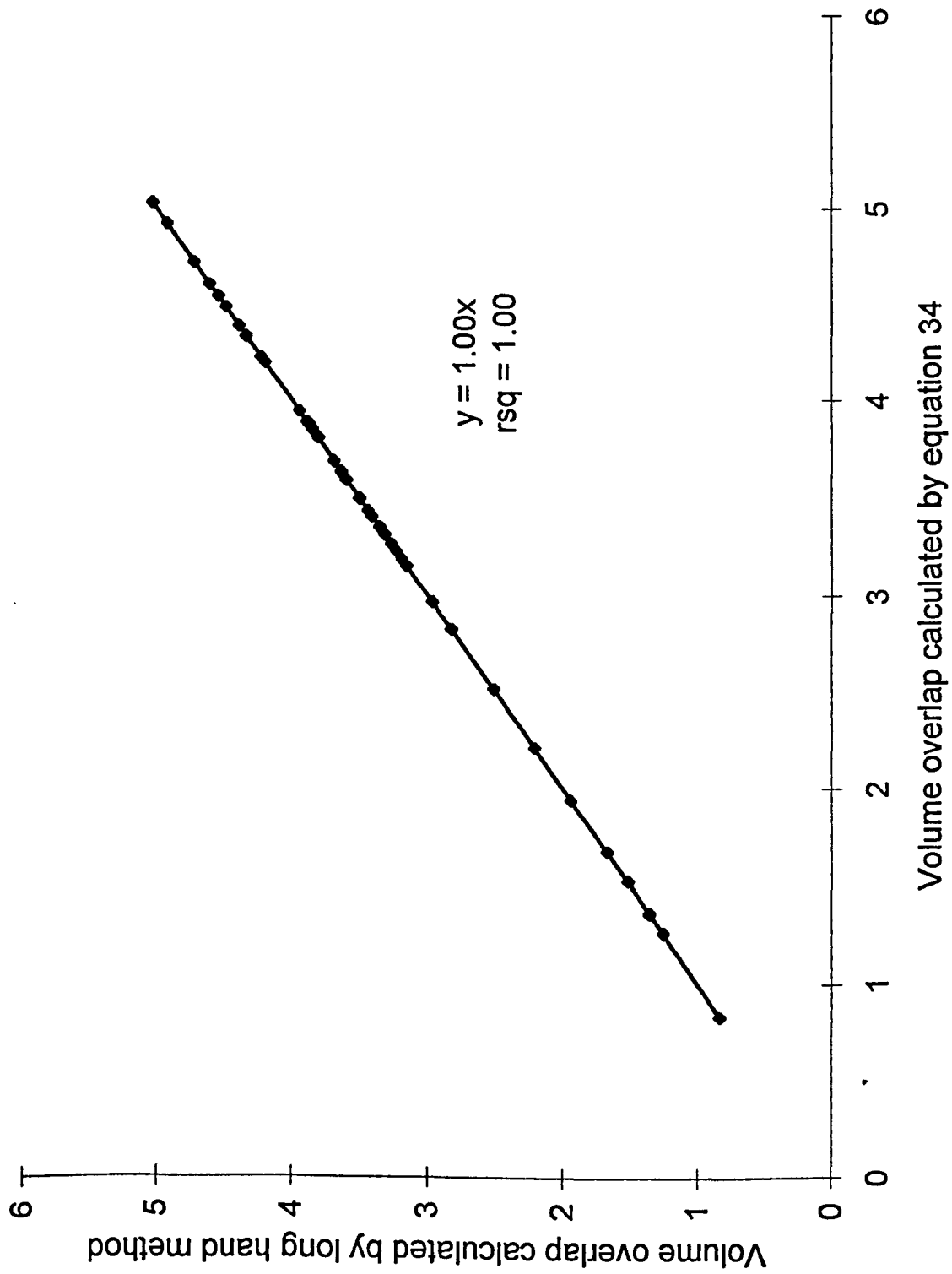


Fig. 29. Plot of proximity factor calculated by equation 35 versus factor calculated by long hand method (equations 25 -33), for fragments at 2 carbon separation

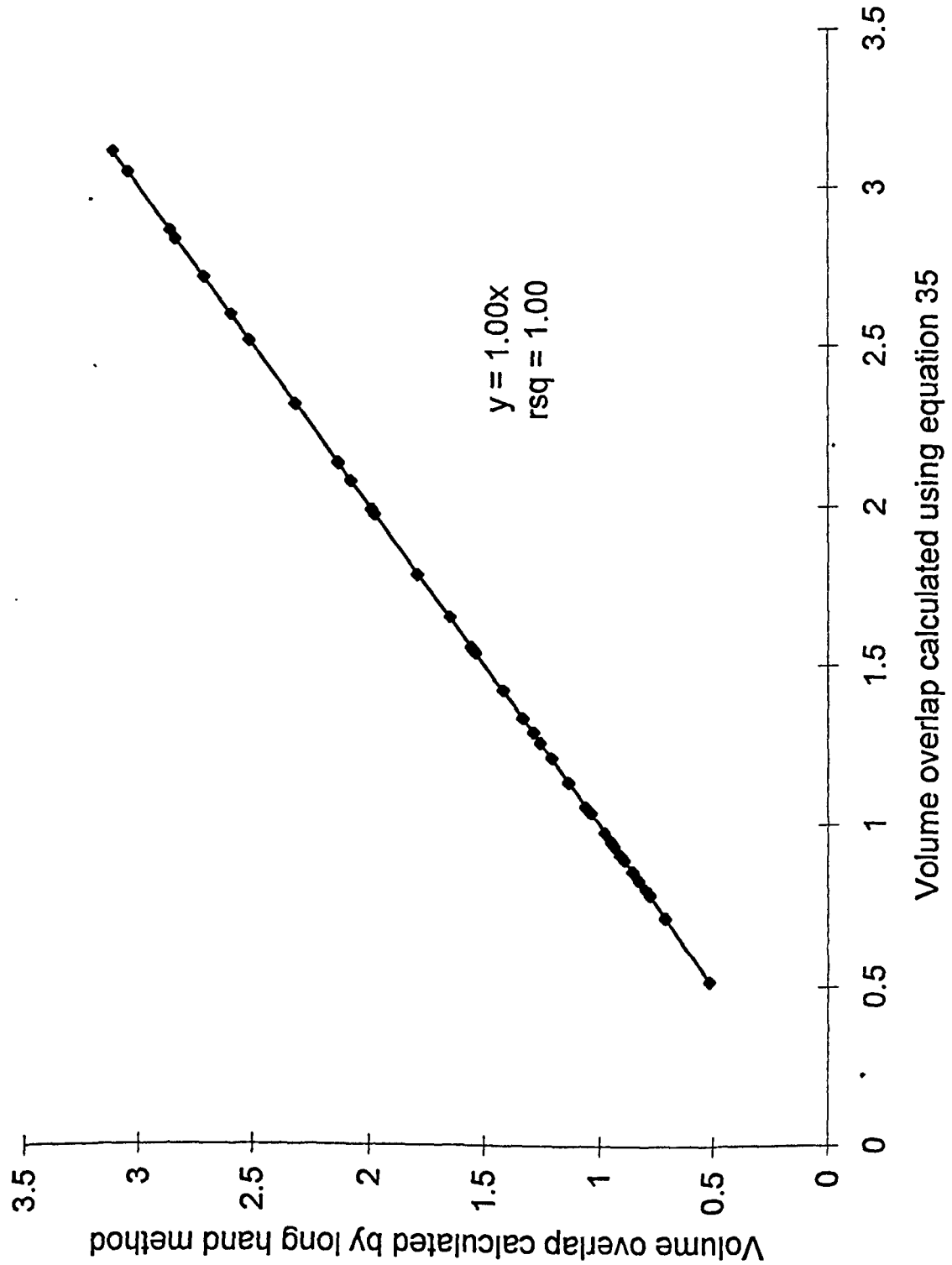
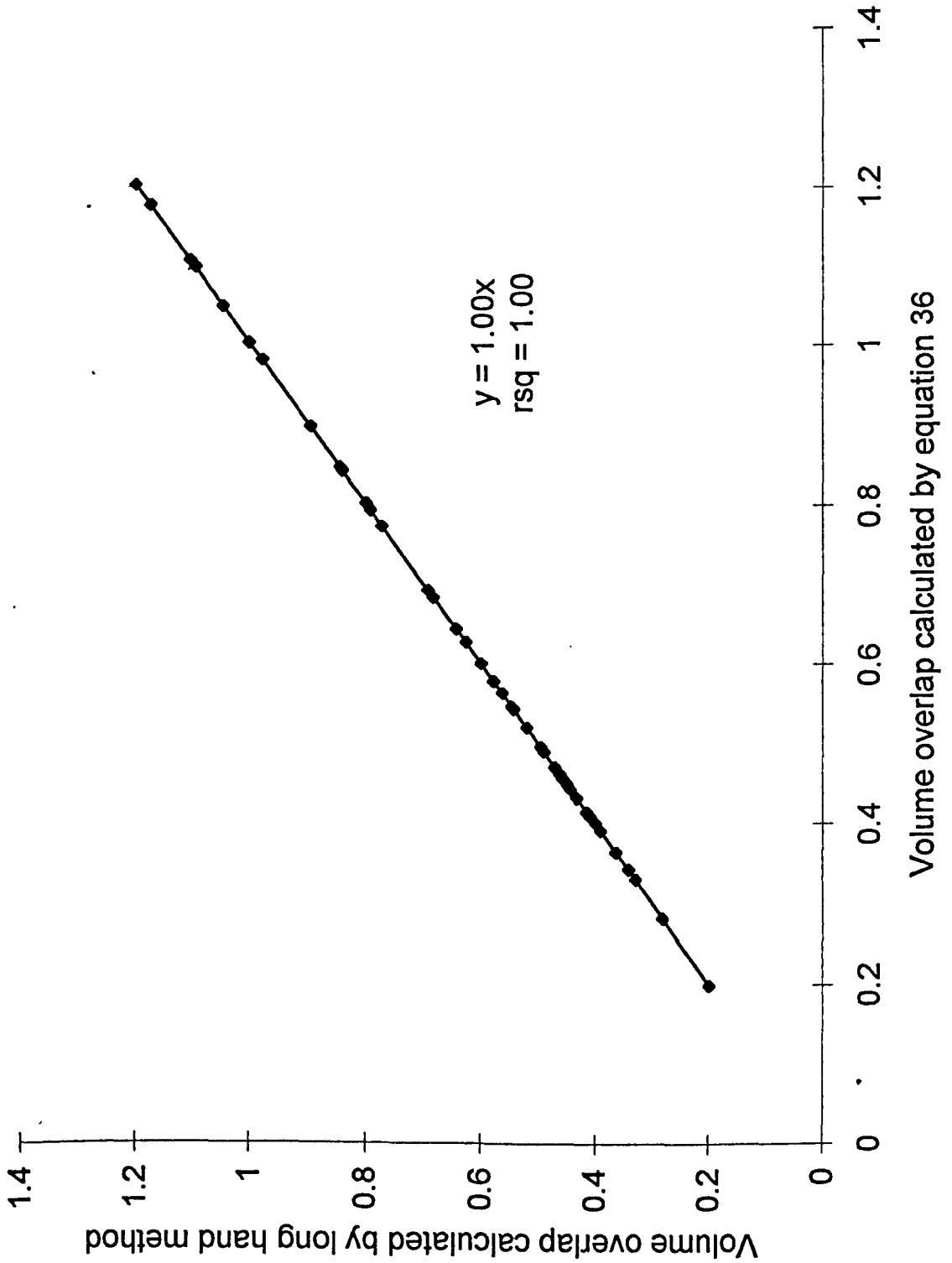


Fig. 30. Plot of proximity factor calculated by equation 36 versus factor calculated by long hand method (equations 25 -33), for fragments at 3 carbon separation



4.3.3 CALCULATION OF LOG P INCORPORATING NEW PROXIMITY FACTORS INTO CONVENTIONAL HANSCH AND LEO METHOD

C12 methyl FAES : $CH_3-(CH_2)_9-CH(SO_3^-Na^+)COO-CH_3$

New proximity factor : $f_L / f_s = f_{SO3}^- / f_{CO2} = 5.87 / 1.49 = 3.94$
: SO_3^- and CO_2 fragments at 1 carbon separation.
: $f_L / f_s > 1.904$.
: Complete overlap of hydration sheaths.

log P = $2f_{CH3} + 9f_{CH2} + f_{CH} + f_{SO3}^- + 13\text{-}1(Fb)$
= $2(0.89) + 9(0.66) + 0.43 + (-5.87) + 12(-0.12)$
= 0.84.

The difference in log P calculated with the new proximity factor and calculated by the Hansch and Leo method is 2.22 (old method) - 0.84 (new method) = 1.38. This difference is the same for all FAES substances (Table 56).

SEMA : $CH_3-(CH_2)_{12}-CONH-CH_2-CH_2-SO_3^-Na^+$

New proximity factor : $f_L / f_s = f_{SO3}^- / f_{CONH} = 5.87 / 2.71 = 2.17$
: SO_3^- and CONH fragments at 2 carbon separation.
: $f_L / f_s < 7.928$.
: Incomplete overlap of hydration sheaths.
: New Fp-2 required.

log P = $f_{CH3} + 14f_{CH2} + f_{CONH} + f_{SO3-} + 16\text{-}1(Fb)$ + New Fp-2.
= $0.89 + 14(0.66) + (-2.71) + (-5.87) + 15(-0.12)$ +
$((10E[-0.0599(\log 2.17)^3 - 0.385(\log 2.17)^2 - 0.0111(\log 2.17) - 0.585]) \times (2.71+5.87))$.
= 1.74.

SMMA : $CH_3-(CH_2)_{12}-CONH-CH_2-SO_3^-Na^+$

New proximity factor : $f_L / f_s = f_{SO3}^- / f_{CONH} = 5.87 / 2.71 = 2.17$

: SO_3^- and CONH fragments at 1 carbon separation.

: $f_L / f_s > 1.904$.

: Complete overlap of hydration sheaths.

$\log P = f_{CH3} + 13f_{CH2} + f_{SO3-} + 15\text{-}1(Fb)$

$= 0.89 + 13(0.66) + (-5.87) + 14(-0.12)$

$= 1.92.$

Geropon T77: $CH_3-(CH_2)_7-CH=CH-(CH_2)_7-CON(CH_3)-CH_2-CH_2-SO_3^-Na^+$

New proximity factor : $f_L / f_s = f_{SO3}^- / f_{CON} = 5.87 / 3.04 = 1.93$

: SO_3^- and CON fragments at 2 carbon separation.

: $f_L / f_s < 7.928$.

: Incomplete overlap of hydration sheaths.

: New Fp-2 required.

$\log P = 2f_{CH3} + 18f_{CH2} + f_{CON} + f_{SO3-} + 21\text{-}1(Fb) + PDBF + Fdou +$ New Fp-2

$= 2(0.89) + 18(0.66) + (-3.04) + (-5.87) + 20(-0.12) + (-1.44\log(1+1)) +$ -0.55) +

$((10E[-0.0599(\log 1.93)^3 - 0.385(\log 1.93)^2 - 0.0111(\log 1.93) - 0.585]) \times (3.04+5.87))$

$= 3.50$

DLAS: CH_3-$(CH_2)_{11}$-CO_2-CH_2-$CH(SO_3^-Na^+)$-$CO_2^-Na^+$

New proximity factor : 1) $f_L / f_s = f_{SO3}^- / f_{CO2} = 5.87 / 1.49 = 3.94$.

: 2) $f_L / f_s = f_{SO3}^- / f_{CO2}^- = 5.87 / 5.19 = 1.13$.

: 3) $f_L / f_s = f_{CO2}^- / f_{CO2} = 5.19 / 1.49 = 3.48$.

: 1) SO_3^- and CO_2 fragments at 2 carbon separation.

: 2) SO_3^- and CO_2^- fragments at 1 carbon separation.

: 3) CO_2^- and CO_2 fragments at 2 carbon separation.

: 1) $f_L / f_s < 7.928$.

: 2) $f_L / f_s < 1.904$.

: 3) $f_L / f_s < 7.928$.

: New Fp-1 required for 2)

: New Fp-2 required for 1) and 3).

$\log P = f_{CH3} + 12(f_{CH2}) + f_{CH} + f_{SO3-} + f_{CO2-} + 16\text{-}1(Fb) + 1(\text{New Fp-1}) + 2(\text{NewFp-2})$

$= 0.89 + 12(0.66) + 0.43 + (-5.87) + (-5.19) + 15(-0.12)$

$+$

$((10E[-0.448(\log 1.13)^3 - 0.962(\log 1.13)^2 - 0.0042\log(1.13) - 0.377]) \times (5.19 + 5.87))$

$+$

$((10E[-0.0599(\log 3.94)^3 - 0.385(\log 3.94)^2 - 0.0111(\log 3.94) - 0.585]) \times (1.49 + 5.87))$

$+$

$((10E[-0.0599(\log 3.48)^3 - 0.385(\log 3.48)^2 - 0.0111(\log 3.48) - 0.585]) \times (1.49 + 5.19))$.

$= 2.13$.

DSDS: CH_3-$(CH_2)_{15}$-$CH(OSO_3^-Na^+)CH_2$-$OSO_3^-Na^+$

New proximity factor : $f_L / f_s = f_{SO4}^- / f_{SO4}^- = 5.23 / 5.23 = 1.0$

: SO_4^- and SO_4^- fragments at 2 carbon separation.

: $f_L / f_s < 7.928$.

: Incomplete overlap of hydration sheaths.

: New Fp-2 required.

log P = $f_{CH3} + 16f_{CH2} + f_{CH} + 2f_{SO4^-}$ + 19-1(Fb) + Fp-2

= 0.89 + 16(0.66) + 0.43 + 2(-5.23) + 18(-0.12)

+

((10E[-0.0599(log1.0)^3 -0.385(log1.0)^2 -0.0111(log1.0) -0.585]) x (5.23 + 5.23)).

= 1.98

log P value for DSDS calculated using new proximity factor differs from that calculated by the Hansch and Leo method only by +0.22 where the FbBr is no longer applied.

SLDI: CH_3-$(CH_2)_{10}$-CO_2-CH_2-CH_2-(O-CH_2-CH_2)-(O-CH_2-CH_2)-$SO_3^-Na^+$

Due to the partially empirical method of calculating log P of structures containing EO groups, such that full calculation applies only to the first EO group, proximity factors need be considered only between f_{SO3^-} / f_O and f_O / f_{CO2}.

New proximity factor : 1) $f_L / f_s = f_{SO3^-} / f_O$ = 5.87 / 1.82 = 3.23.

: 2) $f_L / f_s = f_O / f_{CO2}$ = 1.82 / 1.49 = 1.22.

: 1) SO_3^- and O fragments at 2 carbon separation.

: 2) O and CO_2 fragments at 2 carbon separation.

: 1) f_L / f_s < 7.928.

: 2) f_L / f_s < 7.928.

: 1) and 2) : Incomplete overlap of hydration sheaths.

: New Fp-2 required for 1) and 2).

log P = $f_{CH3} + 14f_{CH2} + f_{SO3^-} + f_{CO2} + f_O$ + 17-1(Fb) + (-0.1) + 2(Fp-2)

= 0.89 + 14(0.66) + (-5.87) + (-1.49) + (-1.82) + 16(-0.12) + (-0.1)

+

((10E[-0.0599(log3.23)^3 -0.385(log3.23)^2 -0.0111(log3.23) -0.585]) x (1.82 + 5.87))

+

((10E[-0.0599(log1.22)^3 -0.385(log1.22)^2-0.0111(log1.22) -0.585]) x (1.49 + 1.82)).

= 1.32

Table 56. Log P values calculated using the new proximity factor (new log P) and by the conventional Hansch and Leo method (old log P) (with the addition of PDBF where appropriate) for FAES substances and substances used in chapter 4

Substance[a]	old log P*	new log P*	Substance[a]	old log P*	new log P*
C12 methyl	2.22	0.84	C9 sec amyl	2.33	0.95
C12 butyl	3.84	2.46	C10 butyl	2.76	1.38
C12 sec butyl	3.41	2.03	C10 sec butyl	2.33	0.95
C12 amyl	4.38	3.00	C10 iso butyl	2.33	0.95
C14 methyl	3.30	1.92	C11 propyl	2.76	1.38
C14 ethyl	3.84	2.46	C12 ethyl	2.76	1.38
C14 iso propyl	3.95	2.57	C13 methyl	2.76	1.38
C14 butyl	4.92	3.54	DLAS	2.97	2.13
C14 amyl	5.46	4.08	SLDI	1.79	1.32
C16 methyl	4.38	3.00	Geropon T77	3.69	3.50
C7 heptyl	2.76	1.38	SMMA	2.81	1.92
C8 hexyl	2.76	1.38	SEMA	1.98	1.74
C8 sec hexyl	2.33	0.95	DSDS	1.76	1.98
C9 amyl	2.76	1.38			

* Values to 2 d.p.

[a] FAES unless otherwise stated.

4.4 RESULTS AND DISCUSSION

4.4.1 TEST SUBSTANCE SOLUBILITY STUDIES

DSDS proved soluble at 1000mg/l in standard Elendt M7 medium (approximate TH of 240mg/l as $CaCO_3$) and required no further solubility studies.

Geropon T77 proved soluble at 560mg/l but formed a slightly cloudy solution at 1000mg/l in standard Elendt M7 medium.

SLDI proved soluble at 1000mg/l in standard Elendt M7 medium.

DLAS proved soluble at 100mg/l in distilled water. Precipitation occurred at all concentrations when prepared in standard Elendt M7 medium. No solutions were prepared with solvents.

SMMA proved soluble at 100mg/l in distilled water. It dissolved at all concentrations in distilled water when hot (*ca.* 60°C) with large amounts of precipitate in all concentrations >100mg/l on cooling, although only slight at 180mg/l. Large amounts of precipitate were present at all concentrations when prepared in standard Elendt medium on cooling. It was soluble in all solvents except DMSO at 10g/l when hot, with precipitation on cooling or following dispersion in distilled water at concentrations >100mg/l.

SEMA proved soluble at 320mg/l in distilled water. It dissolved at all concentrations in distilled water when hot (*ca.* 60°C) with large amounts of precipitate in all concentrations >320mg/l on cooling, although only slight at 560mg/l. Large amounts of precipitate were present at all concentrations when prepared in standard Elendt medium on cooling. It was soluble in all solvents except DMSO at 10g/l when hot, with precipitation on cooling or following dispersion in distilled water at concentrations >320mg/l.

It was clear that a number of test substances (DLAS and particularly SMMA, SEMA) could not be dissolved in standard Elendt M7 medium without inducing large amounts of precipitate and consequent loss of soluble test substance. It was necessary, therefore, to investigate the use of medium at low hardness.

4.4.2 SOLUBILITY OF SMMA IN THE PRESENCE OF A SERIES OF Ca^{2+} AND Mg^{2+} IONS

4.4.2.1 SOLUBILITY STUDY 1.

Measured test solution SF concentrations by MBAS and TH values are presented in Table 57. The measured start concentration was only 88mg/l compared to a nominal 100mg/l. This was probably attributable to either: 1) the probably negligible effect of the warming required to dissolve the test substance resulting in an increased volume and hence reducing apparent concentration, or 2) the more likely result of unaccounted reduced activity. Exposure of SMMA to a series of TH values from nominally 0mg/l to 240mg/l as $CaCO_3$ was seen to result in a considerable drop in soluble measured test substance with increase in hardness of test solution (Fig. 31). Even the presence of only low [Ca^{2+}] and [Mg^{2+}] levels (13 and 23 mg/l as $CaCO_3$) resulted in a loss of 64% and 88% respectively of soluble substance. The greatest hardness tested (nominally 211mg/l as $CaCO_3$) reduced the concentration of soluble test substance from 88mg/l to 2.5mg/l (a loss of >97% soluble substance).

The predicted EC50 values for this test substance using conventional log P calculation (section 4.2.16.1) with the polar narcosis equation (equation 10) gave a value of 17.6mg/l. If based on a modified log P value then the EC50 would be expected to be even higher. It was obvious that testing at low hardness would be required to maintain sufficient soluble test substance to induce an effect on test organisms. Considering that the solubility of SMMA in medium of hardness 0mg/l as $CaCO_3$ was approximately 100 - 180mg/l then it was necessary to test at the lowest studied hardness of 13mg/l as $CaCO_3$.

The disadvantage of testing at low hardness, as previously mentioned, is the additional stress imposed on the test organisms. In addition, TH of each test solution was further reduced as a result of precipitation (Table 57). At nominally 13mg/l as $CaCO_3$ the TH was reduced to 2.8mg/l as $CaCO_3$. It was essential to

establish survivability of *D. magna* neonates at this hardness (sections 4.2.7.3 and 4.4.2.3).

4.4.2.2 SOLUBILITY STUDY 2.

Results of study 2 are presented in Table 58. Restriction in time prevented MBAS analysis of all concentrations. As for study 1, the measured start concentration was 88mg/l. Obvious formation of precipitate occurred almost immediately after addition of Stock A. Maximum loss of soluble test substance was achieved after 10 minutes (Fig. 32) although SF concentration could be seen to rise after this time before equilibrium was reached in the highest concentration (nominally 180mg/l) at between 3 and 6 hours. Solutions appeared to reach equilibrium between 1 and 3 hours in the lower concentrations although any observed slight fluctuations in measured concentrations would be indistinguishable from inherent errors in the test and MBAS systems. Between 6 and 24 hours there was no significant change in SF concentration indicating stable equilibrium. Stable saturated SF concentration could be assumed to be reached after 6 hours stirring.

TF concentration measurement proved inconclusive (Fig. 33). Whilst measured values were higher than for those of the SF, values were always less than start concentration. The MBAS method of measurement of anionic substances is not designed to cope with precipitated material (Abbott, 1962) and the lower than expected values for TF were undoubtedly attributable to this fact.

4.4.2.3 SOLUBILITY STUDY 3

Results of study 3 are presented in Table 59. Time restrictions prevented MBAS analysis of all concentrations. Results of nominally 100mg/l test solution concentration, however, showed very similar trends to study 2 although reduction in SF concentration proved to be slightly greater (Fig. 34). Evidence from study 2 indicated that other concentrations could be assumed to follow the same trend as

Table 57. Solubility study 1: measured concentrations by MBAS of nominally 100mg/l SMMA in the presence of various $[Ca^{2+}]$ and $[Mg^{2+}]$ ions

Measured TH (mg/l as $CaCO_3$) at time t = 0	Measured concentration at time t +300 minutes	
	SMMA (mg/l) *	TH (mg/l as $CaCO_3$)
0	88 [100]	0 [-]
13	32 [36]	2.8 [22]
23	11 [13]	8.5 [37]
41	4.7 [5.3]	28 [68]
72	5.3 [6.0]	55 [76]
121	4.5 [5.1]	103 [85]
211	2.5 [2.8]	195 [92]

* Values to 2 s.f. Measured concentration SMMA = 88mg/l at time t = 0

[ ] value in brackets = % of start.

Table 58. Solubility study 2: measured concentrations by MBAS of various SMMA concentrations in the presence of $[Ca^{2+}]$ and $[Mg^{2+}]$ ions at 13mg/l as $CaCO_3$

Time from addition of Stock A (minutes)	Nominal Concentration (mg/l)					
	32		100		180	
	SF (mg/l)*	TF(mg/l)*	SF(mg/l)*	TF(mg/l)*	SF(mg/l)*	TF(mg/l)*
t = 0	28	28	88	88	170	170
t + 10	7.0	24	31	50	80	140
t + 30	8.5	24	31	54	95	140
t + 60	8.5	21	33	31	99	150
t + 180	7.5	16	30	60	98	140
t + 360	8.0	12	31	58	110	140
t + 540	6.9	12	35	49	110	140
t +1440	7.6	15	32	44	110	130

* Values to 2 s.f.

Table 59. Solubility study 3: measured concentrations by MBAS of various SEMA concentrations in the presence of $[Ca^{2+}]$ and $[Mg^{2+}]$ ions at 13mg/l as $CaCO_3$

Time from addition of Stock A (minutes)	Nominal Concentration (100 mg/l)	
	SF(mg/l)*	TF(mg/l)*
t = 0	78	78
t + 10	25	67
t + 30	20	58
t + 60	19	70
t + 180	24	67
t + 360	21	60
t +1440	18	59

* Values to 2 s.f.

Fig. 31. Change in measured SMMA (mg/l) and TH concentrations (mg/l as $CaCO_3$) as percent of start concentration at various water hardness concentrations (mg/l as $CaCO_3$) for SMMA, solubility study 1

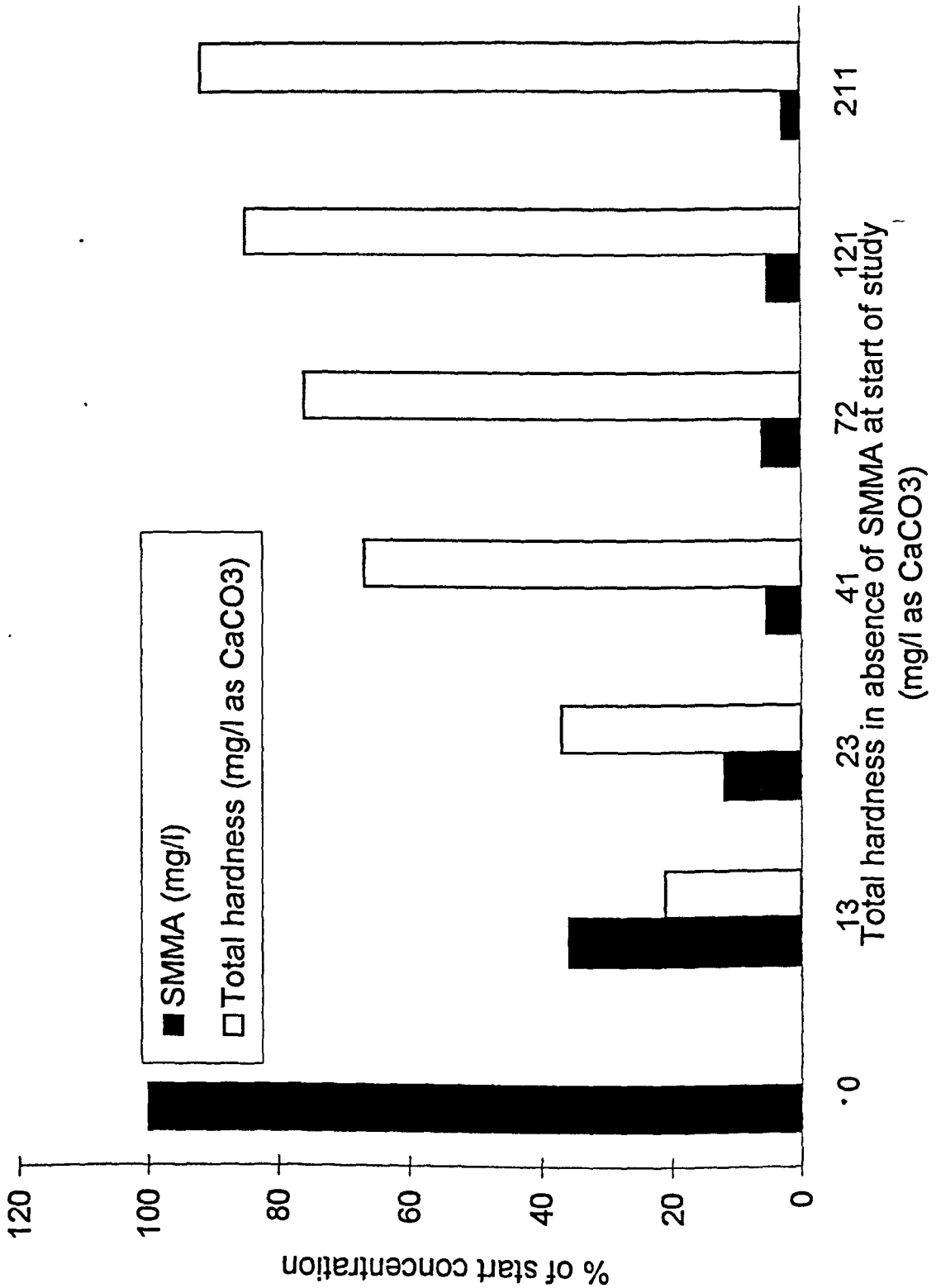


Fig. 32. Change in measured SF fraction SMMA concentrations (mg/l) over 24 hour period for solubility study 2

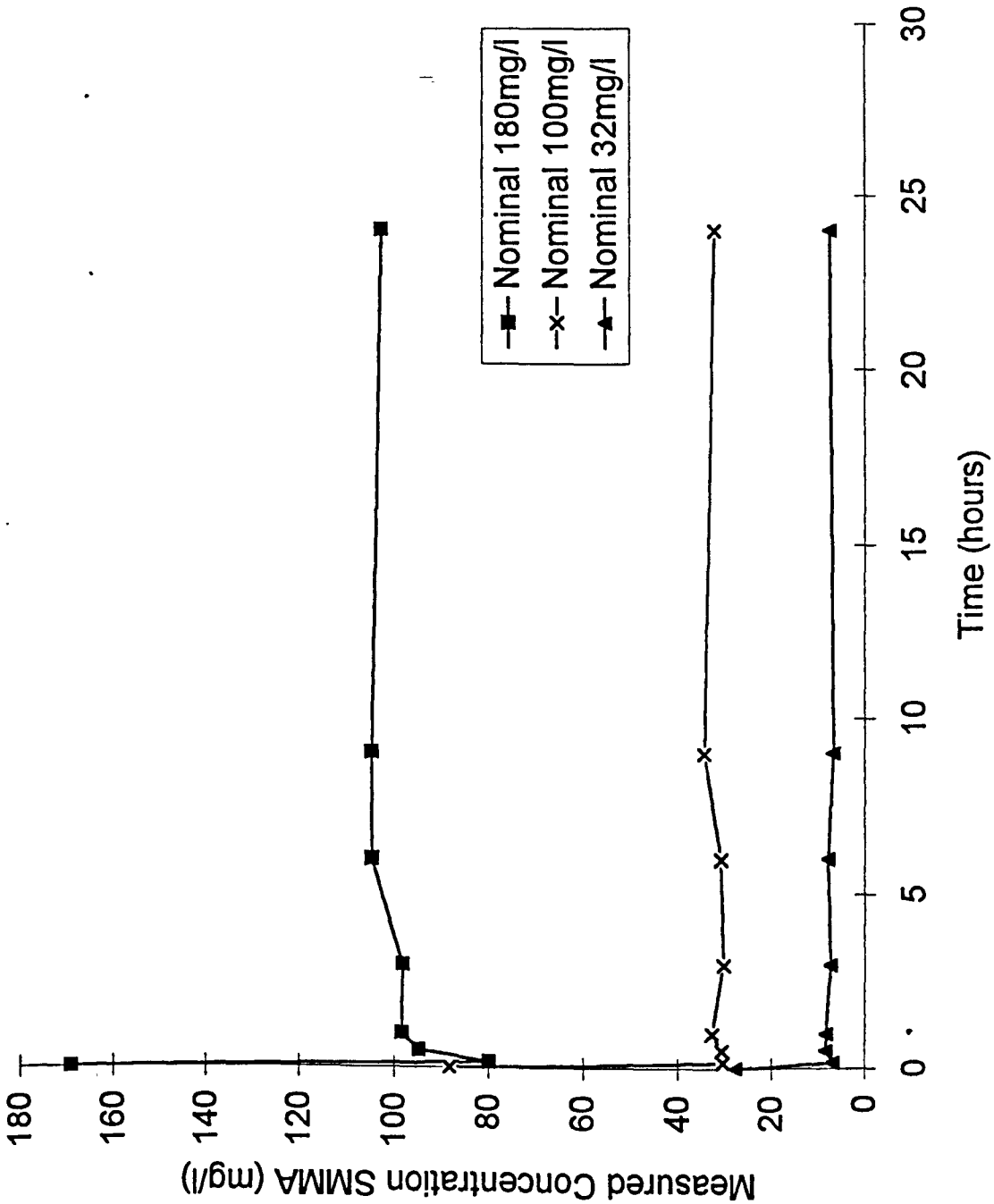


Fig. 33. Change in measured TF fraction SMMA concentrations (mg/l) over 24 hour period for solubility study 2

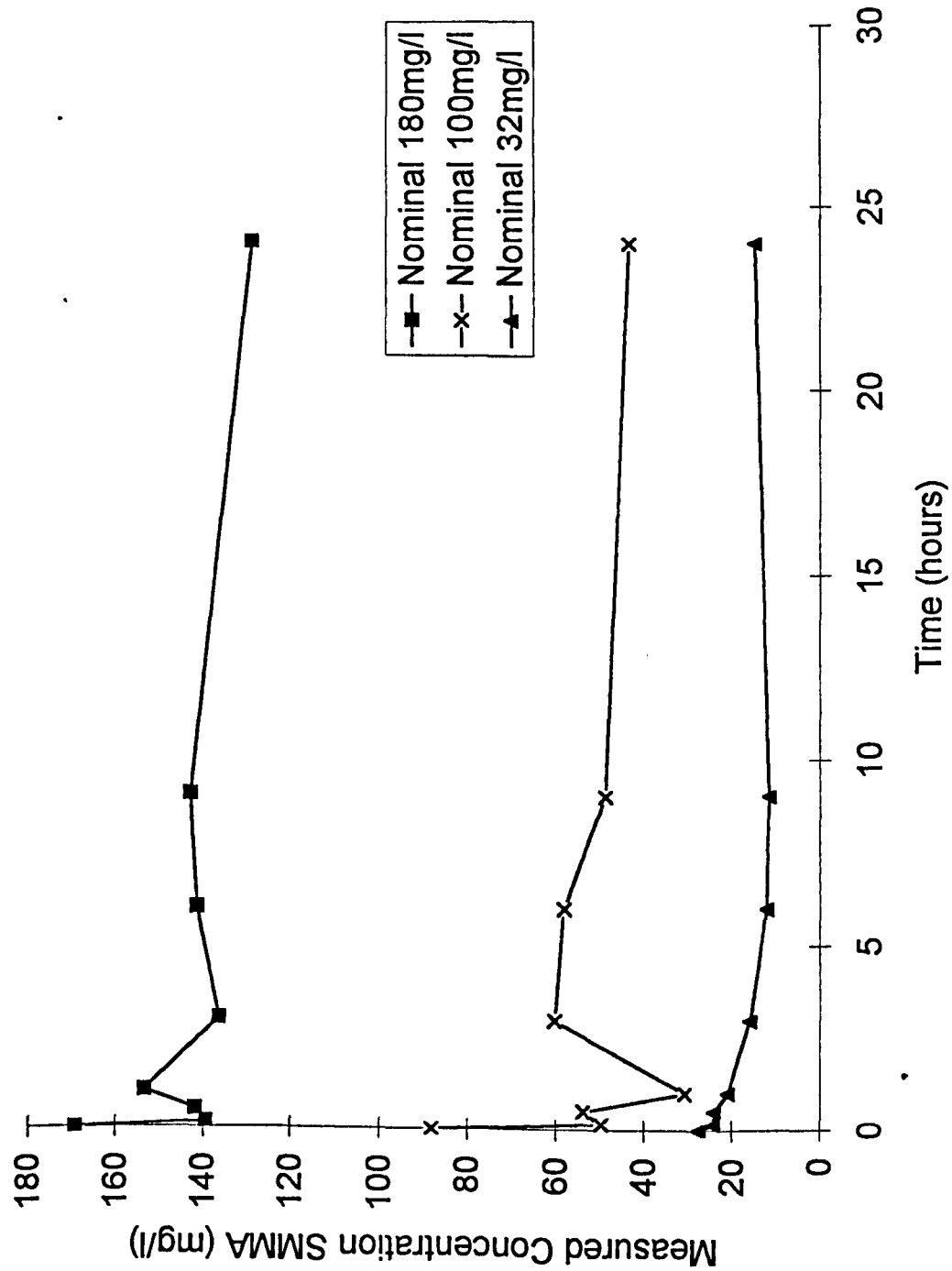
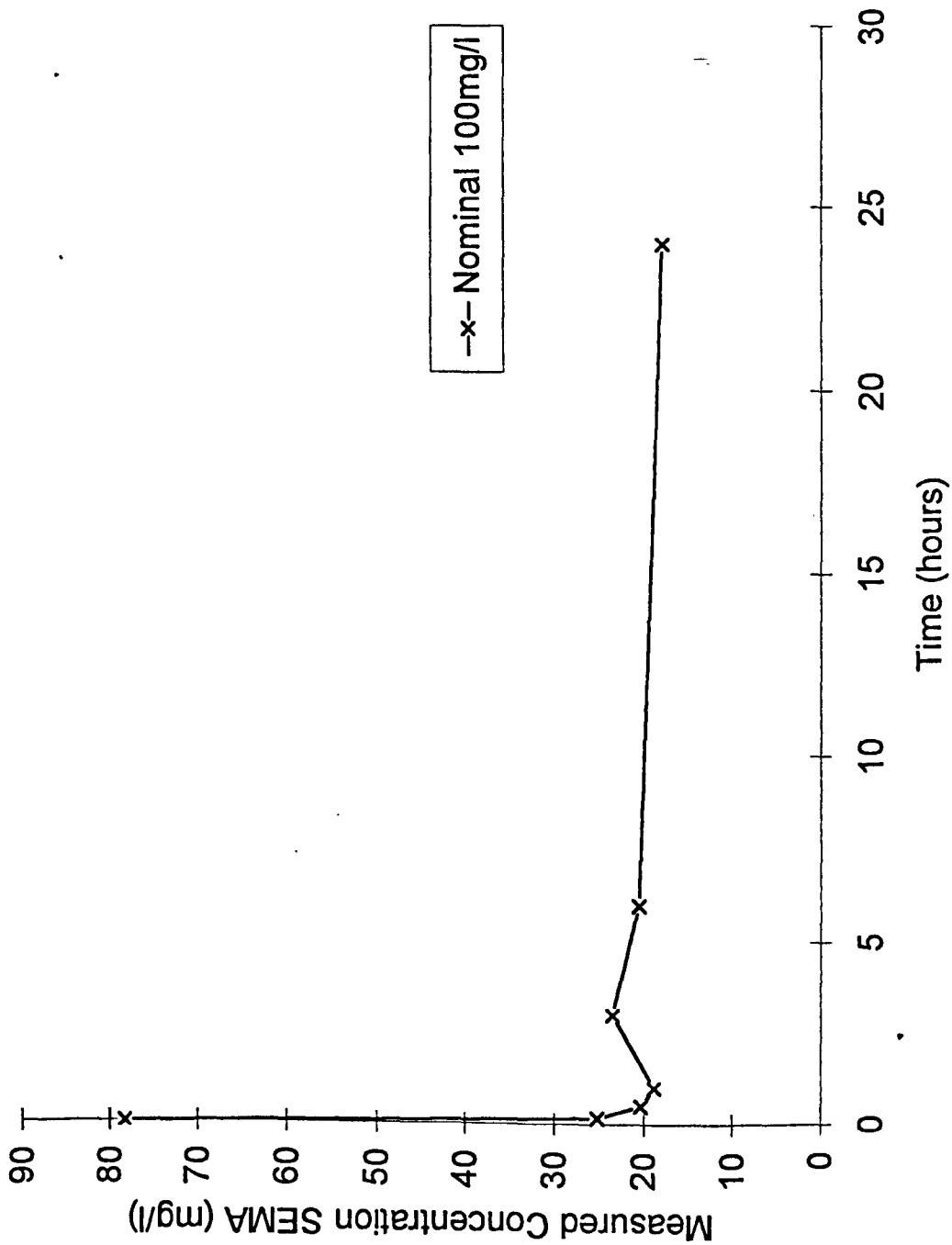


Fig. 34. Change in measured SF fraction SEMA concentrations (mg/l) over 24hour period for solubility study 3



nominal 100mg/l. Equilibrium again appeared to be reached after 6 hours stirring with no significant change between 6 - 24 hours. Stable saturated SF concentration could be assumed to be reached after 6 hours stirring.

4.4.3 EXPOSURE OF *D. MAGNA* TO LOW TOTAL HARDNESS

Results of *D. magna* exposed to a series of hardness concentrations (mg/l as $CaCO_3$), are presented in Table 60, 62 and 64. Water quality data are presented in Table 124, Appendix II. Analysis of variance of 48 hour immobility values at each hardness concentration (Tables 61, 63 and 65) show calculated F < tabulated F for studies 1 and 3 indicating no significant difference in the mean immobility at each hardness concentration at the 5% level. Analysis of variance for study 2 showed calculated F > tabulated F indicating significant difference in the means at the 5% level. This, however, results mainly from immobility at 0mg/l and 130mg/l as $CaCO_3$. High immobility would not be expected due to the change in hardness, at 130mg/l as $CaCO_3$, because no immobility was observed at lower hardness concentrations (0mg/l excepted). It is probable that immobility observed at this hardness concentration was a result of the weakened individuals due to some environmental stress prior to transfer from culture vessels.

It can be assumed that no significant immobility would result from neonate exposure to low hardness after a short acclimatisation period over any 48 hour period. In addition all test substance and reference substance toxicity tests were conducted following the same method and any errors would have been constant.

4.4.4 TOXICITY OF INDIVIDUAL SUBSTANCES

All mean EC50 values for varying numbers of replicates for substances tested individually are presented in Tables 66 and 67. EC50 values for SMMA and SEMA were calculated using mean measured concentrations over the 48 hour period for each concentration in place of nominal concentrations (Tables 68 and 69). All test replicate data and associated confidence limits are presented in

Table 60. Study 1: 48 hour immobility data as percent immobile for neonates exposed to a series of measured TH concentrations (mg/l as $CaCO_3$)

Nominal TH concentration (mg/l as $CaCO_3$)	% immobile at 48 hours	Measured TH (mg/l as $CaCO_3$).			
		0 hour	24 hour old	24 hour new	48 hour
0	15	0.7	0.9	0.7	0.7
13	5	13	14	14	13
24	15	24	24	25	25
42	10	43	43	42	43
75	10	74	73	75	76
130	5	126	126	126	130
240	5	233	235	234	238

Table 61. Analysis of variance for % immobility at 48 hours for total hardness study 1

Source	DF	Sum of squares	Mean square	Calculated F	Tabulated F (0.05)
Among	6	0.243	0.040	0.466	2.170
Within	133	11.550	0.087		
Total	139	11.793			

Table 62. Study 2: 48 hour immobility data as percent immobile for neonates exposed to a series of measured TH concentrations (mg/l as $CaCO_3$)

Nominal TH concentration (mg/l as $CaCO_3$)	% immobile at 48 hours	Measured TH (mg/l as $CaCO_3$).			
		0 hour	24 hour old	24 hour new	48 hour
0	35	0.4	0.4	0.3	0.4
13	0	13	14	13	15
24	0	23	25	23	23
42	5	42	42	41	42
75	0	74	75	75	75
130	30	127	128	125	126
240	0	232	235	234	235

Table 63. Analysis of variance for % immobility at 48 hours for total hardness study 2

Source	DF	Sum of squares	Mean square	Calculated F	Tabulated F (0.05)
Among	6	2.900	0.483	6.627	2.170
Within	133	9.700	0.073		
Total	139	12.600			

Table 64. Study 3: 48 hour immobility data as percent immobile for neonates exposed to a series of measured TH concentrations (mg/l as $CaCO_3$)

Nominal TH concentration (mg/l as $CaCO_3$)	% immobile at 48 hours	Measured TH (mg/l as $CaCO_3$).			
		0 hour	24 hour old	24 hour new	48 hour
0	5	0.2	0.3	0.0	0.3
1.3	0	1.3	1.6	1.4	1.4
2.4	0	2.5	2.3	2.5	2.5
4.2	0	4.1	4.0	4.1	4.0
7.5	0	7.3	7.5	7.5	7.5
13	0	13	13	13	13

Table 65. Analysis of variance for % immobility at 48 hours for total hardness study 3

Source	DF	Sum of squares	Mean square	Calculated F	Tabulated F (0.05)
Among	5	0.042	0.008	1.000	2.370
Within	114	0.950	0.008		
Total	119	0.992			

Table 66. Mean observed EC50 values and log P values calculated using the conventional method of Hansch and Leo (1979) for all test substances

Substance	TH (mg/l as $CaCO_3$)	Observed EC50 (mg/l)*[a]	log P#	MW
DLAS	13	8.3 [7.4]	2.97	442
SLDI		510	1.79	410
Geropon T77		6.5 [5.9]	3.69	426
SMMA		[33]	2.81	343
SEMA		[44]	1.98	357
DSDS		28 [18]	1.76	490
C11.8 LAS		14	-[b]	345
C14 amyl FAES		3.8	5.46	400
C10 sec butyl FAES		350	2.33	330
C12 n butyl FAES		43	3.84	358
DLAS	240	8.4 [7.5]	2.60	442
Geropon T77		6.3 [5.7]	3.69	426
DSDS		15 [9.6]	1.76	490

TH : Nominal total hardness of test solutions.

* Values to 2 s.f..

[a] Mean values. All replicate values presented in Table 80, Appendix I.

[b] No isomer distribution data, unable to calculate log P.

[ ] value in brackets based on measured concentrations (Table 96, Appendix I).

Values to 3 s.f.

Table 67. Observed EC50 values for LAS substances at 13mg/l as $CaCO_3$

Chain length	log P[b]	Observed EC50 value (mg/l)*[a]	Observed EC50 value at 240mg/l $CaCO_3$ (mg/l)[b]
C9	1.63	85 [71]	53
C10	2.15	47	28
C11	2.60	27	11
C12	3.17	13	4.3
C13	3.62	5.3	2.7
C14	4.19	2.1 [1.7]	0.67

* Values to 2 s.f.

[a] Mean values. All replicate values presented in Table 81, Appendix I.

[b] Values from Table 7, Chapter2.

[ ] values in brackets based on measured concentrations (Table 96, Appendix I).

Table 67a. Comparison of observed EC50 values for C11.8 LAS and FAES substances at 13 mg/l as $CaCO_3$ with those at 240 mg/l as $CaCO_3$

Substance	log P[d]	Observed EC50 value (mg/l)*[ad]	Observed EC50 value at 240 mg/l $CaCO_3$ (mg/l)[b]
C11.8 LAS	-[c]	14	5.7
C14 amyl FAES	5.46	3.8	1.3
C10 sec butyl FAES	2.33	350	220
C12 n butyl FAES	3.84	43	16

* Values to 2 s.f.

[a] Mean values. All replicate values presented in Table 80, Appendix I.

[b] Values from Tables 3 and 5, Chapter 2 and Table 20, Chapter 3.

[c] No isomer distribution data, unable to calculate log P value.

[d] Values from Table 66, Chapter 4.

Table 68. Measured concentrations by MBAS of SMMA for acute toxicity study at 13mg/l $CaCO_3$

Nominal Concentration (mg/l)	Time of measurement (hours)				Mean measured Conc. Over 48 hours (mg/l)
	0	24 old	24 new	48	
0	0.0	0.0	0.0	0.0	0.0
32	6.1	5.8	7.4	7.3	6.7
56	11.8	9.0	10.0	10.0	10.2
100	30.7	29.8	25.5	30.1	28.8
180	106	40.5[a]	97.8	104.5	102.8
320	165.5	162.0	-[b]	-[b]	163.8

Mean concentration values used for replicate study also.

[a] Spurious value - ignored.

[b] 100% mortality in highest concentration at 24 hours- no test solution renewal at this concentration at 24 hours.

Table 69. Measured concentrations by MBAS of SEMA for acute toxicity study at 13mg/l $CaCO_3$

Nominal Concentration (mg/l)	Time of measurement (hours)				Mean measured Conc. Over 48 hours (mg/l)
	0	24 old	24 new	48	
0	0.16	0.0	0.1	0.0	0.1
32	7.9	8.3	8.8	7.4	8.1
56	13.8	14.0	14.3	10.7	13.2
100	31.0	34.1	38.0	34.4	34.4
180	101.0	106.0	107.0	106.0	105.0
320	252.0	236.0	-[a]	-[a]	244.0

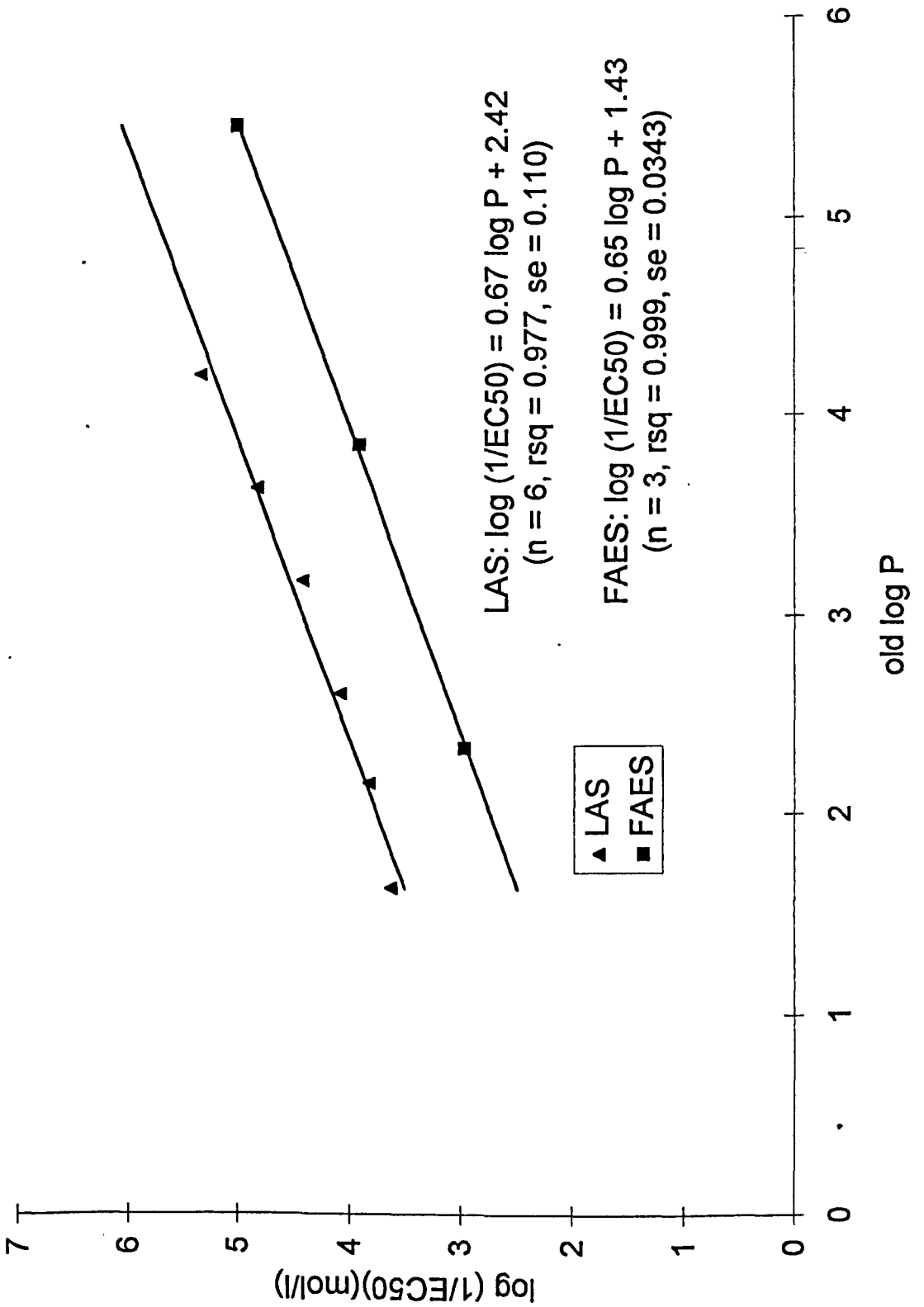
[a] 100% mortality in highest concentration at 24 hours- no test solution renewal at this concentration at 24 hours.

Tables 80 and 81, Appendix I. Water quality data are presented in Tables 125 - 130, Appendix II. Of the selected studies for which test solution concentration analysis was performed (Table 96, Appendix I), only DSDS showed >20% mean difference between nominal and measured concentrations 65%, with the obvious exceptions of SMMA and SEMA. No appreciable drop in concentration was observed over any 24 hour period for any of the test solutions measured, indicating no loss of test substance due to degradation or adsorption onto vessel surfaces. As all substances were either sparing soluble in medium at a hardness of 240mg/l as $CaCO_3$, or tested at low hardness affecting the dissociation of the surfactant, or both, it was considered acceptable to adjust all observed EC50 values for mean measured concentrations even where difference between nominal and mean measured concentrations was <20%.

Where replicate EC50 values were observed the maximum and minimum EC50 values were within ±14% of the mean (Table 89, Appendix I).

As expected, toxicities of LAS and FAES substances were observed to be reduced at low hardness. This is consistent with results of Lewis and Perry (1981) and Lewis (1992) and with in-house Unilever data. Of great importance, however, is that the reduction in toxicity is consistent for both LAS and FAES. Whilst regression slopes of both series of substances plotted as log P v log (1/EC50)(mol/l) have altered due to testing at lower hardness, they remain nearly parallel (Fig. 35). LAS can be considered to remain suitable reference substances for comparison of other anionic surfactants tested at low hardness, including FAES, provided the substances being compared can be assumed to exhibit a reduction in toxicity at lower hardness similar to that observed for LAS. In solutions of surfactant substances, an increase in hardness of the medium causes an increase in competition of Ca^{2+} ions with Na^{+} ions as an alternative counterion to the electrolyte. This causes molecular weight to increase for the larger number of surfactant electrolytes associated with Ca^{2+} ions. The consequent reduction in solubility of these molecules results in an increase of the log P for each of these molecules (equation 9) and an increase in observed toxicity.

Fig. 35. Old log P versus log (1/EC50) for LAS and FAES substances at a hardness of *ca.* 13mg/l as $CaCO_3$



It is reasonable to assume that toxicity for those substances used in this chapter which possess one counterion will behave in a similar manner at reduced hardness to LAS. It is probable that the difference in solubility and log P in media of different hardness for those substances with two counterions, such as DLAS and DSDS, will be vary considerably from that for substances with one counterion. The EC50 value for DLAS was observed to exhibit no difference in toxicity at either hardness although DSDS was observed to exhibit a difference in toxicity at different hardness similar to that observed for substances with one counterion (Table 66). Due to the potential spurious nature of the EC50 values for these substances they were not used for the main comparisons with LAS EC50 values.

4.4.5 TOXICITY OF MIXTURES OF SUBSTANCES

Table 70, shows results of mixture toxicity studies of binary components with anionic surfactants used in this chapter with reference substance of phenol and 1-hexanol. Associated confidence limits are presented in Table 82, Appendix I. Water quality data are presented in Tables 131 and 132, Appendix II. Time restriction prevented a full analysis of each test substance.

Test solution analysis of phenol components suggested concentrations were more consistent with nominal concentrations than in studies in chapter 3 (Table 97, Appendix I). Only three surfactant test components were analysed for DSDS, Geropon T77 and DLAS. Results showed mean difference between measured with nominal concentrations of 58%, 84% and 82% respectively (Table 98, Appendix I). Low concentrations for DSDS solutions were consistent over the 48 hour test period (see confidence limits, Table 98, Appendix I), suggesting that stock solution preparation was in error. All TU values were adjusted for measured concentrations due to the subtlety of differences involved in mixture toxicity observations.

Repeated values showed good replication with observed EC50 values within ±3.7% of the mean (Table 90, Appendix I).

Table 70. Observed EC50 and TU values, adjusted for measured component concentrations by MBAS and GC-MS, for each test replicate mixture with substances used in chapter 4 with phenol and 1-hexanol as the reference substances at hardness *ca.* 240mg/l as $CaCO_3$

Mixture		% # (A,B)	TR	EC50 (mg/l)*[a]	TU of component at EC50 of mixture*		Total TU*
A	B				A	B	
Phenol	DLAS	58,42	1	8.9	0.70[0.60]	0.65[0.53]	[1.1]
Phenol	DSDS	59,41	1	10	0.80[0.74]	1.1[0.64]	[1.4]
		57,43	2	9.6	0.74[0.70]	1.1[0.64]	[1.3]
Phenol	GeroponT77	54,46	1	9.3	0.68[0.58]	0.75[0.64]	[1.2]
Hexanol	SLDI	35,65	1	290	0.78[b]	0.76[b]	1.5
		35,65	2	290	0.78[b]	0.76[b]	1.5

A: Reference substance component, B: Test substance component .

TR: Test Replicate.

nominal % component to give equitoxic or near equitoxic concentrations in mixture.

* Values to 2 s.f..

[ ] Values in brackets based on measured concentrations - adjusted according to measured concentrations as mean % of nominal (Tables 97 and 98, Appendix II).

[a] Observed values - not adjusted for measured concentrations.

[b] No test solution concentration analysis performed - no adjustment made.

TU totals for mixtures with phenol as the reference component were expected to exhibit concentration addition (Table 70). Total TU values at the EC50 of the mixture of 1.1, 1.2 and 1.4 for DLAS, Geropon T77 and DSDS as the test substance component, indicate that these substances are acting by concentration addition with phenol when TU values are considered as a sliding scale as discussed in chapter 3. This suggests a similar mode of action to phenol and LAS. From the result of Geropon T77, it is reasonable to assume that SMMA and SEMA also act as polar narcotics as they are also sulphonated amides.

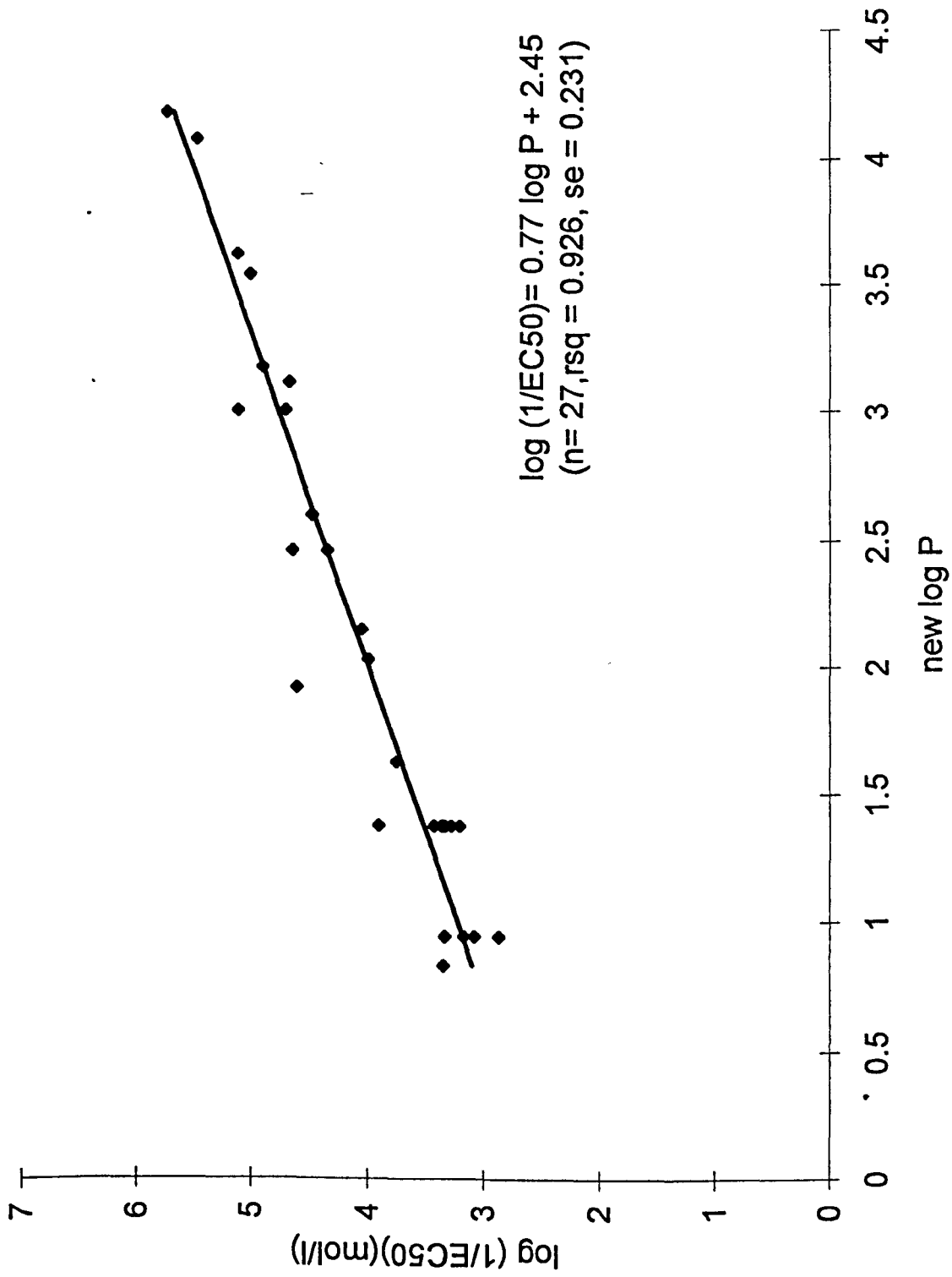
TU total values of 1.5 for replicates of SLDI / 1-hexanol mixture studies are less distinct. Whilst response addition would have been expected, the observed values cannot be considered as concentration addition. Further studies would ideally be required to fully establish a non-additive response with alcohol general narcotics. As the weight of evidence suggests that all other anionic surfactants so far tested in this thesis exhibit polar narcosis mode of action, then It is reasonable to assume that SLDI will also exhibit polar narcosis.

In the same way as in chapter 3, the anionic surfactants in this chapter are shown, on the weight of evidence, to behave as polar narcotics and EC50 values for these substances would, as a result, be expected to behave by a similar mode of action to LAS.

4.4.6 VALIDITY OF NEW PROXIMITY FACTOR

Table 56 summarises new log P values calculated using the Hansch and Leo method in conjunction with the new proximity factor. These will be termed 'new log P' values in order to differentiate between log P values calculated with the proximity method of Hansch and Leo (old log P) and that described in this thesis. Fig. 36 shows EC50 values (Tables 3, 5 and 67), observed at a hardness of 240mg/l as $CaCO_3$, plotted as new log P versus log(1/EC50)(mol/l) for FAES and LAS substances (equations 37 and 38). The slope of the regression is unchanged from that derived when using old log P values (Fig. 11) but now has

Fig. 36. New log P versus log (1/EC50) for LAS and FAES substances at a hardness of *ca.* 240mg/l as $CaCO_3$



an intercept very similar to that of LAS, for which old log P values apply as no proximity values are required in their calculation.

LAS : $\log (1/EC50) = 0.77 \log P + 2.47$ Equation 37.
$(n = 6, rsq = 0.991, se = 0.0759)$

FAES: $\log (1/EC50) = 0.78 \log P + 2.45$ Equation 38.
$(n = 21, rsq = 0.896, se = 0.262)$

Equations 37 and 38 can now be considered identical given the error inherent in the EC50 observation system and is it a reasonable assumption that EC50 values for both FAES and LAS substances can be predicted by the same QSAR (Fig. 36) given by :

$\log (1/EC50) = 0.77 \log P + 2.45$ Equation 39.
$(n = 27, rsq = 0.926, se = 0.231)$

This is now consistent with results of chapter 3 which indicated that both FAES and LAS behave by a similar mode of action and thus share a similar regression equation for log P versus log (1/EC50)(mol/l).

The correlation between EC50 values and new log P values is good, with a high rsq value, and is indicative of the validity of the application of the new proximity factor in log P calculation. Given inherent experiment error and variability in EC50 values, it is unlikely that a much greater rsq value could be achieved.

In a similar way, for EC50 values observed at a hardness of 13 mg/l as $CaCO_3$, for LAS (Table 67) and FAES (Table 66) substances when plotted as old log P versus log(1/EC50)(mol/l), regression slopes differ by a constant amount (equations 40 and 41)(Fig. 35).

LAS : log (1/EC50) = 0.67 log P + 2.42 Equation 40.
(n = 6, rsq = 0.977, se = 0.110)

FAES: log (1/EC50) = 0.65 log P + 1.43 Equation 41.
(n = 3, rsq = 0.999, se = 0.0343)

It should be noted that the regression slope for FAES is based on only 3 values. Confidence in rsq and standard error values, therefore, is not as high as for EC50 values observed at higher water hardness which were based on larger numbers of values. However, rsq and se are good and indicate almost perfect correlation for the three values.

When plotted as new log P versus log (1/EC50)(Tables 56 and 67), LAS and FAES substances can again be seen to share the same regression equation (equation 42)(Fig. 37).

log (1/EC50) = 0.67 log P + 2.38 Equation 42.
(n = 9, rsq = 0.982, se = 0.107)

Correlation between EC50 values and new log P values is high with an rsq value greater that that observed for EC50 values observed at hardness of 240mg/l as $CaCO_3$. The standard error is also now lower. Again this is indicative of the validity of the application of the new proximity factor in log P calculation.

When regression analysis is performed on EC5O values for SMMA, SEMA, Geropon T77, SLDI and LAS, (observed at a hardness of 13mg/l as $CaCO_3$) (Table 66), as a old log P versus combined log (1/EC50)(mol/l) plot, the slope changes and correlation decreases to rsq = 0.875 and the standard error increases to 0.263 (equation 43)(Fig. 38) compared to LAS alone (equation 40).

log (1/EC50) = 0.74 log P + 2.13 Equation 43.
(n = 10, rsq = 0.875, se = 0.263)

Fig. 37. New log P versus log (1/EC50) for LAS and FAES substances at a hardness of *ca.* 13mg/l as $CaCO_3$

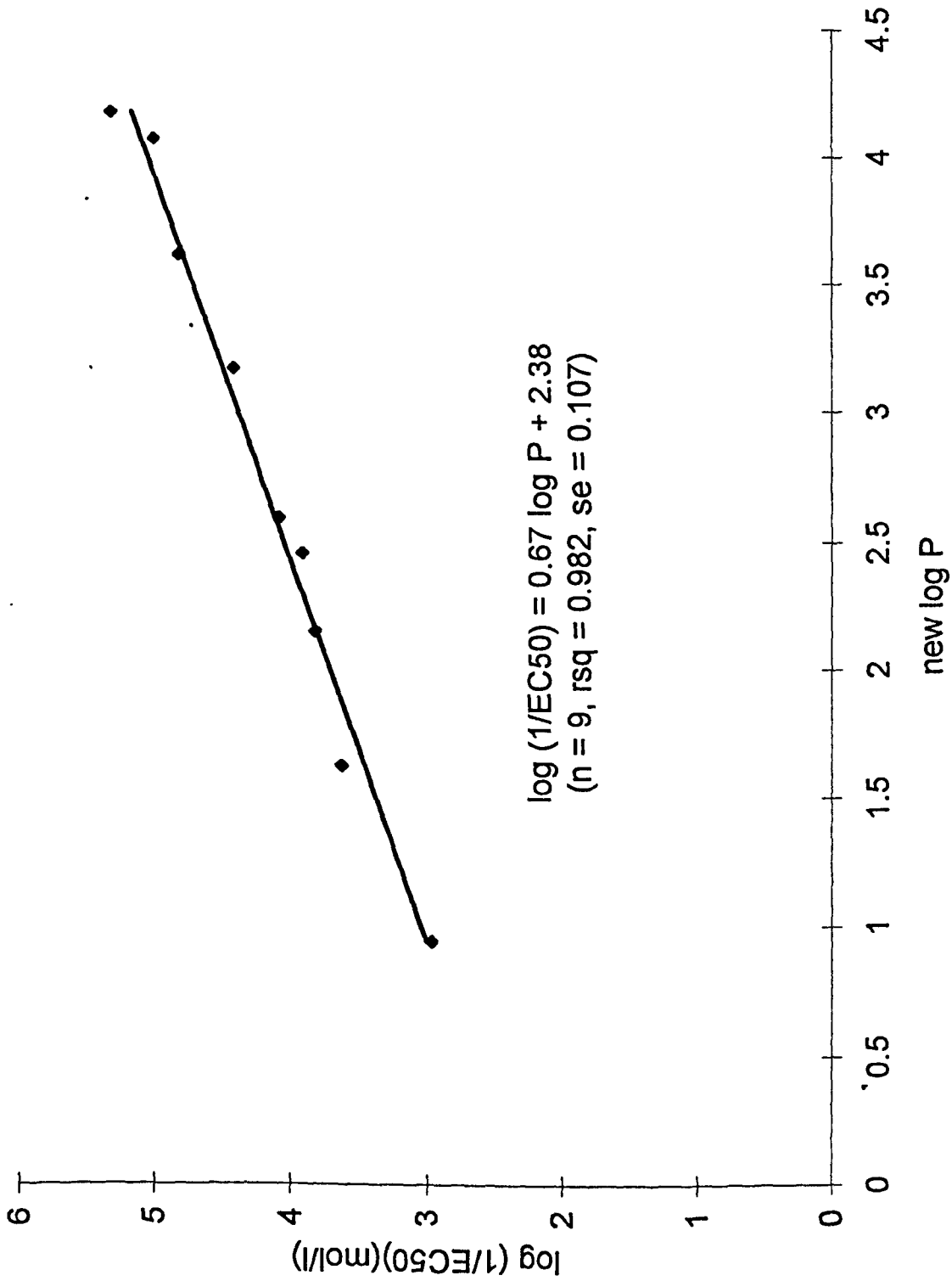
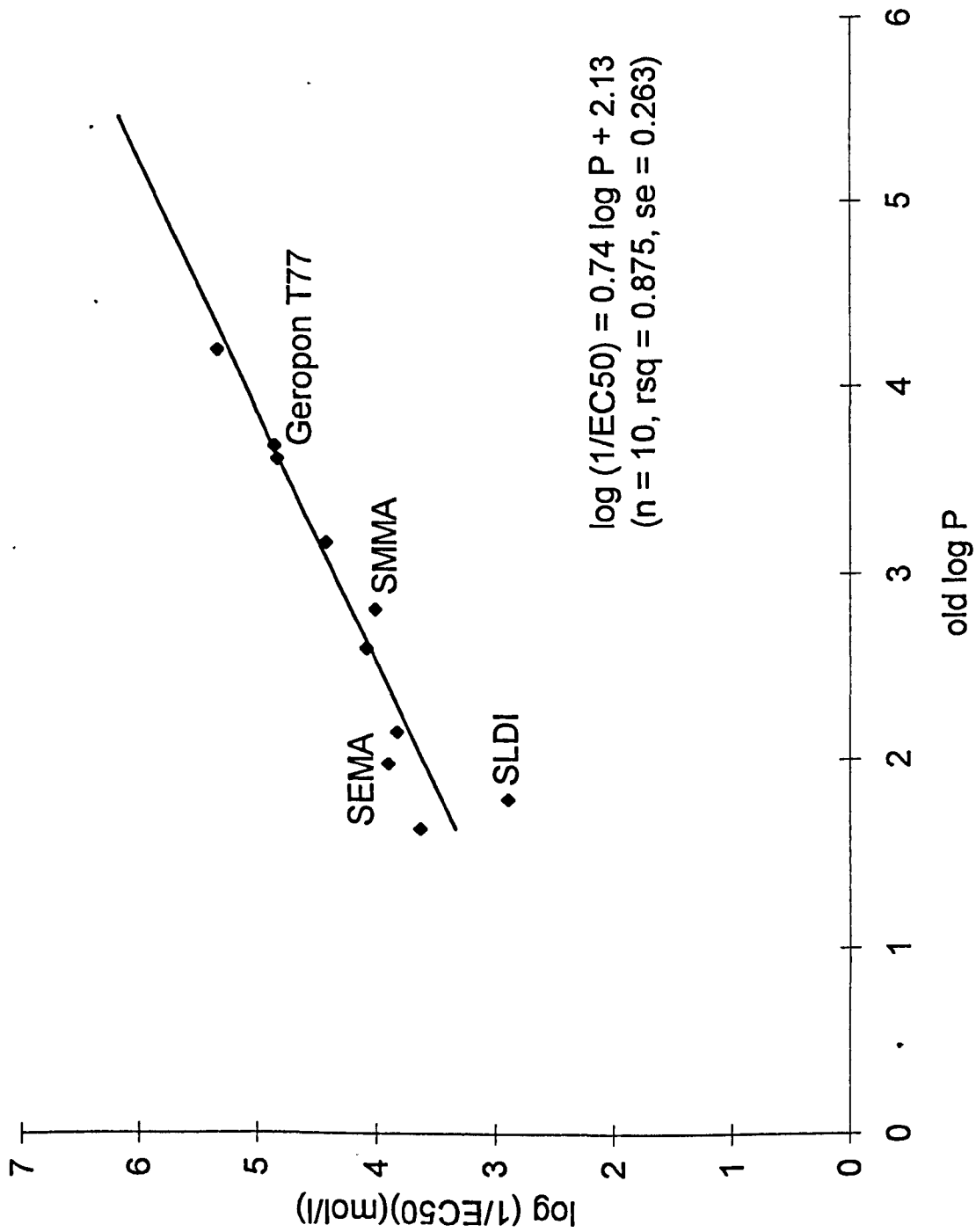


Fig. 38. Old log P versus log (1/EC50) for LAS substances, SMMA, SEMA, Geropon T77 and SLDI at a hardness of *ca.* 13mg/l as $CaCO_3$



Application of new log P values to the same substances and the new log P plot versus combined log (1/EC50)(mol/l) (Fig. 39) now returns a regression of :

log (1/EC50) = 0.68 log P + 2.42 Equation 44.
(n = 10, rsq = 0.909, se = 0.225)

When EC50 values for FAES substances are also applied to the plot of new log P versus log (1/EC50)(mol/l) there is only minor change to the regression and rsq and the standard error are improved slightly (equation 45)(Fig. 40).

log (1/EC50) = 0.68 log P + 2.41 Equation 45.
(n = 13, rsq = 0.931, se = 0.205)

Equations 42 and 44 show good correlation between EC50 values and log P values calculated with the new proximity factor. Equation 45 is also very similar to that of FAES and LAS alone (observed at a hardness of 13mg/l as $CaCO_3$)(equation 42) suggesting that all the substances observed behave by a similar mode of action to LAS. This is consistent with results of mixture toxicity studies (section 4.4.5). EC50 values (at a hardness of 13mg/l as $CaCO_3$) can now be predicted using an equation similar to equation 45 which is comparable to the polar narcosis equation (equation 10) when the new log P calculation method is applied.

EC50 values for DLAS, Geropon T77 and DSDS were plotted as old log P versus log (1/EC50)(Table 66) with EC50 values for LAS obtained as *ca.* 240 mg/l as $CaCO_3$ (Table 67)(Fig. 41). The plot shows some scatter about the LAS reference regression line with poor correlation (rsq = 0.742). When plotted in combination with FAES as new log P versus log (1/EC50)(mol/l), the combined regression equation for LAS, FAES, DLAS, Geropon T77 and DSDS (equation 46)(Fig. 42) is similar to that of LAS and FAES alone (equation 39).

Fig. 39. New log P versus log (1/EC50) for LAS substances, SMMA, SEMA, Geropon T77 and SLDI at a hardness of *ca.* 13mg/l as $CaCO_3$

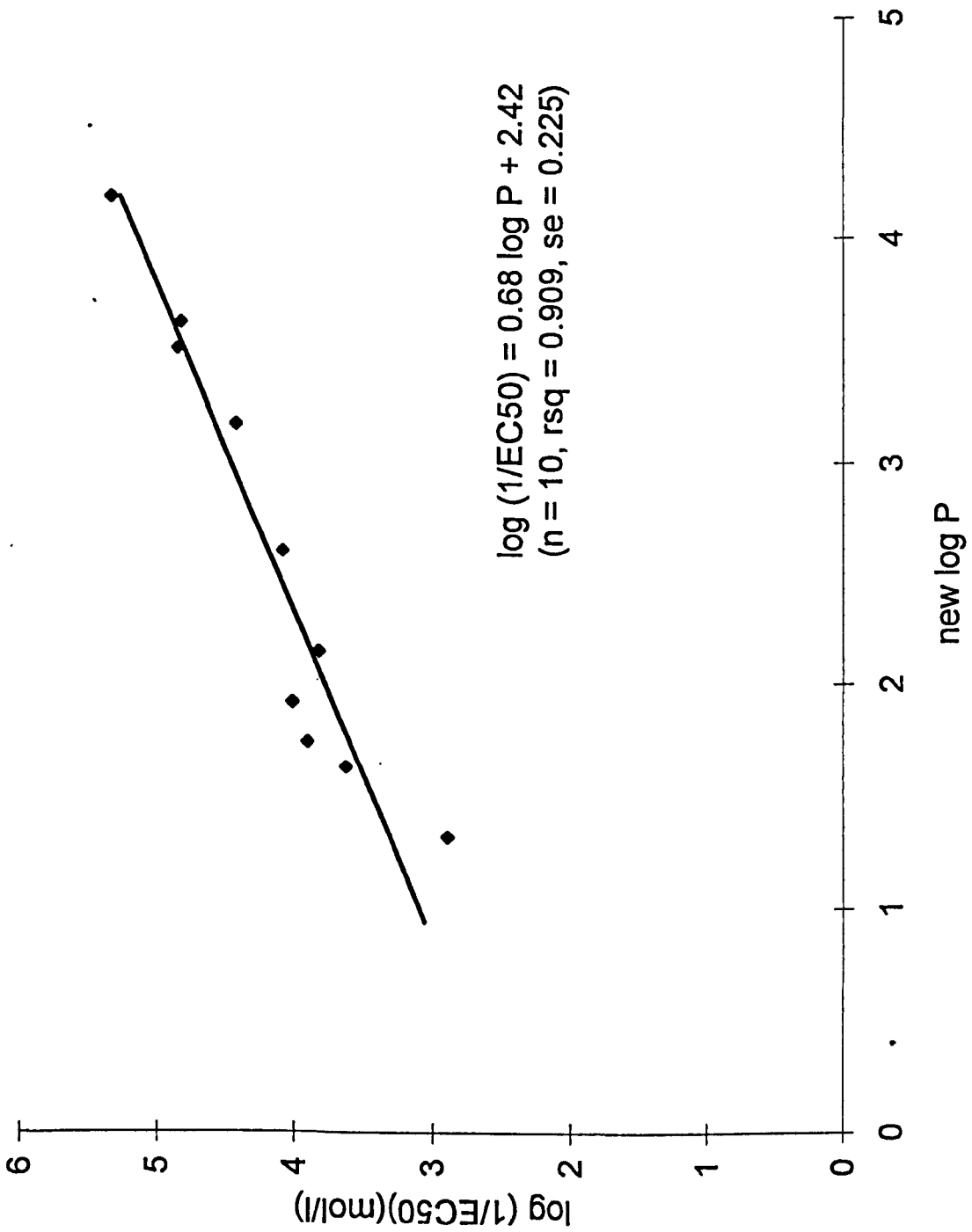


Fig. 40. New log P versus log (1/EC50) for LAS substances FAES substances, SMMA, SEMA, Geropon T77 and SLDI at a hardness of *ca.* 13mg/l as $CaCO_3$

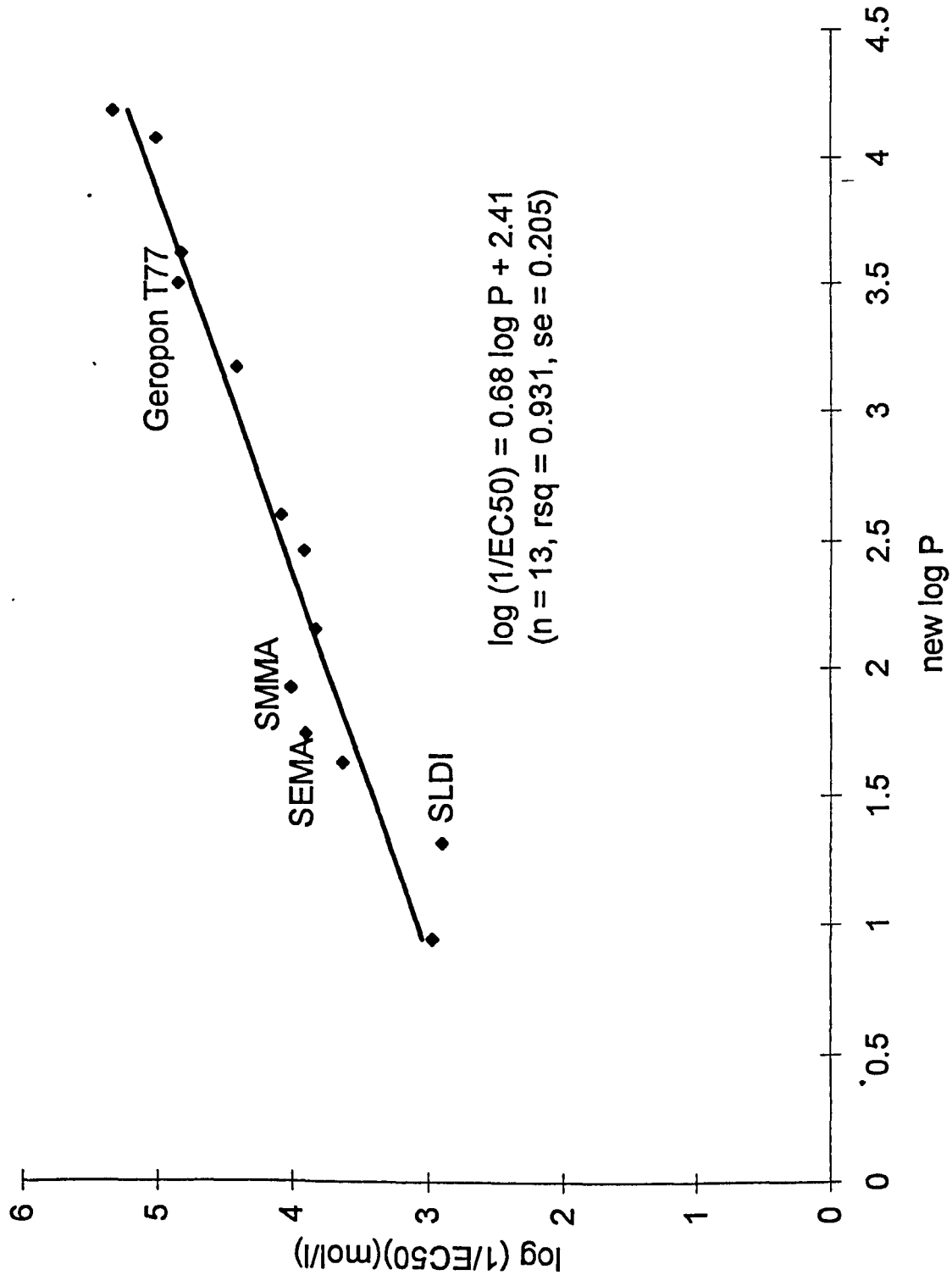


Fig. 41. Old log P versus log (1/EC50) for LAS substances, DLAS, DSDS and Geropon T77 at a hardness of *ca.* 240mg/l as $CaCO_3$

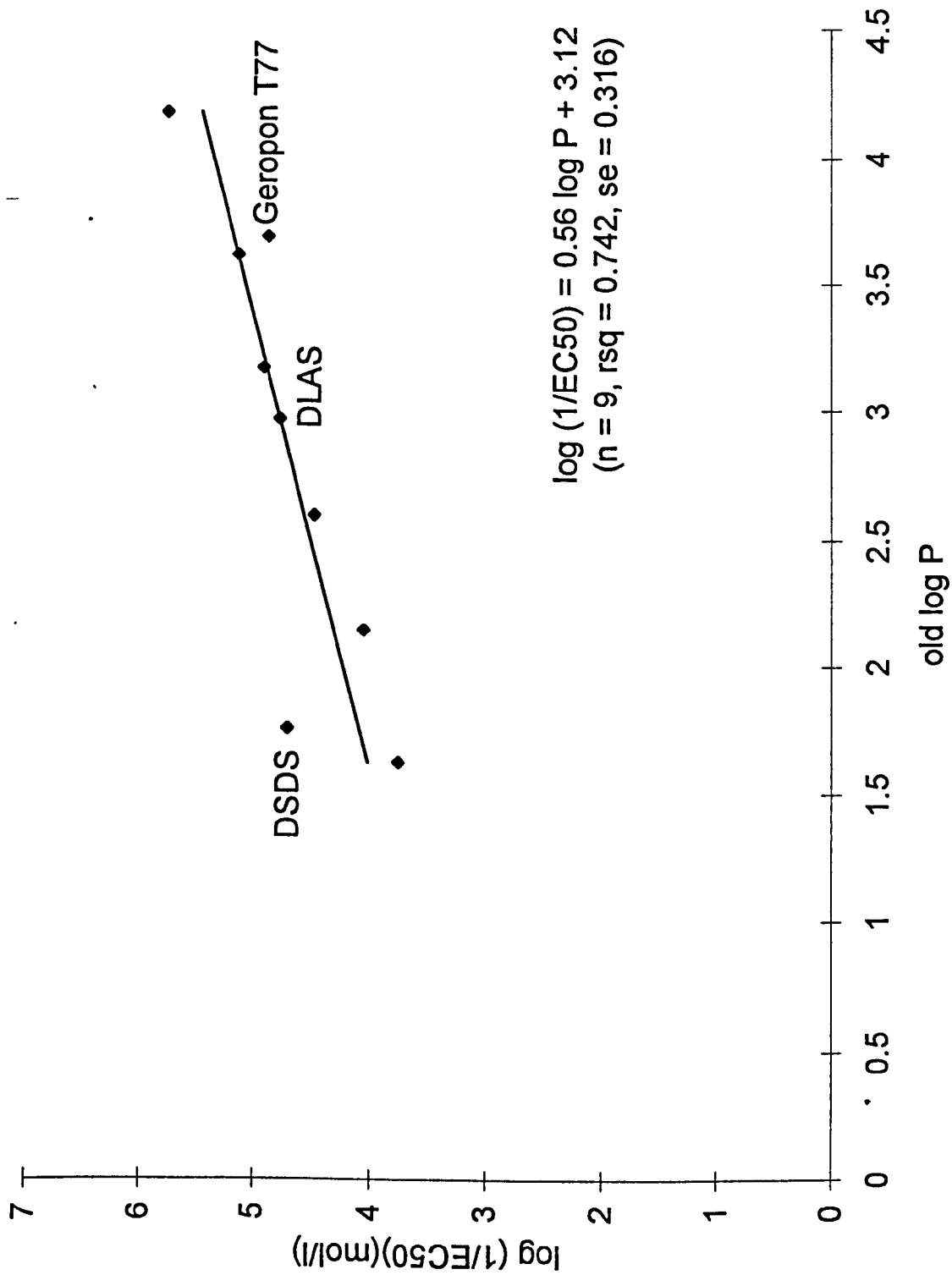


Fig. 42. New log P versus log (1/EC50) for LAS substances, FAES substances, DLAS, DSDS and Geropon T77 at a hardness of *ca.* 240mg/l as $CaCO_3$

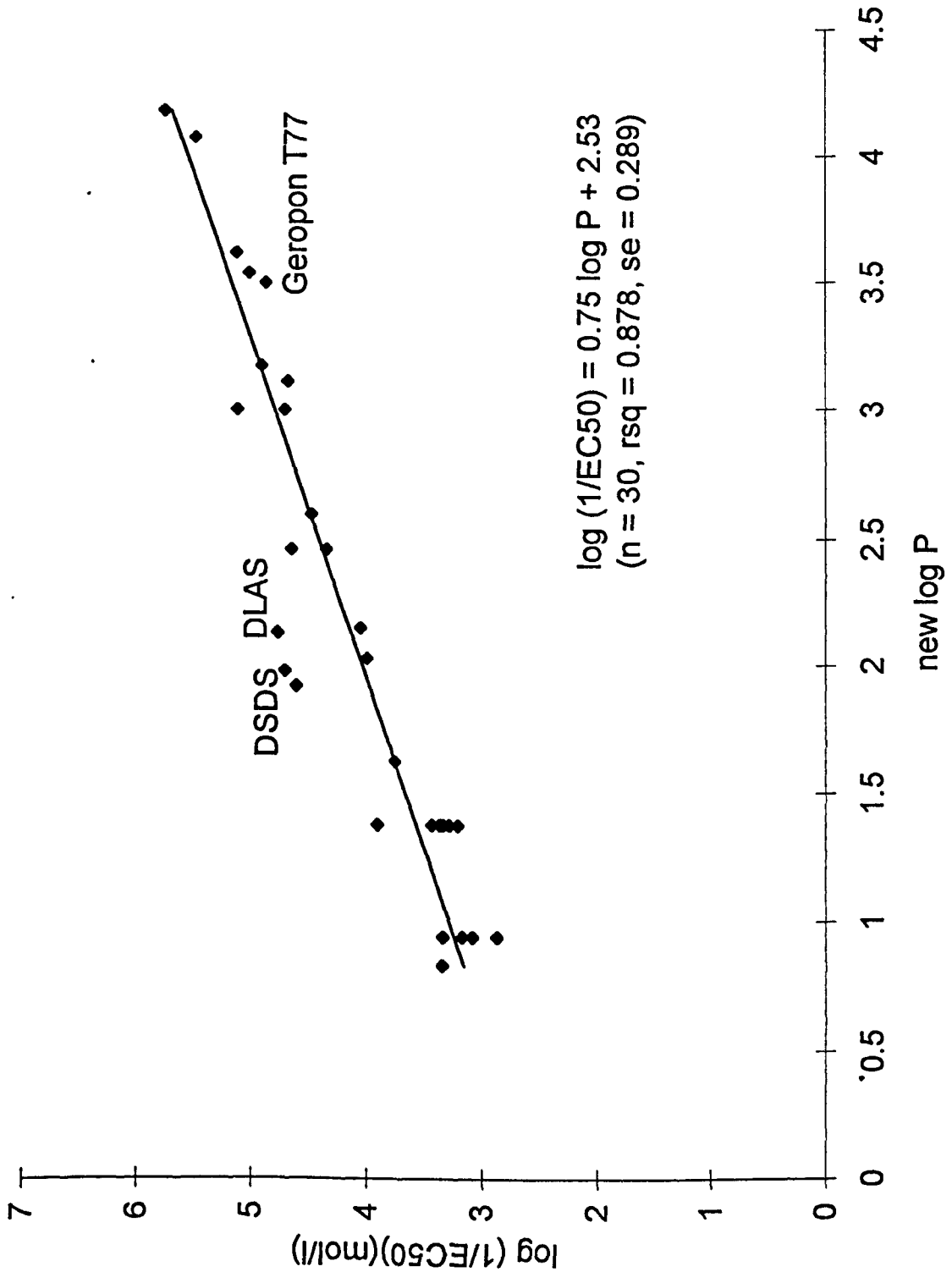
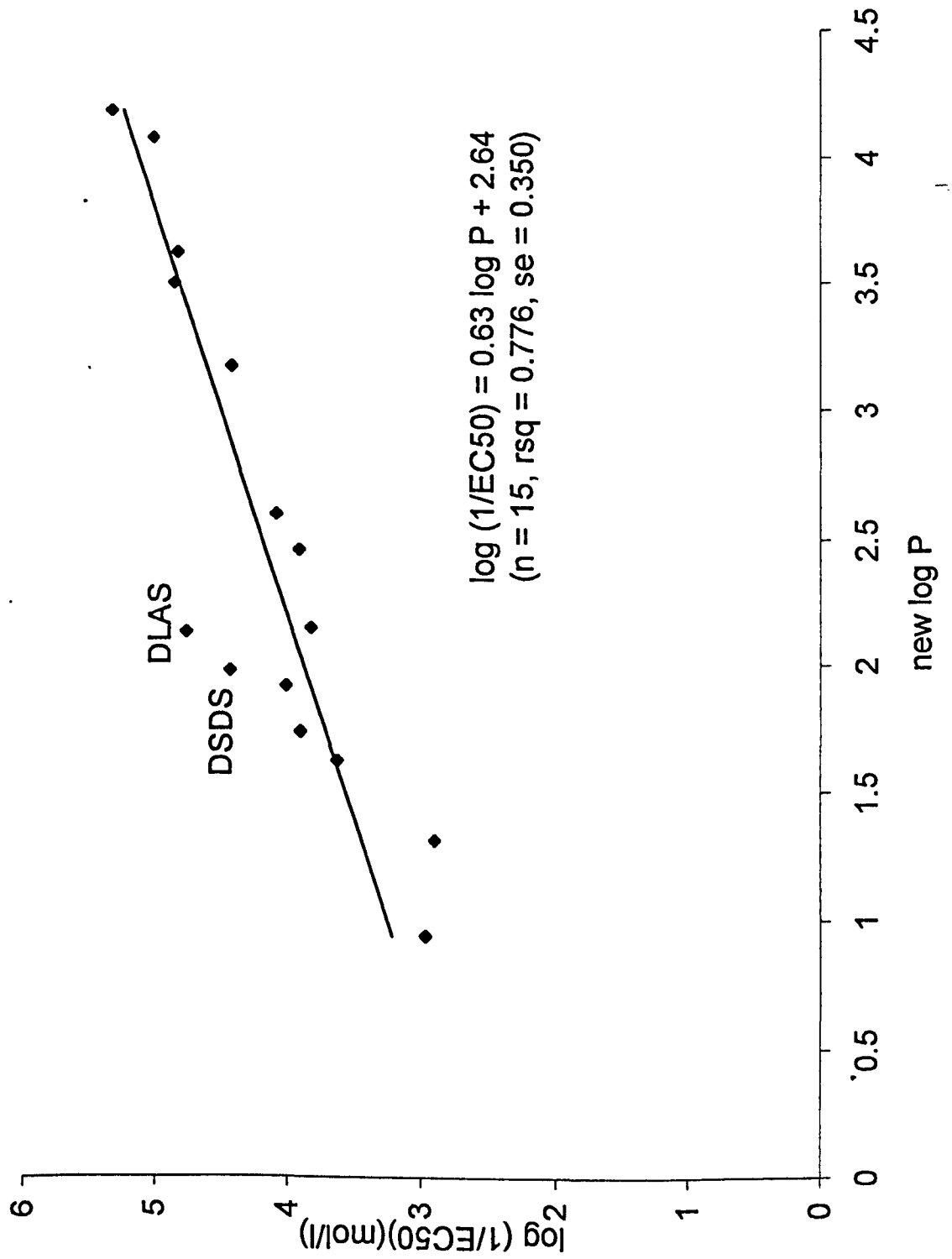


Fig. 43. New log P versus log (1/EC50) for LAS substances, FAES substances, DLAS, DSDS, SMMA, SEMA, SLDI and Geropon T77 at a hardness of *ca.* 13 mg/l as $CaCO_3$



Log (1/EC50) = 0.75 log P + 2.53 Equation 46.

(n = 30, rsq = 0.878, se = 0.289)

Although the correlation (rsq = 0.875) is not as good as that observed for LAS and FAES alone (rsq = 0.926)(Fig. 36) it is improved from that using old log P (rsq = 0.742)(Fig. 41).The lower correlation (rsq = 0.875) is partly due to DLAS and DSDS appearing as slight outliers. Of the three substances DLAS, Geropon T77 and DSDS, the EC50 value for Geropon T77 is the best predicted. Similarly at a hardness of 13mg/l as $CaCO_3$, when plotted as log P versus log (1/EC50) with SMMA, SEMA, SLDI, and FAES and LAS substances, DLAS and DSDS appear as outliers reducing correlation (rsq = 0.776)(Fig. 43). This may indicate a need for a slight modification to the new proximity factor to account for two large proximal polar groups. However, it is probably due, at least in part, to the anomalies in the observed EC50 values for DLAS and DSDS, and because of these anomalies it is not possible to establish any necessary changes without toxicity data for further substances.

4.4.7 CONCLUSION

There are strong similarities between the equation describing the mode of action for LAS and FAES with that for LAS alone at a hardness of both 13mg/l as $CaCO_3$ and 240mg/l as $CaCO_3$, (equations 37, 39, 40 and 42), when applying the new proximity factor to the calculation of log P for these substances. Regression analysis of new log P versus log (1/EC50) for SMMA, SEMA, SLDI, Geropon T77 and LAS at a hardness of 13mg/l as $CaCO_3$, also shows strong similarities with that of LAS alone and in combination with FAES at this hardness (equations 40, 44 and 45). Whilst equation 46 is fairly similar to equation 37, EC50 values as log (1/EC50)(mol/l) of DLAS and DSDS, at a hardness of 240mg/l as $CaCO_3$, do not correlate quite as well with LAS and FAES as those for other substances (equations 44 and 45). This is probably due to the effects of the twin counterion on the behaviour of these substances in varying water hardness (and the relative

insolubility of DLAS) and is likely, therefore, to be due to problems associated with the EC50 value rather than the log P calculation. The two values are closely positioned near the centre of the log P versus log (1/EC50) plot and would not necessarily be expected to give a clear picture of other similar substances over the full range of log P values represented on the plot. It must also be considered that whilst application of the new proximity factor provides improved toxicity prediction of substances over the old factor, the lower correlation with DLAS and DSDS may indicate that it requires further improvement to cope with two large proximal polar fragments. Ultimately further modification may be preferable so as not to calculate 'd' in terms of 'r', although actual bond lengths between fragments do vary with the particular fragments involved and any errors associated with this calculated 'd' values are small since differences between 'r' values of different fragments are also small.

Whilst the range of substances for which the new proximity factors has been applied is limited, similarities in regression equations would suggest on weight of evidence that the new proximity factors, as described by equations 34, 35 and 36, are appropriate for those substances to which they have been applied in this chapter. They provide a relatively simple improvement in log P calculation to account for proximal fragments. For further validation of the new proximity factors, it will be necessary to apply the new calculations to other substances for which good quality acute toxicity data are observed under the same conditions as values obtained in this thesis. This in itself poses problems due to the variation in EC50 values which can occur for single substances when using inter-laboratory data (Table 22). Time restrictions have unfortunately prevented further EC50 values being obtained for other structures with a variety of polar groups.

CHAPTER 5

GENERAL SUMMARY AND CONCLUSIONS

Detergents are complex mixtures of various ingredients which include surfactants, builders, bleaches and other subsidiaries all combined to promote the detergency of the product (Hennes-Morgan and de Oude, 1994). They are to be distinguished from soaps which are sodium or potassium salts of fatty acids (Swisher, 1970).

Since their introduction into detergent formulations in both domestic consumer products and industrial applications in the 1930s, synthetic surfactants have experienced a large increase in output (Falbé, 1987). The resulting increase in usage causes discharge to municipal sewage where most are degraded through treatment (Ahel *et al.*, 1994a; Dorn *et al.*, 1993; Stephanou and Giger, 1982). Small amounts, however, do reach surface waters (Ahel *et al.*, 1994b; Naylor *et al.*, 1992; Rapaport and Eckhoff, 1990). Even though these are at low concentrations, since most surfactants have significant inherent toxicity to aquatic organisms their presence in surface waters is considered undesirable.

In recent years with the advent of greater public awareness and tighter legislation, industry has moved to address the issues of environmental impact of their products with the establishment of task forces by such bodies as the European Chemical Industry Ecology and Toxicology Centre (ECETOC). The EC Council Regulation 793/93/EEC on the Evaluation and Control of Risks of Existing Substances requires data in the form of dossiers to be submitted on all existing substances listed in the European Inventory of Existing Commercial Substances (EINECS) which are produced or imported in quantities exceeding 10 tonne / year (Feijtel, 1995; USES, RIVM, 1994). All data regarding the Harmonised Electronic Data SET (HEDSET), covering ecotoxicological, toxicological and physicochemical properties of substances, are to be included on the EC database which will then be used to rank substances according to relative risk.

All new substances not appearing in EINECS have to be notified to the competent authority in one of the member states accompanied by data equivalent to that defined by the 'base-set'. The size of this data set is dependent on marketed volume. For ecotoxicological information this comprises short term toxicity data for algae, *Daphnia* and fish for the aquatic compartment (USES, RIVM, 1994). Considering the large numbers of existing and new substances in regular commercial use, the acquisition of such large volumes of data is an impossible problem to cope with on an experimental level (Donkin, 1994). The use of QSAR analysis as a tool for filling in data gaps and acting as a safety net for non-valid data of both existing and new substances, has been accelerated by such legislation (Feijtel, 1995). The use of QSAR in industry has been limited to a narrow range of chemical classes for the design of active substances of less toxicity etc. (Feijtel, 1995). In the US, however, QSAR analysis, principally of new substances, has been used as an integral regulatory method for the estimation of aquatic toxicity by the Environmental Protection Agency (EPA) for over 15 years (Zeeman *et al.*, 1995; Auer *et al.*, 1995). The EPA QSARs have been routinely checked and corrected over this time resulting in class specific QSARs which are fairly good at predicting aquatic toxicity to fish, invertebrates and algae.

One of the main limitations of QSAR is that they can be used as prediction tools only for substances with a common mode of action (Verhaar *et al.*, 1992). In assigning mode of action to a particular substance, several experimental approaches have been used. McKim *et al.* (1987) identified six modes of action which related to grouped observed physiological responses in rainbow trout known as fish acute toxicity syndromes (FATS) when exposed to a number of substances; non-polar narcosis, polar narcosis, uncoupling oxidative phosphorylation, respiratory membrane irritation, acetylcholinesterase inhibition and central nervous seizure. The more generalised classification of Hermens (1989) defines substances as inert (non-polar, nonreactive), less inert (polar narcosis), reactive and specifically acting. The method of Veith and Broderius (1990) used joint toxicity theory to show that substances acting strictly additively with phenol were generally more toxic than predicted by the non-polar model

(equation 9) and could be characterised by a different mode of action similar to that of Saarikoski and Viluksela (1982) (equation 10):

$$\log LC50 = -0.65 \log P - 2.29 \qquad \text{Equation 47.}$$

(n = 39, rsq = 0.90)

The joint toxicity of mixtures with similar mode of action can be predicted with the concentration addition model, even for mixtures of more than two substances (De Wolf *et al.*, 1988; Hermens *et al.*, 1985).

The concentration addition model can also be applied to surfactants. Commercial alcohol ethoxylates are mixtures of homologues. Good correlation was observed between predicted EC50 values to *D. magna*, using calculated log P values assuming concentration addition, and observed EC50 values (Roberts and Marshall, 1995). Lewis and Perry (1981) found response addition between mixes of anionic and non-ionic and between cationic and non-ionic surfactants when testing with bluegills. When testing with *D. magna,* five of the twelve mixtures exhibited either response or concentration addition with response addition predominating, although the anionic / non-ionic mixture exhibited concentration addition. Synergism was observed, however, between anionic and cationic surfactants when testing with both these species.

Results of mixture studies presented here (chapter 3) between two anionic surfactants (FAES and LAS) indicate that concentration addition is applicable for these substances. It must be emphasised, however, that observed differences in toxicity can be small and any changes in concentration due to degradation etc. may alter the apparent response. Care must also be taken to include substances of similar individual toxicity when observing binary mixture toxicity to avoid problems associated with the overwhelming effects of the more toxic substance (Tables 26, 27 and 28). This problem can be observed in commercial mixtures of PAS (primary alcohol sulphates) which contain small amounts of the parent alcohol. The toxicity of the alcohol alone, however, accounts for the majority of the toxicity observed. Calculation of predicted toxicity using the concentration addition

model and the response addition model using equations 20 21 and 22 shows that it can be impossible to differentiate between either model (unpublished Unilever data).

In mixtures where only one of the two components of the mixture is a surfactant, results tend to be mixture specific where the other component is either a metal or a pesticide (Lewis, 1982). This makes generalisation difficult. There is some evidence for synergistic behaviour between metals and anionic surfactants (Calamari and Marchetti, 1973). None of the mixtures studies reported in this thesis exhibited greater than additive response and considering other mixture toxicity data for involving other non-specific common substances in mixtures with and without surfactants, there is little evidence for significantly more toxic response than concentration addition of these substances (Alabaster, 1981; Broderius, 1992; Broderius and Kahl, 1985; Broderius *et al.*, 1995; Hermens and Leeuwangh, 1982; Roberts and Marshall, 1995; Shirazi and Linder, 1991).

Thus in binary mixtures where one component (the reference substance) is of known mode of action and where concentration addition is observed, it is reasonable to assume that the second substance (test substance) behaves with the same or a similar mode of action. This is ultimately reliant upon accurate toxicity data and correctly assigning mode of action to the reference substances. EC50 values for alcohols reported in this thesis are consistent with the wealth of toxicity data for alcohols and there is no doubt as to the non-polar nature of the narcosis of these substances; selected here are a few examples of the many references to such data (Blum and Speece, 1990; Broderius, 1992; Broderius and Kahl, 1985; Cronin *et al.* 1991; De Wolf *et al.*, 1988; Ikemoto *et al.*, 1982; Könemann, 1980; Könemann, 1981; McCarty *et al.*, 1992; Schild *et al.*, 1993; Schultz and Tichy, 1993; Veith and Broderius, 1990).

Whilst there is evidence that the more highly chlorinated phenols act as uncouplers of oxidative phosphorylation and respiration (Jaworska and Schultz, 1994; Penttinen, 1995), there are also large numbers of data for phenol and mono-chlorinated phenols in the literature which support a polar narcosis mechanism (Broderius, 1991; Furay and Smith, 1995; McKim *et al.*, 1987;

Penttinen, 1995; Saarikoski and Viluksela, 1982; Veith and Broderius, 1990; Schultz *et al.*, 1986). EC50 values for phenols reported in this thesis suggest that a QSAR similar to that of Saarikoski and Viluksela (1982)(equation 10) is developing (chapter 3). The fact that phenol behaves by response addition in mixtures with alcohol is good evidence that it is acting by a different mode of action to alcohols.

A number of other conclusions can be drawn from results presented in chapters 3 and 4 with anionic surfactants in mixtures with phenols and alcohols.

1) Anionic surfactants can be observed to exhibit concentration addition with surfactant and non-surfactant substances.
2) Anionic surfactants can be observed to exhibit response addition with non-surfactant substances.
3) By exhibiting concentration addition with phenols (polar narcotics) and response addition with alcohols (non-polar narcotics), FAES substances have been shown to act by polar narcosis in line with LAS and other anionic surfactants (Roberts, 1991).

Esters are usually excluded from narcosis QSARs with the rationale that hydrolysis of these substances results in an increase in the observed toxicity (predicted by general narcosis - equation 9) (Kamlet *et al.*, 1987).

FAES substances, however, have been found to be highly resistant to hydrolysis (chapter 2)(Stein and Baumann, 1975; Stirton *et al.*, 1965). FAES can, therefore, be assumed to behave as a polar narcotic with toxicities which would be expected to be predicted by a QSAR similar to that of LAS (equation 37). Consequently apparent deviation from the equation is likely to be as a result of the need to modify the proximity factor used in the log P calculation. In addition, many of the FAES substances exhibit toxicity even less than predicted by the general (baseline) narcosis equation (equation 9)(Tables 3 and 5). By definition clearly this is not possible, indicating a need to modify the log P calculation.

Comparison of data in the EU data base and values determined by EPA QSAR predictions between 1991 - 1993 showed some of the QSARs using parameters such as water solubility and vapour pressure not to be as reliable as hoped while others using log P proved reasonably accurate (Zeeman *et al.*, 1995). Agreement between EPA predicted values and EU measured values was reported as 87% for fish and 79% for daphnid acute toxicity data. It is the issue of reliability and accuracy of QSARs for use in risk assessment which is difficult to assess. Certainly log P, as a parameter simulating the transport of a substance from the aqueous environment to the biophase site of action, is a key parameter controlling effect, accumulation and fate models (Calleja *et al.*, 1994). With its wide applicability, many reliable log P based QSARs have been developed (Foster and Tullis, 1984; Govers *et al.*, 1984; Ikemoto *et al.*, 1992; Isnard and Lambert, 1988; Könemann, 1981; Könemann and Musch, 1981; Lipnick, 1988; McCarty, 1986; Saarikoski and Viluksela, 1982; Schultz and Cormeaux, 1996; van Leeuwen *et al.*, 1990; Veith and Broderius, 1990; Verhaar *et al.*, 1994; Zaroogian *et al.*, 1985). As nonreactive substances the toxicity of surfactants is often directly proportional to hydrophobicity (Könemann and Musch, 1981; Veith *et al.*, 1983) and is often modelled by log P alone (Marshall and Roberts, 1995; Roberts, 1991).

The ease of manual calculation of log P values of relatively simple surfactant structures using the Hansch and Leo (1979) method with some correction factors (Roberts, 1991), and its established use in QSAR, make it arguably a more flexible and valuable alternative to calculated values for these substances over those derived from other methods available such as the AUTOLOGP (Devillers *et al.*, 1995) and KOWWIN (Syracuse Research). Mannhold and Dross (1996) found log P values calculated using KOWWIN to be slightly more accurate than those calculated by ClogP based on a data set of 138 substances. Müller (1996) reported that ClogP calculated more accurate estimates of log P the KOWWIN based on a data set of 1217 substances. There is very little to choose between the accuracy of log P values calculated by these two approaches.

No empirically or non empirically derived log P estimation model is perfect as the calculated log P values remain estimates. All the above mentioned methods have limitations brought about largely from missing values and inability to cope with charge. Log P values as described by Hansch and Leo (1979) show some anomalies. Failure to describe the hydrophilic effect of increasing EO groups and consequent failure to describe acute toxicity of these substances is a good example (Roberts, 1988; Schüürman, 1990). Saarikoski and Vilusela (1982) also found a few irregularities in the approach for some phenols. However, log P values calculated by this method have been used successfully in many QSAR analyses, including surfactants with the inclusion of PDBF and EO correction factors (Broderius, 1992; Broderius and Kahl, 1985; Ikemoto *et al.*, 1992; Pirselova *et al.*, 1996; Roberts, 1991; Roberts and Marshall, 1995; Saarikoski and Viluksela, 1982; Schultz and Tichy, 1993; Veith and Broderius, 1990).

Results presented here (chapter 4) indicate that an empirically derived modified proximity factor, based on volume of overlap of hydration sheaths, can significantly improve the Hansch and Leo (1979) method for calculating log P values for substances with two polar groups of widely different sizes. Correlation between toxicity and log P values calculated with the new factor (newlog P) is improved for substances tested. It must be remembered that the number of substances is relatively few and further validation is required. It may also be the case that other parameters such as solvation energies may also be appropriate. Time restrictions unfortunately have prevented further investigation into either of these areas.

There is certainly a need for estimated log P values for substances of interfacial nature which make measurement of such values extremely difficult. That of Hansch and Leo (1979) has proved of immeasurable value in the development of QSARs for these and other substances. With an empirically derived method there is always scope for improvement as new substances become available. With such developments as reported in this thesis, correlation within aquatic toxicity QSAR analysis will hopefully be improved and help to

secure the growing acceptance of the usefulness of QSAR at the regulatory risk assessment level in the near future.

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APPENDIX I

Replicate observed EC50 values, confidence limits, EC50 values as maximum and minimum percent of mean, and MBAS / GC-MS analysis of test solution concentrations.

Table 71. Observed EC50 values and 95% confidence limits for FAES substances

Substance	Test Replicate	Observed EC50 (mg/l)*	95% Confidence Interval[a]
C12 methyl	1	170	129 - 244
	2	120	96.9 - 137
C12 butyl	1	17	14.9 - 20.0
	2	16	12.9 - 18.5
C12 sec butyl	1	40	31.9 - 50.4
	2	32	32 - 56[c]
C12 amyl	1	7.6	6.18 - 9.21
	2	6.7	5.43 - 8.24
C14 methyl	1	11	9.29 - 13.4
	2	5.7	4.67 - 6.90
	3	8.6	7.40 - 10.1
C14 ethyl	1	7.9	6.40 - 9.76
	2	8.1	5.6 - 10[c]
C14 iso propyl	1	9.5	8.09 - 11.3
	2	7.6	6.21 - 9.30
C14 butyl	1	3.7	3.2 - 5.6[c]
	2	3.6	3.2 - 5.6[c]
C14 amyl	1	1.5	1.25 - 1.71
	2	1.0	0.842 - 1.25
C16 methyl	1	3.2	2.65 - 3.85
	2	2.4	2.3 - 3.1[c]

* Values to 2 s.f..

[a] Values to 3 s.f.

[c] EC50 value calculated from non-linear interpolation between presented limits.

Table 72. Observed EC50 values and 95% confidence limits for FAES substances.

Substance	Test Replicate	Observed EC50 (mg/l)*	95% Confidence Interval[a]
C7 heptyl	1	130	105 - 189
	2	150	100 -180[c]
C8 hexyl	1	190	155- 224
	2	170	140 - 205
C8 sec hexyl	1	400	343 - 469
	2	390	331 - 462
C9 amyl	1	130	103 - 154
	2	150	80.4 - 289
C9 sec amyl	1	270	221 - 342
	2	260	217 - 320
C10 butyl	1	190	153 - 227
	2	150	124 - 169
C10 sec butyl	1	220	182 - 274
C10 iso butyl	1	150	122 - 184
C11 propyl	1	130	112 - 151
	2	110	100 -180[c]
C12 ethyl	1	140	121 - 171
	2	160	137 - 193
C13 methyl	1	38	30.8 - 46.6
	2	43	32 - 56[c]

* Values to 2 s.f..

[a] Values to 3 s.f.

[c] EC50 value calculated from non-linear interpolation between presented limits.

Table 73. Observed EC50 values and 95% confidence limits for LAS substances

Substance	Test Replicate	Observed EC50 (mg/l)*	95% Confidence Interval[a]
C9	1	59	50.7 - 69.2
	2	46	38.9 - 55.2
C10	1	28	18 - 32[c]
	2	27	18 - 32[c]
C11	1	11	9.00 - 12.4
	2	11	6.39 - 19.3
C12	1	5.5	4.80 - 6.43
	2	3.1	2.57 - 3.67
C13	1	2.4	2.04 - 2.89
	2	2.9	2.53 - 3.37
C14	1	1.0	0.56 - 1.8[c]
	2	0.90	0.56 - 1.0[c]

* Values to 2 s.f..

[a] Values to 3 s.f.

[c] EC50 value calculated from non-linear interpolation between presented limits.

Table 74. Observed EC50 values and 95% confidence limits for reference substances to be used in mixture toxicity studies

Substance	Replicate	Observed EC50 (mg/l)*	95% Confidence Interval [a]
Phenol	1	6.7	5.6 - 10 [c]
	2	8.4	7.23 - 9.75
	3	6.8	1.40 - 54.2
	4	7.8	6.80 - 8.76
2 -chlorophenol	1	3.6 [1.6][b]	3.0 - 4.21
	2	2.9	1.8 - 3.2[c]
C11.8 LAS	1	6.7	5.6 - 10 [c]
	2	4.7	3.86 - 5.80
1-Pentanol	1	410	230 - 520 [c]
	2	400	349 - 459
1-Hexanol	1	130	100 - 180 [c]
	2	120	107 - 140
1-Octanol	1	21	17.9 - 24.1
	2	25	21.2 - 30.0
1-Nonanol	1	7.7	6.43 - 9.38
	2	7.2	5.6 - 10 [c]
1-Decanol	1	3.4	2.90 - 4.02
	2	3.6	3.05 - 4.32

* Values to 2 s.f.

[a] Values to 3 s.f.

[b] Value in parentheses corrected for test solution concentration analysis.

[c] EC50 value calculated from non-linear interpolation between presented limits.

Table 75. Observed EC50 values and 95% confidence limits for p-cresol and 2,4,6-trichlorophenol studies

Substance	Replicate	Observed EC50 (mg/l)*	95% Confidence Interval [a]
p-cresol	1	7.9	5.2 - 10 [c]
	2	6.0 [4.0] [b]	5.06 - 7.12
2,4,6-	1	0.58 [0.44] [b]	0.505 - 0.676
trichlorophenol	2	0.52 [0.25] [b]	0.32 -0.56 [c]

* Values to 2 s.f.

[a] Values to 3 s.f.

[b] Value in parentheses corrected for test concentration analysis.

[c] EC50 value calculated from non-linear interpolation between presented limits.

Table 76. 95% confidence limits for observed EC50 values for mixture toxicity studies with LAS and 1-decanol as reference substances

Mixture		%# (A,B)	TR	Observed EC50 (mg/l)*[b]	95% Confidence Interval [a]
A	B				
LAS	C13 methyl	30,70	1	12	9.94 - 13.7
LAS	C13 methyl	20,80	1	25	18 - 32[c]
			2	17	10 - 18[c]
LAS	C13 methyl	10,90	1	26	18 - 32[c]
LAS	C12 methyl	20,80	1	37	32 - 56[c]
LAS	C12 ethyl	30,70	1	15	12.9 - 17.5
			2	25	18 - 32[c]
LAS	C7 heptyl	30,70	1	18	10 - 32[c]
			2	22	18 - 32[c]
LAS	C10 n-butyl	30,70	1	17	10 - 18[c]
LAS	C10 n-butyl	20,80	1	28	18 - 32[c]
			2	32	18 - 56[c]
LAS	C10 n-butyl	10,90	1	42	34.8 - 49.7
LAS	C8 hexyl	20,80	1	28	24.0 - 33.1
			2	21	18 - 32[c]
1-decanol	C13 methyl	20,80	1	11	9.76 - 13.0
			2	16	13.3 - 18.0
1-decanol	C12 ethyl	20,80	1	15	12.5 - 17.1
			2	13	10.6 - 16.0
1-decanol	C7 heptyl	20,80	1	18	15.7 - 21.1
			2	12	9.96 - 14.3
1-decanol	C8 hexyl	20,80	1	14	11.8 - 16.6

A: Reference substance component. B: Test Substance component (FAES).

TR: Test replicate. *Values to 2 s.f.. # Nominal % component in mixture.

[a] Values to 3 s.f.. [b] Values based on nominal concentrations.

[c] EC50 value calculated from non-linear interpolation between presented limits.

Table 77. 95% confidence limits for observed EC50 values for mixture toxicity studies where concentration addition predicted

Mixture		TR	Observed EC50 (mg/l)*[a]	95% Confidence Interval [b]
A	B			
Phenol	C14 methyl	1	10	5.6 - 18 [c]
		2	7.1	5.31 - 9.88
Phenol	C12 amyl	1	12	9.94 - 16.2
		2	14	11.7 - 17.9
Phenol	C14 ethyl	1	13	10 - 18 [c]
		2	11	10 - 18 [c]
Phenol	C12 n-butyl	1	16	10 - 32 [c]
		2	18	18 - 32
Phenol	C11.8 LAS	1	6.4	5.14 - 7.91
		2	6.3	3.10 - 13.2
2-CP	C14 n-butyl	1	4.7	2.3 - 5.6 [c]
		2	3.2	2.54 - 4.06

A: Reference substance component. B: Test Substance component (FAES, LAS).

TR: Test replicate.

*Values to 2 s.f. [b] Values to 3 s.f.

[a] Values based on nominal concentrations.

[c] EC50 value calculated from non-linear interpolation between presented limits.

Table 78. 95% confidence limits for 48 hour EC50 values for nominally equitoxic mixture toxicity studies where response addition predicted

Mixture		%# (A,B)	TR	Observed EC50 (mg/l)*[b]	95% Confidence Interval [a]
A	B				
Pentanol	C8 sec hexyl	50,50	1	570	485 - 664
			2	690	560 - 1000[c]
Hexanol	C12 ethyl	50,50	1	290	180 - 320[c]
			2	270	229 - 312
Hexanol	C10 butyl	43,57	1	250	180 - 320[c]
			2	210	180 - 320[c]
Hexanol	C8 hexyl	41,59	1	270	180 - 320[c]
			2	270	229 - 312
Hexanol	C7 heptyl	50,50	1	200	167 - 241
			2	300	259 - 346
Octanol	C12 butyl	60,40	1	35	27.5 - 70.3
			2	39	32 - 56[c]
Octanol	C13 methyl	36,64	1	58	56 - 100[c]
			2	51	32 - 56[c]
Nonanol	C12 amyl	51,49	1	16	10 - 18[c]
			2	14	10 - 18[c]
Nonanol	C14 methyl	47,53	1	18	10 - 32[c]
			2	19	18 - 32[c]
Nonanol	C11.8 LAS	57,43	1	12	10.0 - 13.9
			2	11	9.22 - 12.4
Nonanol	Phenol	51,49	1	18	15.3 - 20.6
			2	14	12.2 - 16.6

A: Reference substance component. B: Test Substance component (FAES, LAS).

TR: Test replicate. *Values to 2 s.f.. # Nominal % component in mixture.

[a] Values to 3 s.f.. [b] Values based on nominal concentrations.

[c] EC50 value calculated from non-linear interpolation between presented limits.

Table 79. 95% confidence limits for observed EC50 values for nominally non-equitoxic mixture toxicity studies where response addition predicted.

Mixture		%# (A,B)	TR	Observed EC50 (mg/l)*[b]	95% Confidence Interval [a]
A	B				
Hexanol	C7 heptyl	73,27	1	180	149 - 205
Hexanol	C7 heptyl	23,77	1	150	56 - 560 [c]
			2	200	159 - 262
Octanol	C12 butyl	50,50	1	32	18 - 56 [c]
Octanol	C13 methyl	16,84	1	82	56 - 100 [c]
			2	73	56 - 100 [c]
Octanol	C13 methyl	50,50	1	41	32 - 56 [c]
			2	44	38.2 - 49.7

A: Reference substance component. B: Test Substance component (FAES).

TR: Test replicate. *Values to 2 s.f..

Nominal % component in mixture.

[a] Values to 3 s.f..

[b] Values based on nominal concentrations.

[c] EC50 value calculated from non-linear interpolation between presented limits.

Table 80. Observed EC50 values and 95% confidence limits for individual substances at 13mg/l as $CaCO_3$.

Substance	TR	TH (mg/l as $CaCO_3$)	Observed EC50 (mg/l)*	95% Confidence Interval[a]
DLAS	1	13	8.3	6.63 - 10.2
SLDI	1		510	320 - 560[c]
Geropon T77	1		6.5	5.6 - 10[c]
SMMA	1		34	29 - 103
	2		32	29 - 103[c]
SEMA	1		44	34.4 - 105[c]
DSDS	1		28	18 - 32[c]
C11.8 LAS	1		12	10 - 18[c]
	2		16	10 - 18[c]
C14 amyl FAES	1		4.0	3.33 - 4.77
	2		3.6	2.89 - 4.41
C10 sec-butyl FAES	1		330	320 - 560[c]
	2		360	320 - 560[c]
C12 n-butyl FAES	1		43	32 - 56[c]
DLAS	1	240	8.4	7.15 - 9.81
Geropon T77	1		6.7	5.6 - 10[c]
	2		5.8	5.6 - 10[c]
DSDS	1		16	10 - 18[c]
	2		14	10 - 18[c]

* Values based on nominal concentrations (to 2 s.f.).

[a] Values to 3 s.f.

[c] EC50 value calculated from non-linear interpolation between presented limits.

Table 81. Observed EC50 values and 95% confidence limits for LAS substances at 13mg/l as $CaCO_3$.

Substance	Test Replicate	Observed EC50 (mg/l)*	95% Confidence Interval[a]
C9	1	100	100 - 180[c]
	2	69	56.9 - 81.3
C10	1	47	40.4 - 55.1
C11	1	27	18 - 32[c]
C12	1	13	10 - 18[c]
C13	1	5.3	3.2 - 5.6[c]
C14	1	1.9	1.65 - 2.21
	2	2.3	1.98 - 2.74

* Values based on nominal concentrations (to 2 s.f.).

[a] Values to 3 s.f.

[c] EC50 value calculated from non-linear interpolation between presented limits.

Table 82. 95% confidence limits for observed EC50 values for mixture toxicity studies with substances in chapter 4

Mixture		%# (A,B)	TR	Observed EC50 (mg/l)*[b]	95% Confidence Interval [a]
A	B				
Phenol	DLAS	58,42	1	8.9	5.6 - 10[c]
Phenol	DSDS	59,41	1	10	8.63 - 12.1
		57,43	2	9.6	8.01 - 11.8
Phenol	Geropon T77	54,46	1	9.3	5.6 - 10[c]
Hexanol	SLDI	35,65	1	290	246 - 345
			2	290	247 - 327

A: Reference substance component. B: Test Substance component.

TR: Test replicate. *Values to 2 s.f.. # Nominal % component in mixture.

[a] Values to 3 s.f.. [b] Values based on nominal concentrations.

[c] EC50 value calculated from non-linear interpolation between presented limits.

Table 83. Maximum and minimum EC50 values for FAES substances as a percentage of the mean

Substance	No. of Test Replicates	Max. / Min. EC50 values (± % of mean)*
C12 methyl	2	19
C12 butyl	2	5.5
C12 sec butyl	2	12
C12 amyl	2	6.3
C14 methyl	3	32 ,1.2, 33[a]
C14 ethyl	2	1.2
C14 iso propyl	2	11
C14 butyl	2	1.4
C14 amyl	2	20
C16 methyl	2	14

* Values to 2 s.f..

[a] Max., Median, Min. respectively.

Table 84. Maximum and minimum EC50 values for FAES and LAS substances as a percentage of the mean

Substance	No. of Test Replicates	Max. / Min. EC50 values (± % of mean)*
C7 heptyl	2	2.9
C8 hexyl	2	4.6
C8 sec hexyl	2	1.3
C9 amyl	2	8.6
C9 sec amyl	2	2.1
C10 butyl	2	13
C10 sec butyl	1	-
C10 iso butyl	1	-
C11 propyl	2	9.5
C12 ethyl	2	5.9
C13 methyl	2	6.8
C9 LAS	2	12
C10 LAS	2	1.8
C11 LAS	2	0.0
C12 LAS	2	28
C13 LAS	2	9.4
C14 LAS	2	5.3

* Values to 2 s.f..

Table 85. Maximum and minimum EC50 values for reference substances as a percentage of the mean

Substance	No. of Test Replicates (n)	Max. / Min. EC50 Value (± % of mean)*
Phenol	4	9.5, 8.1, 5.4, 14
2-chlorophenol	2	28[a]
LAS	2	18
1-Pentanol	2	1.0
1-Hexanol	2	3.2
1-Octanol	2	9.8
1-Nonanol	2	2.7
1-Decanol	2	2.8

* Values to 2 s.f.

[a] Replicate and mean value corrected for measured test solution concentrations (Table 93, Appendix I).

Table 86. Maximum and minimum EC50 values for 2,4,6-trichlorophenol and p-cresol as a percentage of the mean

Substance	No. of Test Replicates (n)	Max. / Min. EC50 Value (± % of mean)*
2,4,6-trichlorophenol	2	28[a]
p-cresol	2	33[a]

* Values to 2 s.f.

[a] Replicate and mean value corrected for measured test solution concentrations (Table 93, Appendix I).

Table 87. Maximum and minimum total TU values at the EC50 as a percentage of the mean for mixtures of FAES with reference substances LAS and 1-decanol

Mixture		% #	No. of Test Replicates	Max. / Min. total TU (± % of mean)*
A	B	(A,B)		
LAS	C13 methyl	30,70	1	-
LAS	C13 methyl	20,80	2	20
LAS	C13 methyl	10,90	1	-
LAS	C12 methyl	20,80	1	-
LAS	C12 ethyl	30,70	2	24
LAS	C7 heptyl	30,70	2	13
LAS	C10 n-butyl	30,70	1	-
LAS	C10 n-butyl	20,80	2	8.3
LAS	C10 n-butyl	10,90	1	-
LAS	C8 hexyl	20,80	2	13
1-decanol	C13 methyl	20,80	2	18
1-decanol	C12 ethyl	20,80	2	7.4
1-decanol	C7 heptyl	20,80	2	20
1-decanol	C8 hexyl	20,80	1	-

A: Reference substance, B: Test substance (FAES).

* Values to 2 s.f..

Nominal % component in mixture.

Table 88. Maximum and minimum total TU values at the EC50 as a percentage of the mean for equitoxic mixtures

Mixture		% #	No. of Test Replicates	Max. / Min. total TU (± % of mean)*[a]
A	B	(A,B)		
Phenol	C14 methyl	46,54	2	9.3[a]
Phenol	C12 amyl	51,49	2	23[a]
Phenol	C14 ethyl	48,52	2	17[a]
Phenol	C12 n-butyl	32,68	2	7.1[a]
Phenol	C11.8 LAS	56,44	2	3.7[a]
2-CP	C14 n-butyl	47,53	2	1.5[a]
Pentanol	C8 sec hexyl	50,50	2	9.7
Hexanol	C12 ethyl	50,50	2	5.0
Hexanol	C10 butyl	43,57	2	9.7
Hexanol	C8 hexyl	41,59	2	0
Hexanol	C7 heptyl	50,50	2	19
Octanol	C12 butyl	60,40	2	5.3
Octanol	C13 methyl	36,64	2	5.9
Nonanol	C12 amyl	51,49	2	7.3
Nonanol	C14 methyl	47,53	2	2.1
Nonanol	C11.8 LAS	57,43	2	2.9
Nonanol	Phenol	51,49	2	3.0[a]
Hexanol	C7 heptyl	73,27	1	-
Hexanol	C7 heptyl	23,77	2	15
Octanol	C12 butyl	50,50	1	-
Octanol	C13 methyl	16,84	2	7.0
Octanol	C13 methyl	50,50	2	3.4

A: Reference substance, B: Test substance.

*Values to 2 s.f.. # Nominal % component in mixture.

[a] Values based on measured concentrations.

Table 89. Maximum and minimum EC50 values as a percentage of the mean for substances used in chapter 4

Substance	TH	No. of Test Replicates	Max. / Min. EC50 values (± % of mean)*
DLAS	13	1	-
SLDI		1	-
Geropon T77		1	-
SMMA		2	3.0
SEMA		1	-
DSDS		1	-
C9 LAS		2	18
C10 LAS		1	-
C11 LAS		1	-
C12 LAS		1	-
C13 LAS		1	-
C14 LAS		2	9.5
C11.8 LAS		2	14
C14 amyl FAES		2	5.3
C10 sec-butyl FAES		2	4.3
C12 n-butyl FAES		1	-
DLAS	240	1	-
Geropon T77		2	7.2
DSDS		2	2.8

* Values to 2 s.f..

Table 90. Maximum and minimum total TU values at the EC50 as a percentage of the mean for mixture studies in chapter 4

Mixture		% #	No. of Test Replicates	Max. / Min. total TU (± % of mean)*[a]
A	B	(A,B)		
Phenol	DLAS	58,42	1	-
Phenol	DSDS	59,41	2	3.7
Phenol	Geropon T77	54,46	1	-
Hexanol	SLDI	35,65	2	0.0

A: Reference substance, B: Test substance.

*Values to 2 s.f.. # Nominal % component in mixture.

[a] Values based on measured concentrations.

Table 91. Measured concentrations of FAES substances as mean percent of nominal

Substance	TR	n*	Measured Concentrations (mean % of nominal)	95% Confidence Interval[a]
C12 methyl FAES	1	6	103	101 - 105
	2	4	106	102 - 109
C12 butyl FAES	1	6	108	106 - 109
	2	6	106	102 - 109
C12 sec butyl FAES	1	7	108	105 - 111
	2	6	107	104 - 110
C12 amyl FAES	1	8	106	103 - 109
	2	8	102	99.2 - 105
C14 methyl FAES	1	8	100	96.4 - 103
	2	8	101	97.5 - 104
	3	8	98	94.6 - 102
C14 ethyl FAES	1	8	106	103 - 109
	2	8	105	101 - 108
C14 iso propyl FAES	1	8	98	84.2 - 112
	2	8	97	83.7 - 111
C14 butyl FAES	1	8	98	90.1 - 107
	2	6	96	91.0 - 102
C14 amyl FAES	1	7	93	79.3 - 107
	2	8	89	78.5 - 99.3
C16 methyl FAES	1	4	87	76.6 - 97.2
	2	8	83	70.1 - 96.7

* Number of measured values used in % of nominal calculation. n excludes four measured control samples which were performed for every test analysed.

[a] Values to 3 s.f.

Table 92. Measured concentrations by MBAS of FAES and LAS substances as mean percent of nominal

Substance	TR	n*	Measured Concentrations (mean % of nominal)	95% Confidence Interval[a]
C7 heptyl FAES	1	8	95	93.2 - 97.8
C8 hexyl FAES	1	8	96	93.5 - 98.6
C8 sec hexyl FAES	1	3	101	90.7 - 110
	2	3	92	84.7 - 98.9
C9 amyl FAES	1	8	95	91.5 - 98.8
C9 sec amyl FAES	1	8	95	92.7 - 97.3
C10 butyl FAES	1	8	99	97.8 - 100
C10 sec butyl FAES	1	8	96	93.9 - 99.0
C11 propyl FAES	1	6	92	85.3 - 97.8
C13 methyl FAES	1	8	103	99.7 - 107
C9 LAS	1	8	90	86.7 - 93.3
C14 LAS	1	7	71	61.2 - 80.1

* Number of measured values used in % of nominal calculation. n excludes four measured control samples which were performed for every test analysed.

[a] Values to 3 s.f.

Table 93. Measured concentrations by GC-MS of phenol reference substances as mean percent of nominal

Substance	TR	n*	Measured Concentrations (mean % of nominal)	95% Confidence Interval[a]
Phenol	2	8	91	80.2 - 101
Phenol	3	8	83	53.8 - 113
2-chlorophenol	1	8	44	40.1 - 48.5
p-cresol	2	8	67	58.0 - 76.0
2,4,6-trichlorophenol	1	8	76	50.2 - 102
2,4,6-trichlorophenol	2	8	52	40.1 - 64.4

* Number of measured values used in % of nominal calculation. n excludes four measured control samples which were performed for every test analysed.

[a] Values to 3 s.f.

Table 94. Measured concentrations by GC-MS of the phenol and 1-nonanol components in nominally equitoxic mixture studies, as mean percent of nominal.

Mixture		Test Replicate	n*	Measured Phenol Concentration (mean % of nominal)#	95% Confidence Interval[a]
A	B				
Phenol	C14 methyl	1	8	70	37.0 - 104
		2	8	86	59.6 - 112
Phenol	C12 amyl	1	8	45	30.9 - 58.3
		2	8	74	51.7 - 96.9
Phenol	C14 ethyl	1	8	55	32.3 - 77.8
		2	8	41	27.4 - 55.1
Phenol	C12 n-butyl	1	8	110	95.0 - 116
		2	8	91	80.1 - 101
Phenol	LAS	1	8	92	79.9 - 104
		2	-	-	-
2-CP	C14 n-butyl	1	8	51	39.5- 62.1
		2	8	78	63.3 - 92.6
Phenol	1-nonanol	1	8	89	79.0 - 99.9
		2	8	66	47.4 - 84.1
1-nonanol	Phenol	1	8	47	23.4 - 70.9
		2	-	-	-

A: Measured component. B: Second component.

*Number of samples - excludes four control samples which were performed for every mixture study analysed.

Values to 2 s.f.. [a] Values to 3 s.f..

Table 95. Measured concentrations by MBAS of the FAES and LAS component in nominally equitoxic mixture studies with phenol and 2-chlorophenol, as mean percent of nominal

Mixture		Test Replicate	n*	Measured Surfactant Concentration (mean % of nominal)#	95% Confidence Interval[a]
A	B				
C14 methyl	Phenol	1	8	92[b]	86.1 - 98.8
		2	-	-	-
C12 amyl	Phenol	1	8	82	78.3 - 85.2
		2	8	98	87.6 - 109
C14 ethyl	Phenol	1	8	110	105 - 110
		2	8	100	96.6 - 102
C12 butyl	Phenol	1	8	82	77.7 - 87.1
		2	8	99	93.8 - 104
C11.8 LAS	Phenol	1	8	93	89.2 - 97.3
		2	-	-	-
C14 butyl	2-CP	1	8	80	73.2 - 85.3
		2	-	-	-

A: Measured component, B: Second component.

*Number of samples - excludes four control samples which were performed for every mixture analysed as no FAES found except where denoted.

Values to 2 s.f., [a] Values to 3 s.f., [b] Some control sample contamination noted.

Table 96. Measured concentrations by MBAS of substances used in chapter 4 as mean percent of nominal

Substance	TR	n*	TH (mg/l as $CaCO_3$)	Measured Concentrations (mean % of nominal)	95% Confidence Interval[a]
Geropon T77	2	8	240	90	81.5 - 98.3
DSDS	1	6	240	65	56.7 - 73.8
C11.8 LAS	2	3	13	113	109 - 117
C14 amyl FAES	1	8	13	90	85.1 - 95.8
C9 LAS	1	8	13	84	77.2 - 89.8
C14 LAS	1	4	13	83	75.1 - 90.3

* Number of measured values used in % of nominal calculation. n excludes four measured control samples which were performed for every test analysed.

[a] Values to 3 s.f.

Table 97. Measured concentrations by GC-MS of the phenol component in nominally equitoxic mixture studies with substances from chapter 4 as mean percent of nominal

Mixture		TR	TH (mg/l as $CaCO_3$	n*	Measured Phenol Concentration (mean % of nominal)#	95% Confidence Interval[a]
A	B					
Phenol	DLAS	1	240	8	85	75.3 - 93.8
Phenol	Geropon T77	1	240	8	86	79.1 - 93.1
Phenol	DSDS	1	240	8	93	88.5 - 98.4
		2	240	8	95	90.4 - 99.7

Table 98. Measured concentrations by MBAS of the surfactant component in nominally equitoxic mixture studies with phenol as mean percent of nominal

Mixture		TR	TH (mg/l as $CaCO_3$	n*	Measured Surfactant Concentration (mean % of nominal)#	95% Confidence Interval[a]
A	B					
DLAS	Phenol	1	240	7	82	78.6 - 86.0
Geropon T77	Phenol	1	240	8	84	75.1 - 93.6
DSDS	Phenol	2	240	8	58	54.0 - 62.1

A: Measured component, B: Second component.

*Number of samples - excludes four control samples which were performed for every mixture analysed.

Values to 2 s.f., [a] Values to 3 s.f..

Table 99. Measured phenol concentrations stored with no formalin in the light and dark by GC-MS for 6 week phenol stability study 1

Sample*	Measured concentration of phenol (mg/l) at time t (days)#						
	t = 0	t + 7	t + 15	t + 22	t + 28	t + 35	t + 43
0.0 L1	0.0	0.0	0.0	0.0	0.04[a]	0.0	0.0
0.0 L2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0 D1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0 D2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.8 L1	0.98	0.0	0.0	0.0	0.0	0.0	0.0
1.8 L2	1.0	0.0	0.0	0.0	0.0	0.0	0.0
1.8 D1	1.1	0.0	0.0	0.0	0.0	0.0	0.0
1.8 D2	-	0.0	0.0	0.0	0.0	0.0	0.0
18.0 L1	10	10	1.0	2.9	7.1	12	4.3
18.0 L2	9.2	10	0.99	2.8	7.2	12	4.3
18.0 D1	7.2	11	0.94	2.7	6.3	10	3.1
18.0 D2	9.6	10	0.96	2.7	6.3	11	3.6

Values to 2 s.f.

* Approximate nominal concentration phenol (mg/l)

L1, L2 Sample stored in light, vessel 1,2

D1,D2 Sample stored in dark, vessel 1,2

[a] Spurious value

Table 100. Measured phenol concentrations stored with 3% formalin in the light and dark by GC-MS for 6 week phenol stability study 1

Sample*	Measured concentration of phenol (mg/l) at time t (days)#						
	t = 0	t + 7	t + 15	t + 22	t + 28	t + 35	t + 43
0.0 L1	0.0	0.0	0.0	0.0	0.04[a]	0.0	0.0
0.0 L2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0 D1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0 D2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.8 L1	1.2	0.97	0.0	0.0	1.0	1.2	0.56
1.8 L2	1.0	0.96	0.0	0.0	0.97	1.2	0.55
1.8 D1	1.2	0.97	0.0	0.0	8.7	1.4	0.52
1.8 D2	1.1	1.0	0.0	0.0	8.7	1.3	0.51
18.0 L1	11	12	1.0	2.9	8.2	14	4.8
18.0 L2	9.1	12	1.0	2.9	8.2	14	4.8
18.0 D1	9.3	12	1.1	3.0	8.1	15	4.7
18.0 D2	9.3	13	1.2	3.2	8.1	15	4.8

Values to 2 s.f.

* Approximate nominal concentration phenol (mg/l)

L1, L2 Sample stored in light, vessel 1,2

D1,D2 Sample stored in dark, vessel 1,2

Table 101. Measured phenol concentrations stored with no added formalin in the light and dark by GC-MS for 8 day phenol preservation study 2

Sample*	Measured concentration of phenol (mg/l) at time t (days)#					
	t = 0	t + 1	t + 2	t + 3	t + 4**	t + 8
0.0 L1	0	0	0	0	0	0.09[a]
0.0 L2	0	0	0	0	0	0
0.0 D1	0	0	0	0	0	0
0.0 D2	0	0	0	0	0	0
1.8 L1	1.3	1.2	0.83	0.80	0.53	0
1.8 L2	1.4	1.2	1.1	1.0	0.92	0.72
1.8 D1	1.4	1.2	0.98	0.23[a]	0.88	0.72
1.8 D2	1.3	1.3	0.61	1.0[a]	0	0
5.6 L1	4.1	4.0	3.4	3.6	3.1	2.7
5.6 L2	4.1	4.0	3.4	3.2	2.4	1.6
5.6 D1	4.1	3.9	3.1	2.9	2.4	1.4
5.6 D2	4.1	3.9	3.1	2.8	2.3	1.4
18.0 L1	13	13	12	13	11	9.8
18.0 L2	13	13	12	13	11	9.8
18.0 D1	13	14	12	12	11	8.9
18.0 D2	13	14	12	12	11	8.9

Values to 2 s.f.

* Approximate nominal concentration phenol (mg/l)

** Quantisation based on average instead of daily calibration

L1, L2 Sample stored in light, vessel 1,2

D1,D2 Sample stored in dark, vessel 1,2

[a] Spurious value

Table 102. Measured phenol concentrations stored with 3% formalin in the light and dark by GC-MS for 8 day phenol preservation study 2

Sample*	Measured concentration of phenol (mg/l) at time t (days)#					
	t = 0	t + 1	t + 2	t + 3	t + 4	t + 8
0.0 L1	0	0	0	0	0	0
0.0 L2	0	0	0	0	0	0
0.0 D1	0	0	0	0	0	0
0.0 D2	0	0	0	0	0	0
1.8 L1	1.4	1.3	1.1	1.3	1.1	1.1
1.8 L2	1.4	1.2	1.0	1.3	1.1	1.1
1.8 D1	1.3	1.3	1.2	1.3	1.1	1.1
1.8 D2	1.3	1.3	1.2	1.3	1.1	1.1
5.6 L1	4.0	3.9	3.7	4.0	3.5	3.3
5.6 L2	4.0	4.0	3.7	4.0	3.6	3.4
5.6 D1	3.9	3.9	3.7	4.0	3.6	3.1
5.6 D2	4.1	4.0	3.8	4.1	3.6	3.3
18.0 L1	13	14	12	14	12	11
18.0 L2	13	14	12	14	12	11
18.0 D1	12	14	12	13	13	11
18.0 D2	13	0.15[a]	12	14	13	11

Values to 2 s.f.

* Approximate nominal concentration phenol (mg/l).

L1, L2 Sample stored in light, vessel 1,2.

D1,D2 Sample stored in dark, vessel 1,2.

[a] Spurious value.

Table 103. Maximum and minimum phenol concentrations stored with 3% formalin as ±% of the mean for 6 week phenol stability study 1

Sample*	Max./Min. phenol concentration values (±% of mean)# [a]						
	t = 0	t + 7	t + 15	t + 22	t + 28	t + 35	t + 43
0.0 L	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0 D	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.8 L	9.1	0.5	0.0	0.0	1.5	0.0	0.9
1.8 D	4.3	1.5	0.0	0.0	0.0	3.7	1.0
18.0 L	9.5	0.0	0.0	0.0	0.0	0.0	0.0
18.0 D	0.0	4.0	4.3	3.2	0.0	0.0	1.1

Table104. Maximum and minimum phenol concentrations stored without formalin as a ±% of the mean for 6 week phenol stability study 1

Sample*	Max./Min. phenol concentration values (±% of mean)# [a]						
	t = 0	t + 7	t + 15	t + 22	t + 28	t + 35	t + 43
0.0 L	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0 D	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.8 L	1.0	0.0	0.0	0.0	0.0	0.0	0.0
1.8 D	-	0.0	0.0	0.0	0.0	0.0	0.0
18.0 L	4.2	0.0	0.5	1.8	0.7	0.0	0.0
18.0 D	14	4.8	1.1	0.0	0.0	4.8	7.5

[a]Sample size n=2.

Values to 2 s.f.

* Approximate nominal concentration phenol (mg/l)

Table 105. Maximum and minimum phenol concentrations stored with 3% formalin as ±% of the mean for 8 day phenol stability study 2

Sample*	Max./Min. phenol concentration values (±% of mean)#[a]					
	t = 0	t + 1	t + 2	t + 3	t + 4	t + 8
0.0 L	0.0	0.0	0.0	0.0	0.0	0.0
0.0 D	0.0	0.0	0.0	0.0	0.0	0.0
1.8 L	0.0	4.0	4.8	0.0	0.0	0.0
1.8 D	0.0	0.0	0.0	0.0	0.0	0.0
5.6 L	0.0	1.3	0.0	0.0	1.4	1.5
5.6 D	2.5	1.3	1.3	1.2	0.0	3.1
18.0 L	0.0	0.0	0.0	0.0	0.0	0.0
18.0 D	4.0	-	0.0	3.7	0.0	0.0

Table106. Maximum and minimum phenol concentrations stored without formalin as a ±% of the mean for 8 day phenol stability study 2

Sample*	Max./Min. phenol concentration values (±% of mean)#[a]					
	t = 0	t + 1	t + 2	t + 3	t + 4	t + 8
0.0 L	0.0	0.0	0.0	0.0	0.0	0.0
0.0 D	0.0	0.0	0.0	0.0	0.0	0.0
1.8 L	3.7	0.0	14	11	27	100
1.8 D	3.7	4.0	23	-	100	100
5.6 L	0.0	0.0	0.0	5.9	13	26
5.6 D	0.0	0.0	0.0	1.8	2.1	0.0
18.0 L	0.0	0.0	0.0	0.0	0.0	0.0
18.0 D	0.0	0.0	0.0	0.0	0.0	0.0

[a]Sample size n=2.

Values to 2 s.f.

* Approximate nominal concentration phenol (mg/l)

APPENDIX II

Water quality data: temperature, dissolved oxygen, pH and total hardness ranges for all studies.

ΔT (°C): refers to largest sudden temperature change to which neonates were subjected during the test period on transfer from culture vessels etc..

Table 107. Preparation of M7 Elendt Medium

Stock	Conc. of Stock ($x10^3$ mg/l)*	Concentration in medium (mg/l)#
H_3BO_4	5.72	0.72
$MnCl_2.4H_2O$	7.21	0.091
LiCl	6.12	0.077
RbCl	1.42	0.018
$SrCl_2.6H_2O$	3.04	0.038
NaBr	0.32	4.0×10^{-3}
Na_2MoO_4	1.26	0.016
$CuCl_2.2H_2O$	0.340	4.2×10^{-3}
$ZnCl_2$	0.260	0.013
$CoCl_2.6H_2O$	0.200	0.010
KI	0.0650	3.3×10^{-3}
Na_2SeO_3	0.0440	2.2×10^{-3}
NH_4VO_3	0.0120	6.0×10^{-3}
$CaCl_2.2H_2O$	294	290
$MgSO_4.7H_2O$	247	120
KCl	58.0	5.8
$NaHCO_3$	64.8	65
$Na_2SiO_3.5H_2O$	3.73	7.5
$NaNO_3$	2.74	0.27
KH_2PO_4	1.43	0.14
K_2HPO_4	1.84	0.18
$FeSO_4.7H_2O$	1.99	0.50
$Na_2EDTA.2H_2O$	5.00	1.3
Thiamine Hydrochloride	0.750	0.075
Cyanocobalamine	0.0100	0.0010
d - Biotin	0.00750	0.00075

* Values to 3 s.f.. # Values to 2 s.f..

Table 108. Test solution quality data ranges over 48 hour test period for FAES substances

Substance	Test Replicate	Dissolved Oxygen (mg/l)	Total Hardness (mg/l as $CaCO_3$)
C12 methyl	1	7.2 - 8.7	242 - 259
	2	7.0 - 8.8	238 - 245
C12 butyl	1	7.5 - 9.1	242 - 255
	2	7.5 - 8.7	230 - 249
C12 sec butyl	1	7.4 - 8.3	233 - 255
	2	6.9 - 8.7	251 - 258
C12 amyl	1	7.1 - 7.9	242 - 253
	2	7.4 - 9.0	234 - 251
C14 methyl	1	7.5 - 8.7	232 - 251
	2	7.4 - 8.9	230 - 248
	3	7.4 - 8.6	233 -249
C14 ethyl	1	7.8 - 9.0	232 - 252
	2	7.4 - 8.9	236 - 254
C14 iso propyl	1	7.7 -8.3	234 - 257
	2	7.5 - 8.5	238 - 260
C14 butyl	1	7.3 - 9.0	245 - 257
	2	7.0 - 9.2	230 - 255
C14 amyl	1	6.9 - 7.9	242 - 258
	2	7.4 - 9.3	232 - 251
C16 methyl	1	7.8 - 8.8	220 - 246
	2	8.1 - 9.6	232 - 256

Table 109. Test solution quality data ranges over 48 hour test period for FAES substances

Substance	Test Replicate	Temperature Range (°C)	ΔT (°C)	pH
C12 methyl	1	20.5 - 21.0	0.5	7.8 - 8.1
	2	20.0 - 21.0	1.0	7.5 - 8.0
C12 butyl	1	20.0 - 20.5	0.5	7.5 - 8.0
	2	20.0 - 21.0	1.0	7.5 - 7.9
C12 sec butyl	1	20.5 - 22.0	1.0	7.5 - 7.9
	2	19.5 - 22.0	2.5	7.0 - 7.5
C12 amyl	1	20.5 - 23.0	1.0	7.5 - 7.8
	2	20.0 - 20.5	0.5	7.5 - 7.8
C14 methyl	1	21.0 - 22.0	2.0	7.7 -8.1
	2	20.0 - 21.0	1.0	7.8 - 8.2
	3	20.0 - 21.0	1.0	7.7 - 8.0
C14 ethyl	1	21.0 - 22.0	0.5	7.7 - 7.9
	2	20.0 - 21.5	1.0	7.7 - 7.9
C14 iso propyl	1	19.5 - 22.0	1.5	7.5 - 7.7
	2	20.5 - 22.0	1.0	7.2 - 7.5
C14 butyl	1	19.5 - 21.0	1.0	7.7 - 8.0
	2	20.0 - 20.5	0.5	7.8 - 8.0
C14 amyl	1	20.0 - 21.5	1.0	7.1 - 7.9
	2	19.5 - 21.0	0.5	7.5 -7.8
C16 methyl	1	19.0 - 21.0	0.5	8.0 - 8.3
	2	20.5 - 21.5	1.0	7.6 - 8.1

Table 110. Test solution quality data ranges over 48 hour test period for FAES substances

Substance	Test Replicate	Dissolved Oxygen (mg/l)	Total Hardness (mg/l as $CaCO_3$)
C7 heptyl	1	8.6 - 9.4	227 - 250
	2	8.3 - 8.9	236 - 247
C8 hexyl	1	7.7 - 9.3	225 - 248
	2	8.0 - 9.9	233 -255
C8 sec hexyl	1	7.8 - 9.9	245 - 250
	2	7.2 - 8.8	247 - 255
C9 amyl	1	7.4 - 8.3	236 - 250
	2	7.0 - 8.6	230 - 252
C9 sec amyl	1	8.0 - 9.0	242 -250
	2	8.0 -10.2	213 - 253
C10 butyl	1	7.6 -10.0	243 - 257
	2	8.5 - 9.3	228 - 249
C10 sec butyl	1	7.8 - 9.6	251 - 270
C10 iso butyl	1	8.3 - 9.5	255 - 280
C11 propyl	1	8.4 - 9.0	230 - 254
	2	8.2 - 9.7	248 - 265
C12 ethyl	1	8.4 - 9.2	240 - 253
	2	7.6 - 8.8	238 - 250
C13 methyl	1	7.4 - 9.1	247 - 255
	2	7.6 - 9.1	230 - 255

Table 111. Test solution quality data ranges over 48 hour test period for FAES substances

Substance	Test Replicate	Temperature Range (°C)	ΔT (°C)	pH
C7 heptyl	1	19.0 - 21.5	1.0	6.9 - 7.5
	2	20.0 - 21.0	1.0	6.9 - 7.2
C8 hexyl	1	19.5 - 21.0	1.0	6.8 - 7.5
	2	18.5 - 20.5	1.5	7.0 - 7.5
C8 sec hexyl	1	18.5 - 20.0	0.5	6.9 - 7.5
	2	18.5 - 20.0	0.5	7.1 - 7.5
C9 amyl	1	18.5 - 20.0	1.0	7.2 - 7.6
	2	18.5 - 20.0	1.5	7.0 - 7.6
C9 sec amyl	1	18.5 - 20.0	1.5	5.8[a] - 7.6
	2	18.5 - 20.0	1.5	5.0[a] - 7.7
C10 butyl	1	18.5 - 20.0	1.5	6.8 - 7.8
	2	19.0 - 20.5	1.0	6.7 - 8.1
C10 sec butyl	1	18.5 - 20.5	1.0	6.9 - 8.0
C10 iso butyl	1	18.5 - 20.0	1.5	4.5[a] - 7.7
C11 propyl	1	19.5 - 20.0	0.5	3.8[a] - 7.8
	2	19.5 - 20.5	1.0	6.2 - 7.8
C12 ethyl	1	18.5 - 21.0	0.5	6.7 - 7.6
	2	19.0 - 20.5	1.0	6.1 - 7.6
C13 methyl	1	19.5 - 20.5	1.0	6.7 - 7.6
	2	19.5 - 20.5	1.0	6.8 - 7.6

[a] Low pH observed in highest concentration only. Test not invalidated as 100% mortality observed in at least adjacent lower concentration where pH resumes acceptable level and mortality can be assumed to be as a result of test compound only.

Table 112. Test solution quality data ranges over 48 hour test period for LAS substances

Substance	Test Replicate	Dissolved Oxygen (mg/l)	Total Hardness (mg/l as $CaCO_3$)
C9	1	8.0 – 8.6	236 – 246
	2	7.7 – 8.2	237 – 250
C10	1	7.9 – 8.2	233 – 249
	2	7.8 – 8.3	236 - 240
C11	1	7.8 – 8.4	240 – 248
	2	7.7 – 8.1	230 - 238
C12	1	8.0 – 8.6	235 – 241
	2	7.9 – 8.2	238 - 242
C13	1	8.2 – 8.4	240 – 248
	2	7.8 – 8.0	228 - 236
C14	1	7.8 – 8.2	238 – 244
	2	8.0 – 8.4	238 - 245

Table 113. Test solution quality data ranges over 48 hour test period for LAS substances

Substance	TR	Temperature Range (°C)	ΔT (°C)	pH
C9	1	17.0 – 18.0	1.0	7.4 – 8.4
	2	17.0 – 18.5	1.0	7.5 – 7.8
C10	1	17.0 – 18.5	1.5	7.5 – 7.8
	2	18.0 – 20.0	1.0	7.5 – 7.9
C11	1	16.5 – 18.0	1.5	7.4 – 7.7
	2	18.5 – 20.0	1.0	7.5 – 7.9
C12	1	16.5 – 19.0	0.5	7.4 – 8.3
	2	17.5 – 19.5	1.5	7.5 – 7.9
C13	1	17.0 – 18.5	1.0	7.5 – 7.8
	2	18.0 – 20.0	1.5	7.5 – 7.9
C14	1	18.0 – 19.0	1.0	7.4 – 7.7
	2	17.0 – 18.5	1.5	7.5 – 7.9

Table 114. Test solution quality data ranges over 48 hour test period for reference substances tested individually

Substance	Test Replicate	Dissolved Oxygen (mg/l)	Total Hardness (mg/l as $CaCO_3$)
phenol	1	7.6 – 8.1	236 – 245
	2	7.5 – 8.2	228 – 240
	3	7.3 – 7.7	233 – 246
	4	7.0 – 7.8	228 – 248
2-chlorophenol	1	7.4 – 7.7	240 – 253
	2	7.5 – 8.1	242 – 250
p-cresol	1	7.5 – 8.2	223 – 248
	2	7.2 – 7.7	233 – 245
2,4,6-trichlorophenol	1	7.0 – 7.7	240 – 248
	2	7.1 – 7.6	244 - 249
	3	7.0 – 8.0	240 - 248
C11.8 LAS	1	6.8 – 7.8	236 – 249
	2	7.1 – 8.3	242 – 250
1-decanol	1	7.7 – 8.6	204 – 249
	2	7.3 – 8.6	210 – 247
1-pentanol	1	7.2 – 8.3	235 – 247
	2	7.2 – 8.1	238 - 246
1-hexanol	1	7.9 – 7.9	224 – 234
	2	7.6 – 8.5	224 – 237
1-octanol	1	7.5 – 8.7	217 – 233
	2	8.0 – 8.9	228 – 245
1-nonanol	1	7.4 – 9.2	228 – 252
	2	7.7 – 8.2	228 - 240

Table 115. Test solution quality data ranges over 48 hour test period for reference substances tested individually

Substance	Test Replicate	Temperature Range (°C)	ΔT (°C)	pH
phenol	1	20.0 – 20.5	0.5	7.3 – 8.0
	2	19.0 – 20.0	0.5	7.3 – 8.0
	3	17.5 – 19.5	1.5	6.9 – 7.3
	4	17.0 – 19.5	0.5	6.9 – 7.2
2-chlorophenol	1	19.5 – 20.5	1.0	7.1 – 7.4
	2	17.5 – 19.5	1.5	7.0 – 7.5
p-cresol	1	18.0 – 20.0	1.0	7.3 – 7.9
	2	17.5 – 20.0	1.0	6.9 – 7.4
2,4,6-trichlorophenol	1	19.0 – 20.5	1.0	7.1 – 7.6
	2	19.0 – 20.5	1.5	6.7 – 7.3
	3	16.0 – 18.5	1.0	6.6 – 7.5
C11.8 LAS	1	19.5 – 21.0	1.5	7.2 – 7.7
	2	20.0 – 21.0	0.5	7.3 – 7.8
1-decanol	1	18.0 – 19.5	1.0	7.2 – 7.5
	2	19.0 – 20.0	0.5	7.1 – 7.6
1-pentanol	1	20.0 – 20.5	0.5	7.3 – 7.9
	2	19.0 – 20.5	0.5	7.2 – 7.8
1-hexanol	1	19.0 – 20.0	0.5	7.2 – 7.6
	2	19.5 – 20.0	0.5	7.1 – 7.7
1-octanol	1	18.0 – 19.5	1.0	7.2 – 7.5
	2	18.5 – 20.0	0.5	7.2 – 7.6
1-nonanol	1	18.5 – 20.5	2.0	7.2 – 7.8
	2	19.0 – 20.5	2.0	7.1 – 7.6

Table 116. Test solution quality data ranges over 48 hour test period for mixture studies with LAS / 1-decanol

Mixture		%# (A,B)	TR	Dissolved Oxygen (mg/l)	Total Hardness (mg/l as $CaCO_3$)
A	B				
LAS	C13 methyl	30, 70	1	7.6 – 8.7	232 – 247
LAS	C13 methyl	20, 80	1	7.3 – 8.6	218 – 234
			2	7.7 – 8.3	228 – 246
LAS	C13 methyl	10, 90	1	7.8 – 8.3	234 – 247
LAS	C12 methyl	20, 80	1	6.4 – 8.1	220 – 237
LAS	C12 ethyl	30, 70	1	7.1 – 8.8	218 – 239
			2	7.3 – 8.0	220 – 236
LAS	C7 heptyl	30, 70	1	7.3 – 8.3	228 – 236
			2	7.9 – 8.3	225 – 236
LAS	C10 n-butyl	30, 70	1	7.6 – 8.4	222 – 245
LAS	C10 n-butyl	20, 80	1	7.2 – 9.0	224 – 245
			2	7.8 – 8.2	222 – 239
LAS	C10 n-butyl	10, 90	1	7.7 – 8.3	232 – 245
LAS	C8 hexyl	20, 80	1	7.5 – 8.2	224 – 242
			2	7.5 – 8.0	219 – 244
1-decanol	C13 methyl	20, 80	1	7.4 – 8.7	223 – 246
			2	7.7 – 8.7	224 – 246
1-decanol	C12 ethyl	20, 80	1	7.6 – 8.2	218 – 245
			2	8.0 – 8.7	218 – 242
1-decanol	C7 heptyl	20, 80	1	7.5 – 8.4	226 – 251
			2	7.7 – 8.4	222 – 255
1-decanol	C8 hexyl	20, 80	1	7.7 – 8.3	224 - 256

A: Reference substance component. B: Test substance component (FAES).

Table 117. Test solution quality data ranges over 48 hour test period for mixture studies with LAS / 1-decanol

Mixture		%# (A,B)	TR	Temperature Range (°C)	ΔT (°C)	pH
A	B					
LAS	C13 methyl	30, 70	1	20.0 – 20.5	0.5	7.5 – 8.0
LAS	C13 methyl	20, 80	1	19.5 – 20.5	0.5	7.2 – 7.5
			2	20.0 – 20.5	0.5	7.3 – 7.7
LAS	C13 methyl	10, 90	1	19.5 – 20.5	0.5	7.4 – 7.7
LAS	C12 methyl	20, 80	1	19.5 – 20.5	0.5	7.2 – 7.5
LAS	C12 ethyl	30,70	1	19.5 – 20.5	1.0	7.0 – 7.6
			2	19.5 – 20.5	0.5	7.2 – 7.8
LAS	C7 heptyl	30, 70	1	19.5 – 20.5	1.0	7.2 – 7.5
			2	20.0 – 20.5	0.5	7.5 – 7.7
LAS	C10 n-butyl	30, 70	1	19.5 – 20.0	0.5	7.4 – 7.8
LAS	C10 n-butyl	20, 80	1	20.0 – 21.0	1.0	7.3 – 8.0
			2	19.5 – 20.5	0.5	7.5 – 7.7
LAS	C10 n-butyl	10, 90	1	19.5 – 20.5	1.5	7.4 – 7.6
LAS	C8 hexyl	20, 80	1	20.0 – 21.0	0.5	7.5 – 7.8
			2	19.5 – 20.5	1.0	7.5 – 7.8
1-decanol	C13 methyl	20, 80	1	19.0 – 19.5	0.5	6.7 – 7.2
			2	19.0 – 19.5	0.0	6.7 – 7.2
1-decanol	C12 ethyl	20, 80	1	19.0 – 19.5	0.5	6.9 – 7.4
			2	19.0 – 20.0	0.5	6.5 – 7.1
1-decanol	C7 heptyl	20, 80	1	19.0 – 20.0	0.5	7.3 – 7.5
			2	19.5 – 20.0	0.5	7.3 – 7.5
1-decanol	C8 hexyl	20, 80	1	19.0 – 20.0	1.0	7.3 – 7.5

A: Reference substance component. B: Test substance component (FAES).

Table 118. Test solution quality data ranges over 48 hour test period for nominally equitoxic mixture toxicity studies where concentration addition predicted.

Mixture		TR	Dissolved Oxygen (mg/l)	Total Hardness (mg/l as $CaCO_3$)
A	B			
Phenol	C14 methyl[b]	1	7.7 – 8.8	240 – 249
		2	7.7 – 8.2	236 - 250
Phenol	C12 amyl[b]	1	8.0 – 8.6	236 – 240
		2	7.6 – 8.2	230 - 244
Phenol	C14 ethyl[b]	1	7.5 – 8.2	229 – 234
		2	7.7 – 8.2	223 - 235
Phenol	C12 n-butyl[b]	1	8.5 – 9.1	215 – 258
		2	7.8 – 8.5	238 - 253
Phenol	C11.8 LAS	1	7.6 – 8.3	235 – 240
		2	7.1 – 8.0	232 - 242
2-CP	C14 n-butyl[b]	1	7.3 – 8.1	237 – 245
		2	7.5 – 8.2	232 - 236

A: Reference substance component. B: Test substance component (FAES[b]).

Table 119. Test solution quality data ranges over 48 hour test period for nominally equitoxic mixture toxicity studies where concentration addition predicted

Mixture		TR	Temperature Range (°C)	ΔT (°C)	pH
A	B				
Phenol	C14 methyl[b]	1	20.0 – 21.5	0.5	6.7 – 7.4
		2	17.5 – 20.5	0.5	7.0 – 7.5
Phenol	C12 amyl[b]	1	17.0 – 21.5	0.5	7.1 – 7.3
		2	19.0 – 21.0	0.5	6.9 – 7.3
Phenol	C14 ethyl[b]	1	20.5 – 21.5	1.0	6.9 – 7.2
		2	18.5 – 19.5	0.5	6.9 – 7.1
Phenol	C12 n-butyl[b]	1	15.5 – 17.5	1.5	7.2 – 7.4
		2	16.0 – 18.0	1.5	7.0 – 7.1
Phenol	C11.8 LAS	1	18.0 – 19.5	0.5	6.9 – 7.1
		2	17.0 – 19.0	0.5	6.7 – 7.1
2-CP	C14 n-butyl[b]	1	19.0 – 21.5	1.0	7.0 – 7.2
		2	16.0 – 21.0	1.0	7.0 – 7.4

A: Reference substance component. B: Test substance component (FAES[b]).

Table 120. Test solution quality data ranges over 48 hour test period for nominally equitoxic mixture toxicity studies where response addition predicted

Mixture		%# (A,B)	TR	Dissolved Oxygen (mg/l)	Total Hardness (mg/l as $CaCO_3$)
A	B				
Pentanol	C8 sec hexyl[b]	50,50	1	7.7 – 8.4	228 – 237
			2	7.4 – 8.1	220 – 245
Hexanol	C12 ethyl[b]	50,50	1	7.4 – 8.2	233 – 246
			2	7.4 – 8.5	222 – 241
Hexanol	C10 butyl[b]	43,57	1	7.7 – 8.5	238 – 245
			2	7.0 – 8.1	230 - 240
Hexanol	C8 hexyl[b]	41,59	1	7.4 – 8.0	230 – 242
			2	7.2 – 8.4	232 – 240
Hexanol	C7 heptyl[b]	50,50	1	7.4 – 8.3	234 – 250
			2	7.2 – 8.3	242 – 259
Octanol	C12 butyl[b]	60,40	1	7.7 – 8.8	234 – 248
			2	7.5 – 8.5	230 – 246
Octanol	C13 methyl[b]	36,64	1	7.2 – 8.0	219 – 248
			2	7.8 – 9.2	237 – 245
Nonanol	C12 amyl[b]	51,49	1	7.3 – 7.9	238 – 248
			2	7.0 – 8.1	238 – 251
Nonanol	C14 methyl[b]	47,53	1	7.3 – 8.1	237 – 246
			2	7.3 – 8.2	241 – 246
Nonanol	C11.8 LAS	57,43	1	8.1 – 8.4	179 – 235
			2	7.2 – 8.4	242 – 250
Nonanol	Phenol	51,49	1	8.0 – 8.6	240 – 248
			2	7.1 – 8.7	240 - 245

A: Reference substance component. B: Test substance component (FAES[b]).

Table 121. Test solution quality data ranges over 48 hour test period for nominally equitoxic mixture toxicity studies where response addition predicted

Mixture		%# (A,B)	TR	Temperature Range (°C)	ΔT (°C)	pH
A	B					
Pentanol	C8 sec hexyl[b]	50,50	1	18.5 – 20.5	2.0	7.1 – 7.8
			2	18.5 – 21.0	1.0	7.2 – 7.6
Hexanol	C12 ethyl[b]	50,50	1	21.0 – 21.5	0.5	6.4 – 7.5
			2	19.5 – 20.5	1.0	6.6 – 7.6
Hexanol	C10 butyl[b]	43,57	1	19.0 – 20.0	0.5	7.2 – 7.5
			2	19.0 – 20.5	0.5	7.1 – 7.9
Hexanol	C8 hexyl[b]	41,59	1	19.0 - 20.5	0.5	7.1 – 7.8
			2	19.0 – 20.0	0.5	7.1 – 7.8
Hexanol	C7 heptyl[b]	50,50	1	21.0 – 21.5	0.5	7.1 – 7.5
			2	21.0 – 22.0	1.0	7.1 – 7.4
Octanol	C12 butyl[b]	60,40	1	18.9 – 19.0	0.5	7.2 – 7.6
			2	19.5 – 20.0	0.5	7.2 – 7.5
Octanol	C13 methyl[b]	36,64	1	19.0 – 20.0	0.5	7.2 – 7.5
			2	20.0 – 21.0	0.5	7.2 – 7.4
Nonanol	C12 amyl[b]	51,49	1	19.5 – 21.0	0.5	7.1 – 7.7
			2	19.5 – 20.5	1.0	7.3 – 7.8
Nonanol	C14 methyl[b]	47,53	1	19.5 – 20.5	0.5	7.2 – 7.8
			2	20.5 – 21.0	0.5	7.1 – 7.6
Nonanol	C11.8 LAS	57,43	1	17.0 – 19.0	0.5	6.9 – 7.3
			2	16.0 – 18.0	2.0	7.0 – 7.7
Nonanol	Phenol	51,49	1	20.0 – 27.5	2.5	7.0 – 7.3
			2	20.0 – 23.0	1.0	6.7 – 7.4

A: Reference substance component. B: Test substance component (FAES[b]).

Table 122. Test solution quality data ranges over 48 hour test period for nominally non-equitoxic mixture toxicity studies where response addition predicted

Mixture		%# (A,B)	TR	Dissolved Oxygen (mg/l)	Total Hardness (mg/l as $CaCO_3$)
A	B				
Hexanol	C7 heptyl	73,27	1	7.6 – 8.4	238 – 250
Hexanol	C7 heptyl	23,77	1	7.2 – 8.2	237 – 256
			2	7.3 – 8.3	240 – 254
Octanol	C12 butyl	50,50	1	7.8 – 8.5	232 – 245
Octanol	C13 methyl	16,84	1	8.0 – 8.4	236 – 249
			2	7.5 – 8.3	234 – 246
Octanol	C13 methyl	50,50	1	7.6 – 9.2	237 – 245
			2	7.7 – 8.3	234 - 243

Table 123. Test solution quality data ranges over 48 hour test period for nominally non-equitoxic mixture toxicity studies where response addition predicted

Mixture		%# (A,B)	TR	Temperature Range (°C)	ΔT (°C)	pH
A	B					
Hexanol	C7 heptyl	73,27	1	21.0 – 21.5	0.5	7.1 – 7.5
Hexanol	C7 heptyl	23,77	1	21.0 – 21.5	0.5	7.1 – 7.5
			2	21.0 – 22.5	0.5	7.1 – 7.4
Octanol	C12 butyl	50,50	1	19.5 – 20.0	0.5	7.2 – 7.5
Octanol	C13 methyl	16,84	1	20.0 – 20.5	0.5	7.2 – 7.4
			2	19.5 – 20.0	0.5	7.1 – 7.4
Octanol	C13 methyl	50,50	1	20.0 – 21.0	1.0	7.1 – 7.4
			2	19.5 – 20.0	0.5	7.1 – 7.5

A: Reference substance component. B: Test Substance component (FAES).

Table 124. Test solution quality data ranges over 48 hour test period for total hardness studies

Total hardness study number	Temperature Range (°C)	ΔT (°C)	pH	Dissolved Oxygen (mg/l)
1	16.5 – 18.5	1.5	7.4 – 8.5	7.8 – 8.4
2	16.5 – 18.0	0.5	6.8 – 7.8	7.0 – 8.3
3	19.5 – 21.0	1.5	7.3 – 8.0	7.6 – 8.3

Table 125. Test solution quality data ranges over 48 hour test period for anionic surfactant test substances used in Chapter 4

Substance	Nominal TH (mg/l as $CaCO_3$)	TR	Dissolved Oxygen (mg/l)	Total Hardness (mg/l as $CaCO_3$)
DLAS		1	7.5 – 8.2	12.0 – 14.0
SLDI		1	8.2 – 8.5	12.3 – 16.0
Geropon T77		1	7.0 – 7.7	12.0 – 14.0
SMMA	13	1	7.0 – 8.0	2.8 – 13.0
		2	7.2 – 7.8	2.3 – 13.0
SEMA		1	7.8 – 8.5	2.8 – 13.0
DSDS		1	7.4 – 8.5	12.0 – 14.0
DLAS		1	8.0 – 9.1	226 – 243
Geropon T77		1	8.0 – 8.4	230 – 241
	240	2	8.0 – 8.5	230 - 238
DSDS		1	7.0 – 8.6	218 – 279
		2	7.8 – 8.2	205 – 250

Table 126. Test solution quality data ranges over 48 hour test period for anionic surfactant test substances used in Chapter 4

Substance	Nominal TH (mg/l as $CaCO_3$)	TR	Temperature Range (°C)	ΔT (°C)	pH
DLAS		1	20.0 – 21.0	0.5	7.5 – 7.9
SLDI		1	18.0 – 19.0	1.0	7.5 – 8.1
Geropon T77		1	18.0 – 19.5	1.0	7.8 – 8.2
SMMA	13	1	17.5 – 18.0	0.5	7.7 – 8.1
		2	18.5 – 19.5	0.5	7.7 – 8.0
SEMA		1	19.0 – 19.0	0	7.8 – 8.5
DSDS		1	17.0 – 18.5	1.5	7.3 – 7.8
DLAS		1	17.0 – 19.0	1.5	6.7 – 7.9
Geropon T77		1	16.0 – 18.0	1.5	7.2 – 7.9
	240	2	17.0 – 18.0	1.0	7.3 – 7.9
DSDS		1	16.5 – 18.0	1.5	7.3 – 8.0
		2	16.5 – 18.0	1.5	7.2 – 8.0

Table 127. Test solution quality data for LAS and FAES substances tested at nominal total hardness of 13 mg/l as $CaCO_3$

Substance	TR	Temperature Range (°C)	ΔT (°C)	pH
C11.8 LAS	1	17.5 – 19.5	1.5	7.3 – 8.0
	2	18.0 – 19.0	0.5	7.3 – 7.9
C14 amyl FAES	1	18.0 – 19.0	1.0	7.4 – 7.8
	2	17.5 – 18.5	1.0	7.6 – 7.9
C10 sec butyl FAES	1	18.0 – 19.0	0.5	4.8 – 7.7
	2	17.5 – 18.0	1.0	7.4 – 7.8
C12 n butyl FAES	1	18.0 – 19.5	1.0	7.8 – 8.0

Table 128. Test solution quality data for LAS and FAES substances tested at nominal total hardness of 13 mg/l as $CaCO_3$

Substance	TR	Dissolved Oxygen (mg/l)	Total Hardness (mg/l as $CaCO_3$)
C11.8 LAS	1	7.5 – 8.5	12.0 – 14.0
	2	7.7 – 8.7	12.0 – 14.0
C14 amyl FAES	1	7.9 – 8.4	12.3 – 14.0
	2	7.8 – 8.4	12.7 – 14.2
C10 sec butyl FAES	1	7.8 – 8.7	13.2 – 16.0
	2	7.9 – 8.7	11.8 – 13.7
C12 n butyl FAES	1	8.1 – 8.4	11.6 – 15.1

Table 129. Test solution quality data ranges over 48 hour test period for LAS substances

Substance	TR	Temperature Range (°C)	ΔT (°C)	pH
C9	1	18.5 – 20.0	1.0	7.4 – 7.8
	2	19.0 – 19.5	1.0	7.5 – 7.9
C10	1	18.5 – 20.0	0.5	7.4 – 7.9
C11	1	19.5 – 20.5	0.5	7.5 – 7.8
C12	1	19.0 – 20.5	1.0	7.3 – 7.9
C13	1	17.0 – 19.0	2.0	7.7 – 8.0
C14	1	18.0 – 19.0	1.0	7.4 – 7.8
	2	17.5 – 18.5	0.5	7.6 – 7.9

Table 130. Test solution quality data ranges over 48 hour test period for LAS substances

Substance	TR	Dissolved Oxygen (mg/l)	Total Hardness (mg/l as $CaCO_3$)
C9	1	7.6 – 8.3	11.8 – 15.0
	2	7.6 – 8.2	12.6 – 15.1
C10	1	8.0 – 8.3	12.7 – 15.0
C11	1	7.8 – 8.3	12.0 – 13.9
C12	1	8.0 – 8.5	12.7 – 13.7
C13	1	7.9 – 8.4	12.4 – 14.0
C14	1	7.6 – 7.9	11.7 – 15.0
	2	7.8 – 8.4	12.4 – 13.8

Table 131. Test solution quality data ranges over 48 hour test period for nominally equitoxic mixture toxicity studies where concentration addition predicted

Mixture		%# (A,B)	TR	Temperature Range (°C)	ΔT (°C)	pH
A	B					
Phenol	DLAS	58, 42	1	21.0 - 21.5	0.5	7.2 – 7.4
Phenol	DSDS	59, 41	1	17.0 – 18.0	1.0	7.0 – 7.7
		57, 43	2	18.0 - 20.5	0.5	7.1 – 7.5
Phenol	Geropon T77	54, 46	1	19.0 - 20.5	1.5	7.3 – 7.7
Hexanol	SLDI	35, 65	1	16.0 – 18.0	1.0	7.2 – 7.7
		35, 65	2	17.0 - 18.5	1.0	7.2 – 7.8

Table 132. Test solution quality data ranges over 48 hour test period for nominally equitoxic mixture toxicity studies where concentration addition predicted

Mixture		%# (A,B)	TR	Dissolved Oxygen (mg/l)	Total Hardness (mg/l as $CaCO_3$)
A	B				
Phenol	DLAS	58, 42	1	7.6 – 8.0	228 – 236
Phenol	DSDS	59, 41	1	7.9 – 8.8	228 – 234
		57, 43	2	7.7 – 8.2	238 – 244
Phenol	Geropon T77	54, 46	1	8.1 – 8.7	230 – 236
Hexanol	SLDI	35, 65	1	7.8 – 8.3	178 – 186
			2	7.2 – 7.9	196 - 203

A: Reference substance component. B: Test substance component.

APPENDIX III

NMR, IR and MS spectra for synthesised substances in chapters 2 and 4.

NMR spectra: reduced in size.

: Trioxan peak at 5.2ppm

IR spectra : Alkyl chain at approximately 2900 cm^{-1}

: Amide at approximately 1550 -1650 cm^{-1}

: Fatty acid at approximately 1700 (1680)cm^{-1}

Table 133. Weight % product for FAES and LAS substances

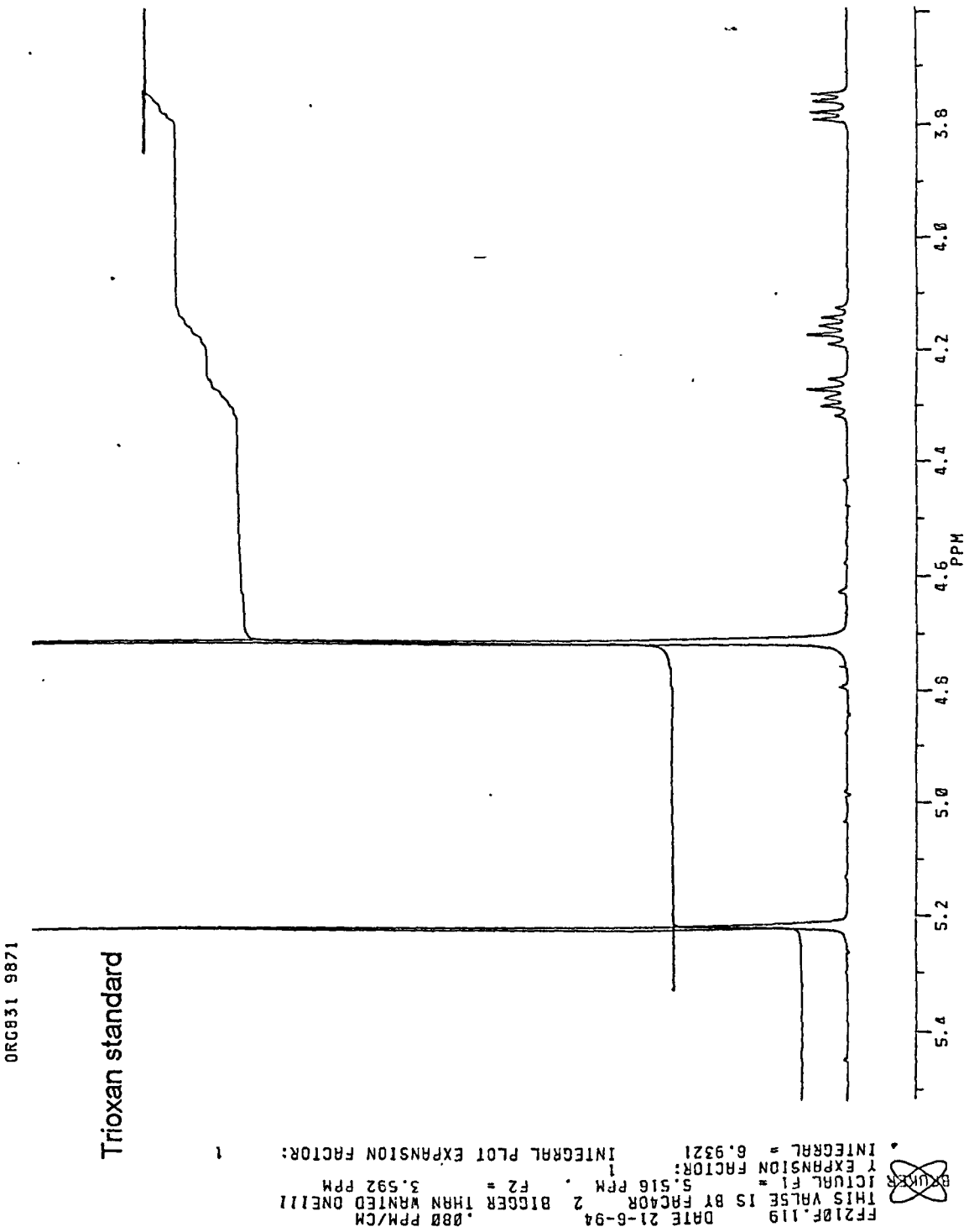
Substance[b]	Weight % product*	Substance[b]	Weight % product*
C12 methyl	>95[a]	C9 sec amyl	>99
C12 butyl	>95[a]	C10 butyl	>94
C12 sec butyl	>99[a]	C10 sec butyl	>99
C12 amyl	>91[a]	C10 iso butyl	>99
C14 methyl	>95[a]	C11 propyl	>99
C14 ethyl	>91[a]	C12 ethyl	>96
C14 iso propyl	>88[a]	C13 methyl	>99
C14 butyl	>99[a]	C9 LAS	>97[a]
C14 amyl	>95[a]	C10 LAS	>98[a]
C16 methyl	>95[a]	C11 LAS	>98[a]
C7 heptyl	>98	C12 LAS	>98[a]
C8 hexyl	>96	C13 LAS	>97[a]
C8 sec hexyl	>99	C14 LAS	>95[a]
C9 amyl	>97		

* Calculated from NMR traces (Appendix III).

[a] Data provided with samples.

[b] FAES substances unless otherwise stated.

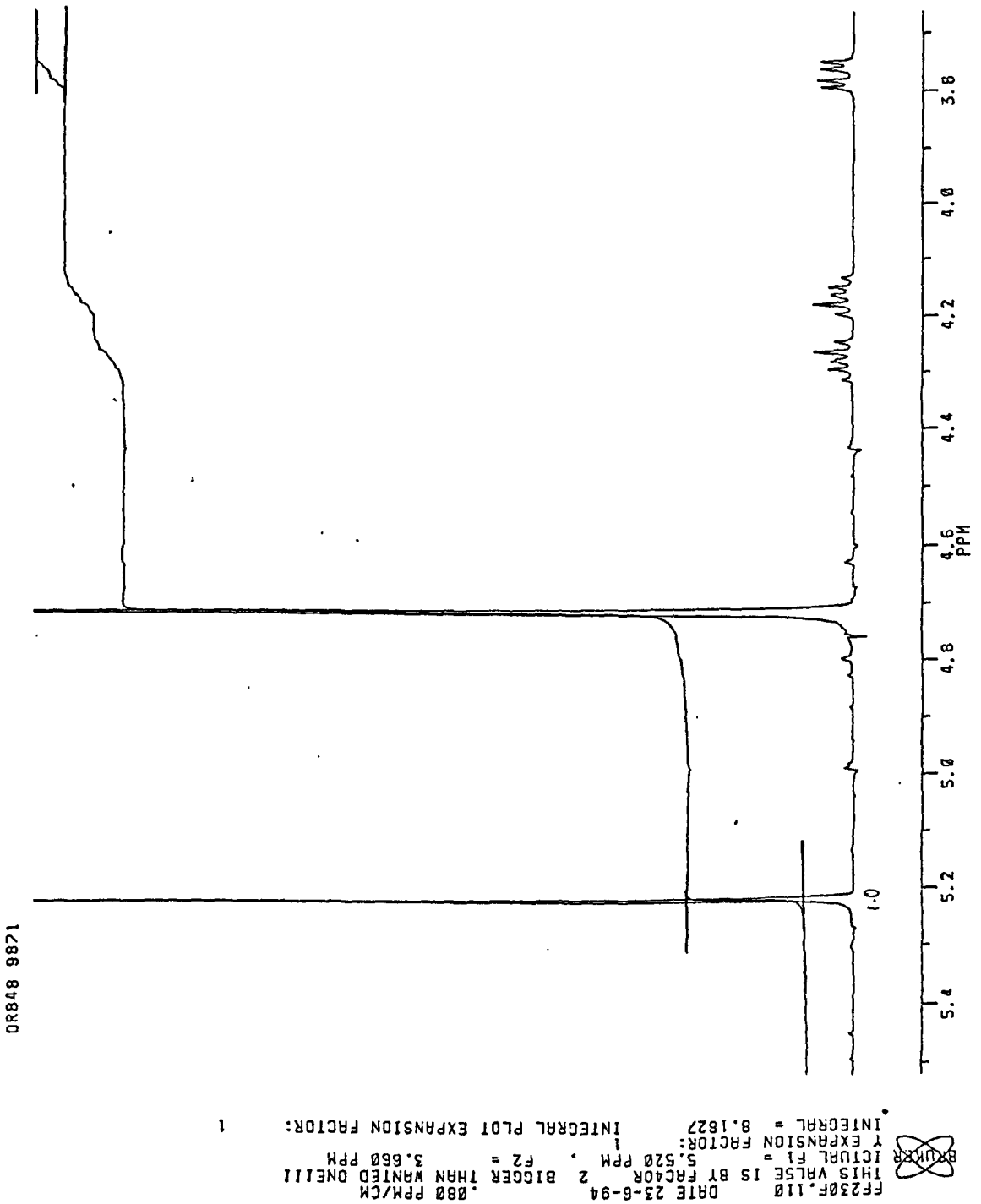
Fig. 44. NMR spectrum for calculation of weight percent product for C7 heptyl FAES



Weight sample : 0.0156g

Weight Trioxan in 1ml solvent : 0.00281g

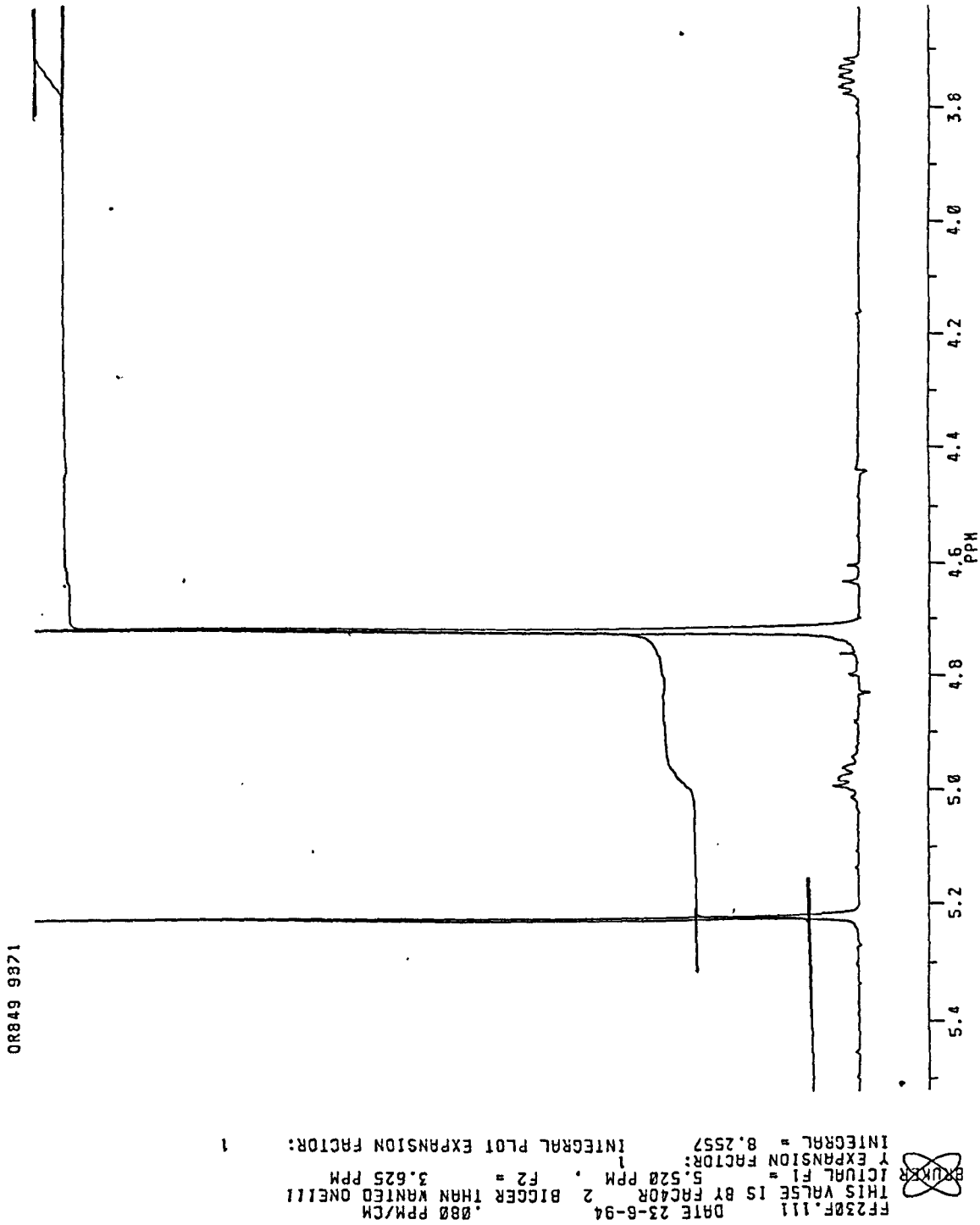
Fig. 45. NMR spectrum for calculation of weight percent product for C8 hexyl FAES



Weight sample : 0.0155g

Weight Trioxan in 1ml solvent : 0.00281g

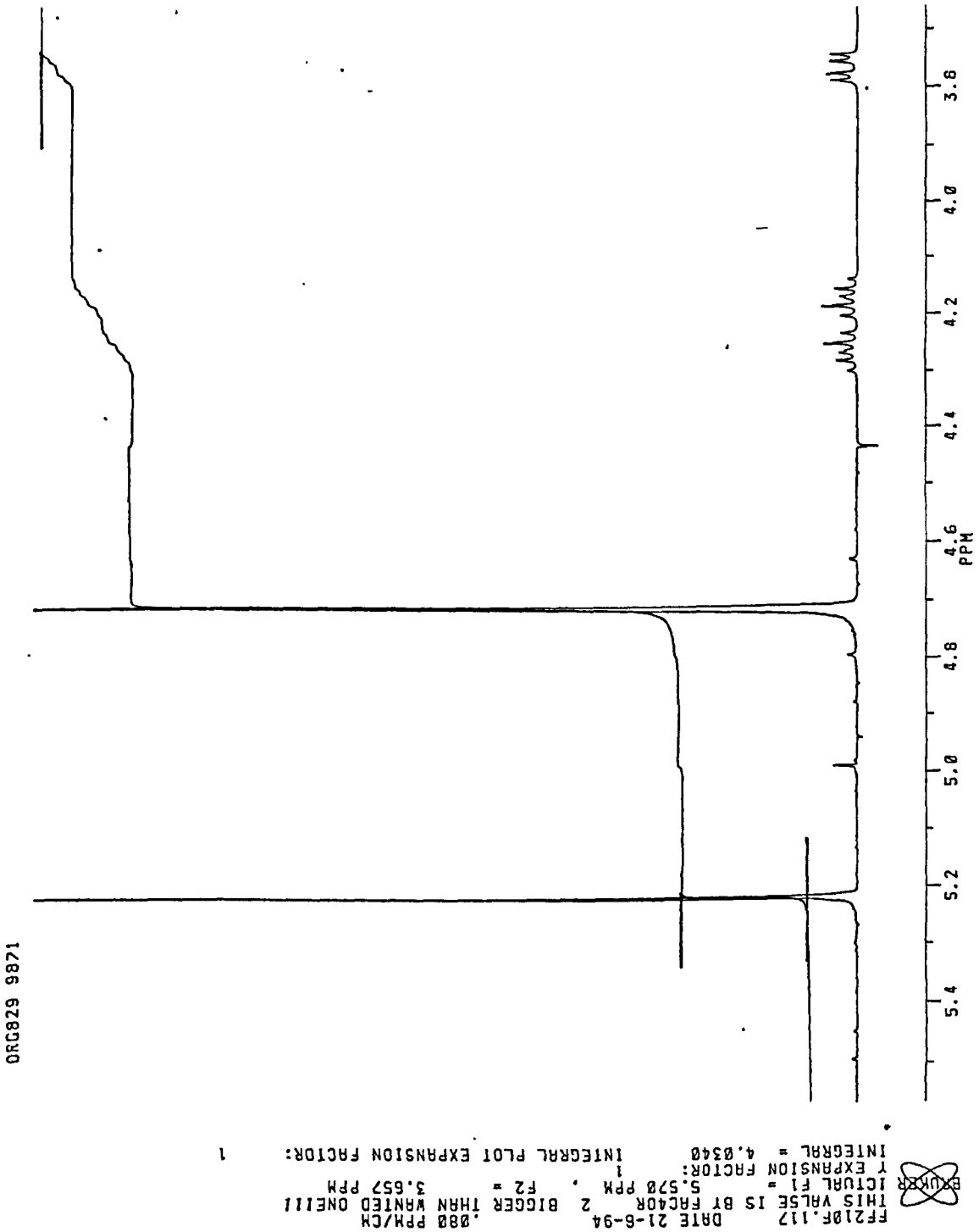
Fig. 46. NMR spectrum for calculation of weight percent product for C8 sec hexyl FAES



Weight sample : 0.0153g

Weight Trioxan in 1ml solvent : 0.00281g

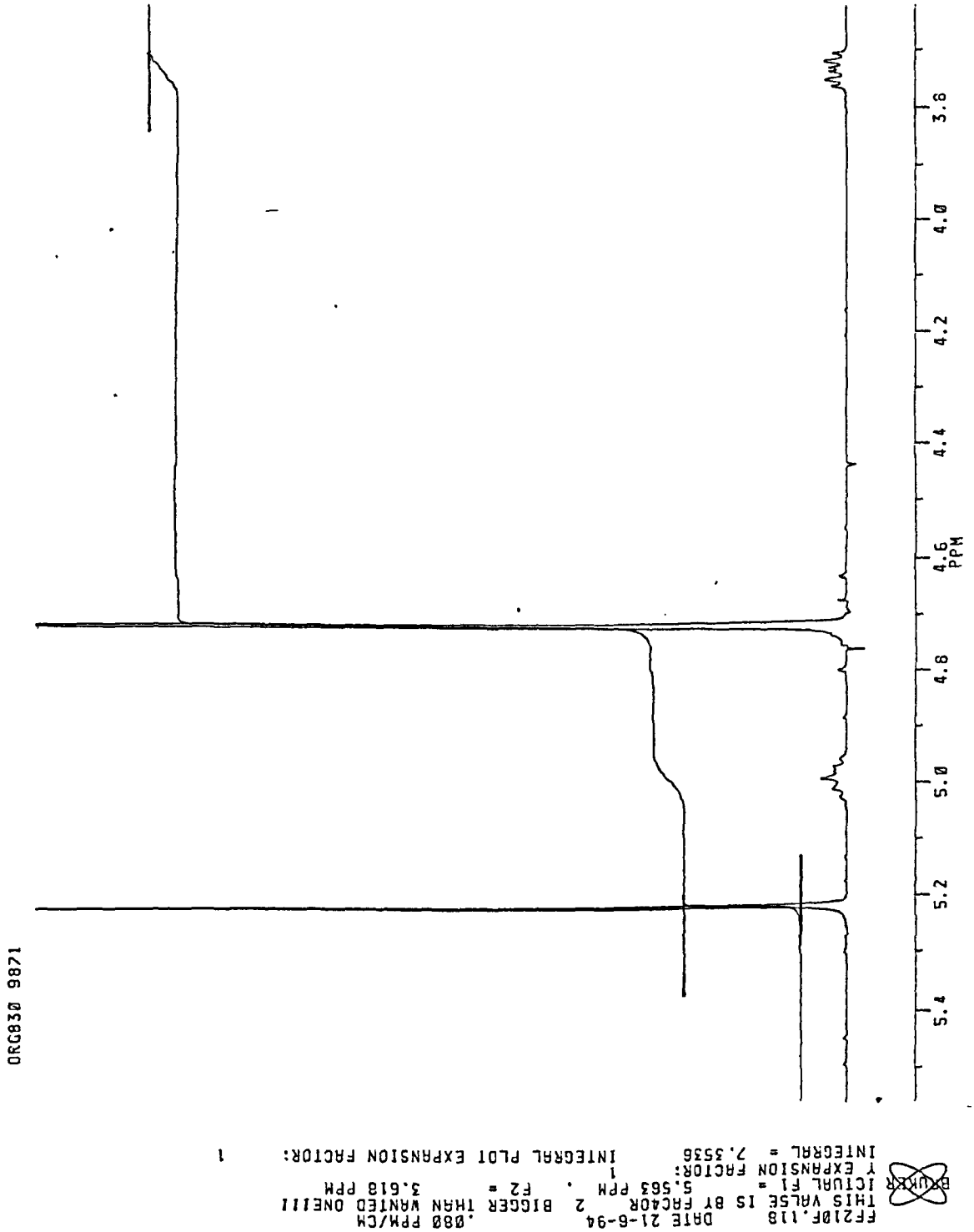
Fig. 47. NMR spectrum for calculation of weight percent product for C9 amyl FAES



Weight sample : 0.0158g

Weight Trioxan in 1ml solvent : 0.00281g

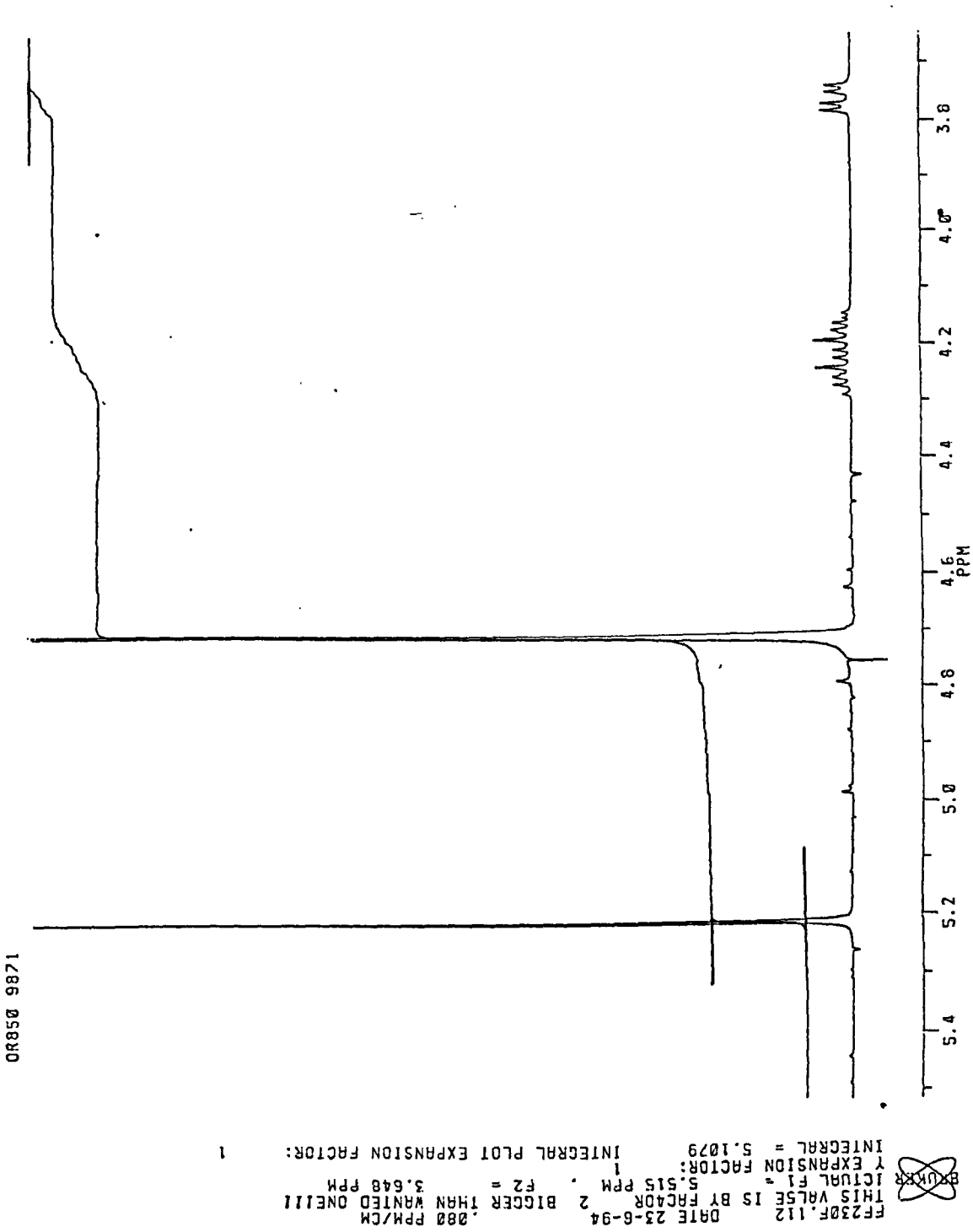
Fig. 48. NMR spectrum for calculation of weight percent product for C9 sec amyl FAES



Weight sample : 0.0156g

Weight Trioxan in 1ml solvent : 0.00281g

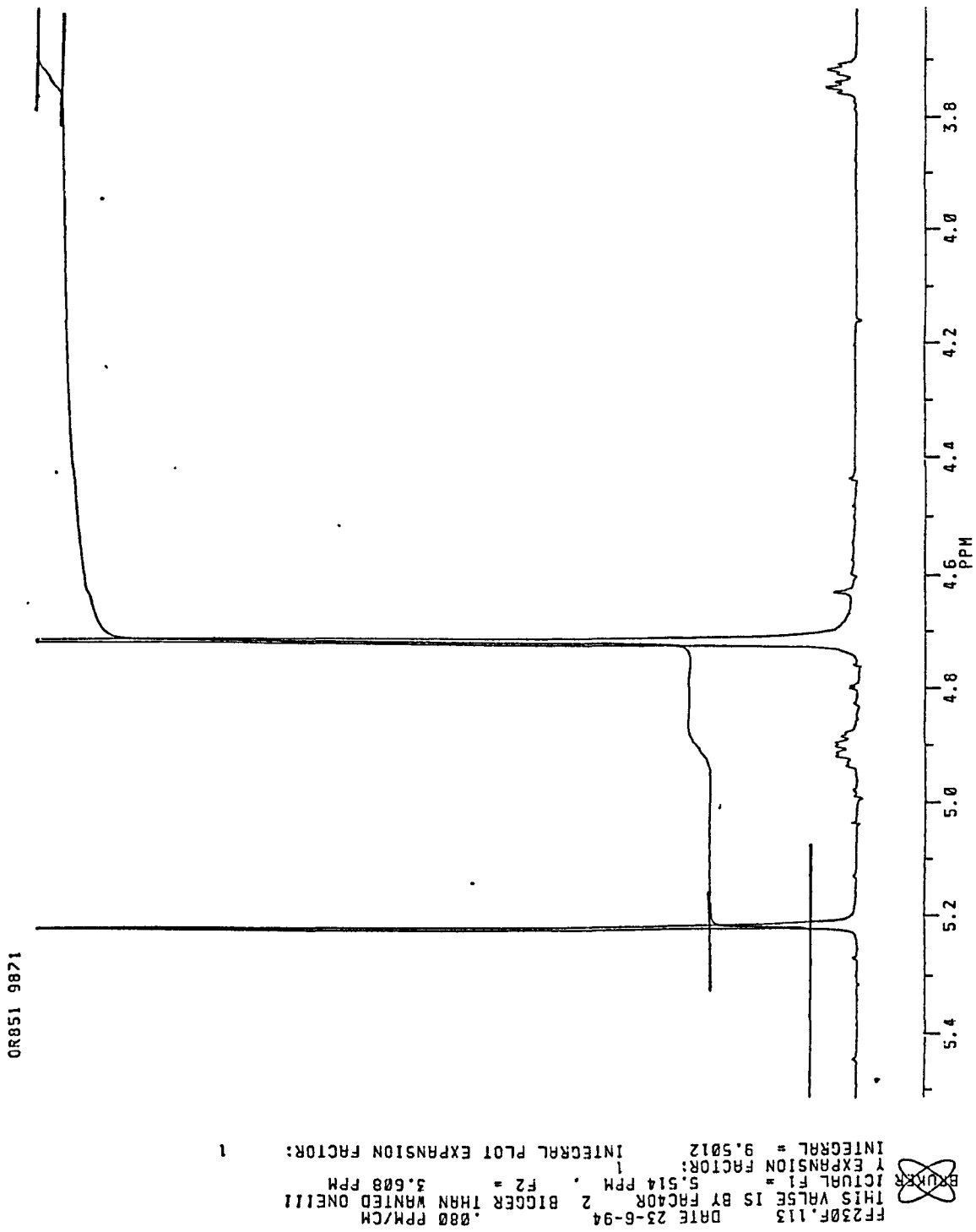
Fig. 49. NMR spectrum for calculation of weight percent product for C10 butyl FAES



Weight sample : 0.0155g

Weight Trioxan in 1ml solvent : 0.00281g

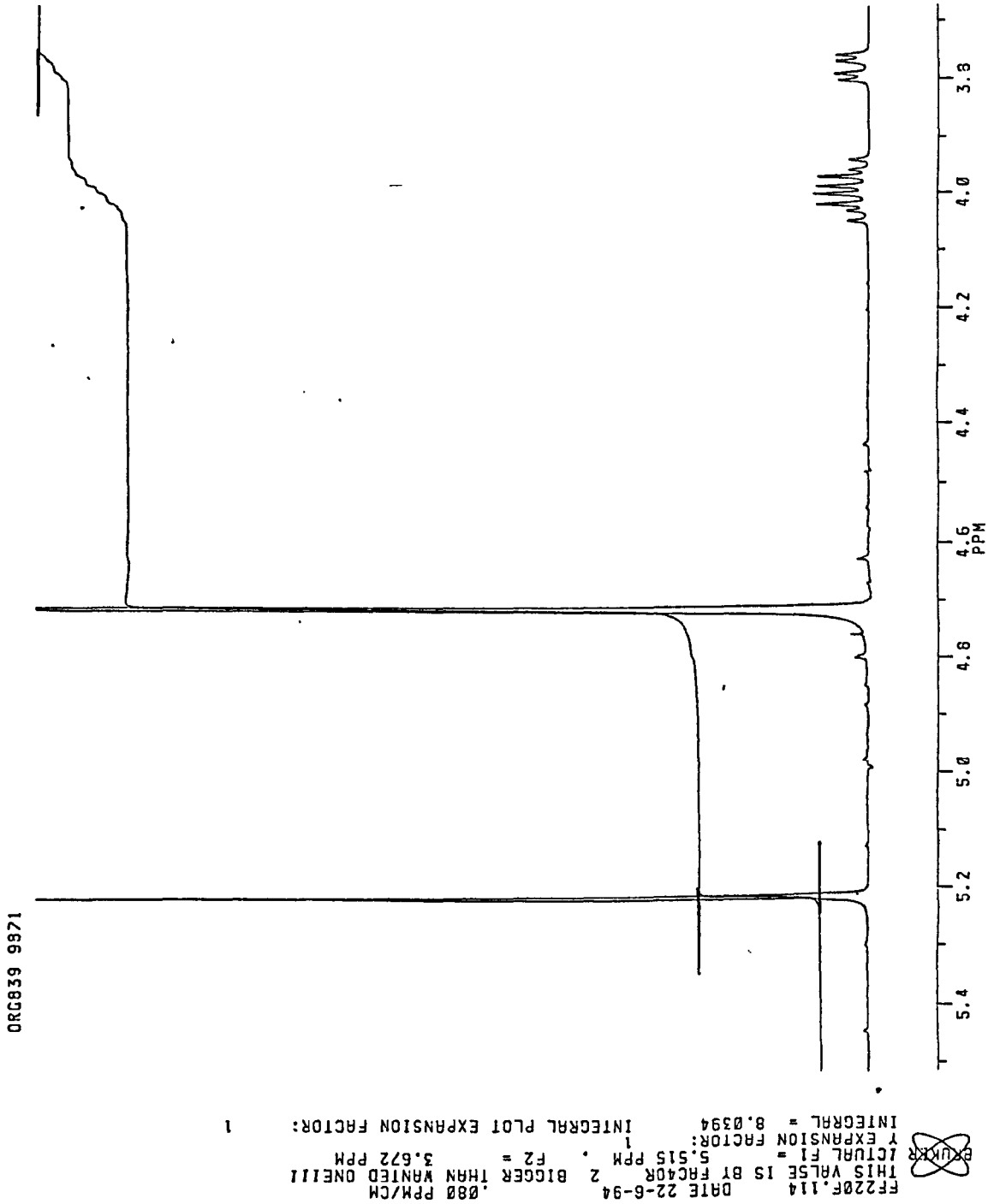
Fig. 50. NMR spectrum for calculation of weight percent product for C10 sec butyl FAES



Weight sample : 0.0153g

Weight Trioxan in 1ml solvent : 0.00281g

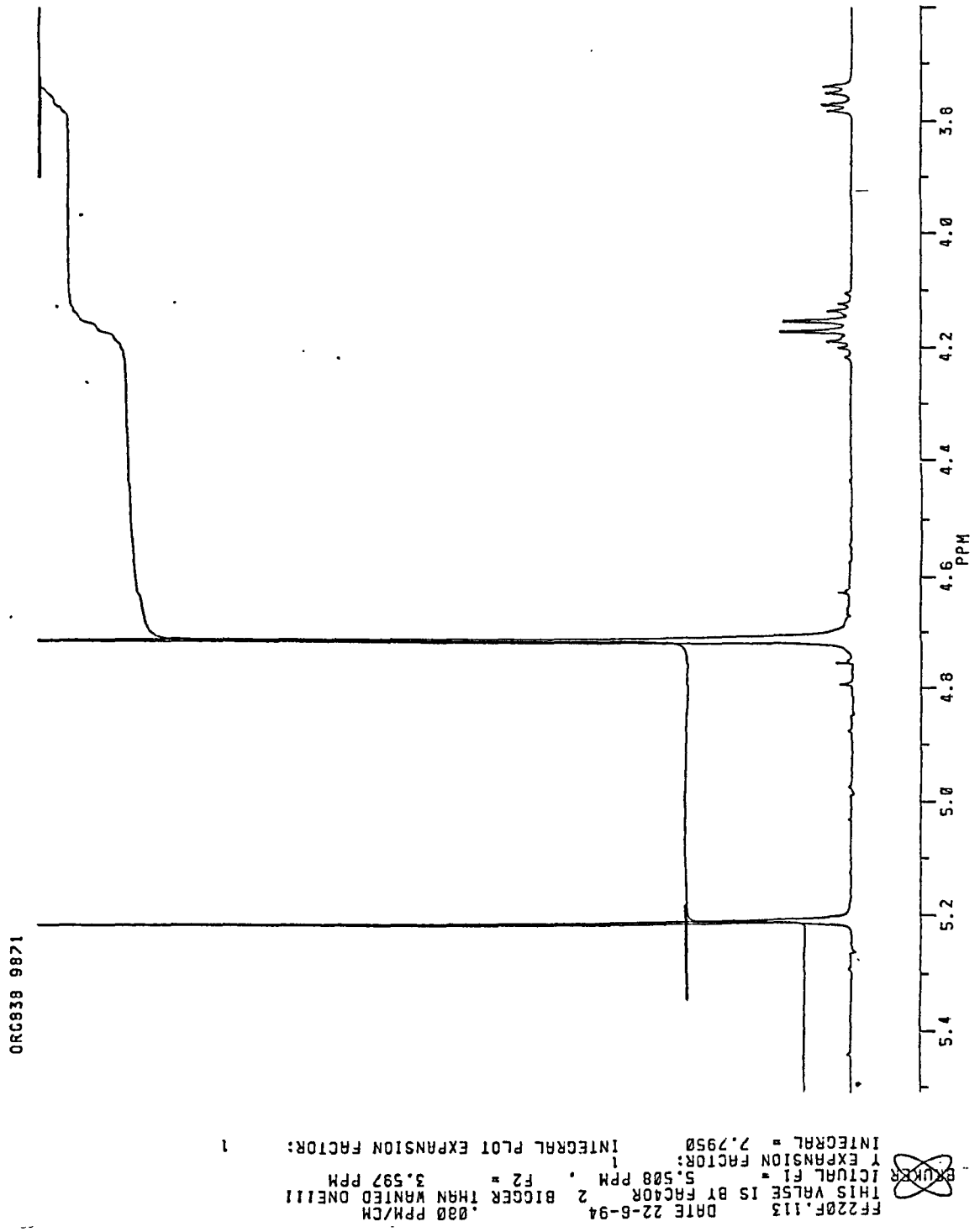
Fig. 51. NMR spectrum for calculation of weight percent product for C10 iso butyl FAES



Weight sample : 0.0153g

Weight Trioxan in 1ml solvent : 0.00281g

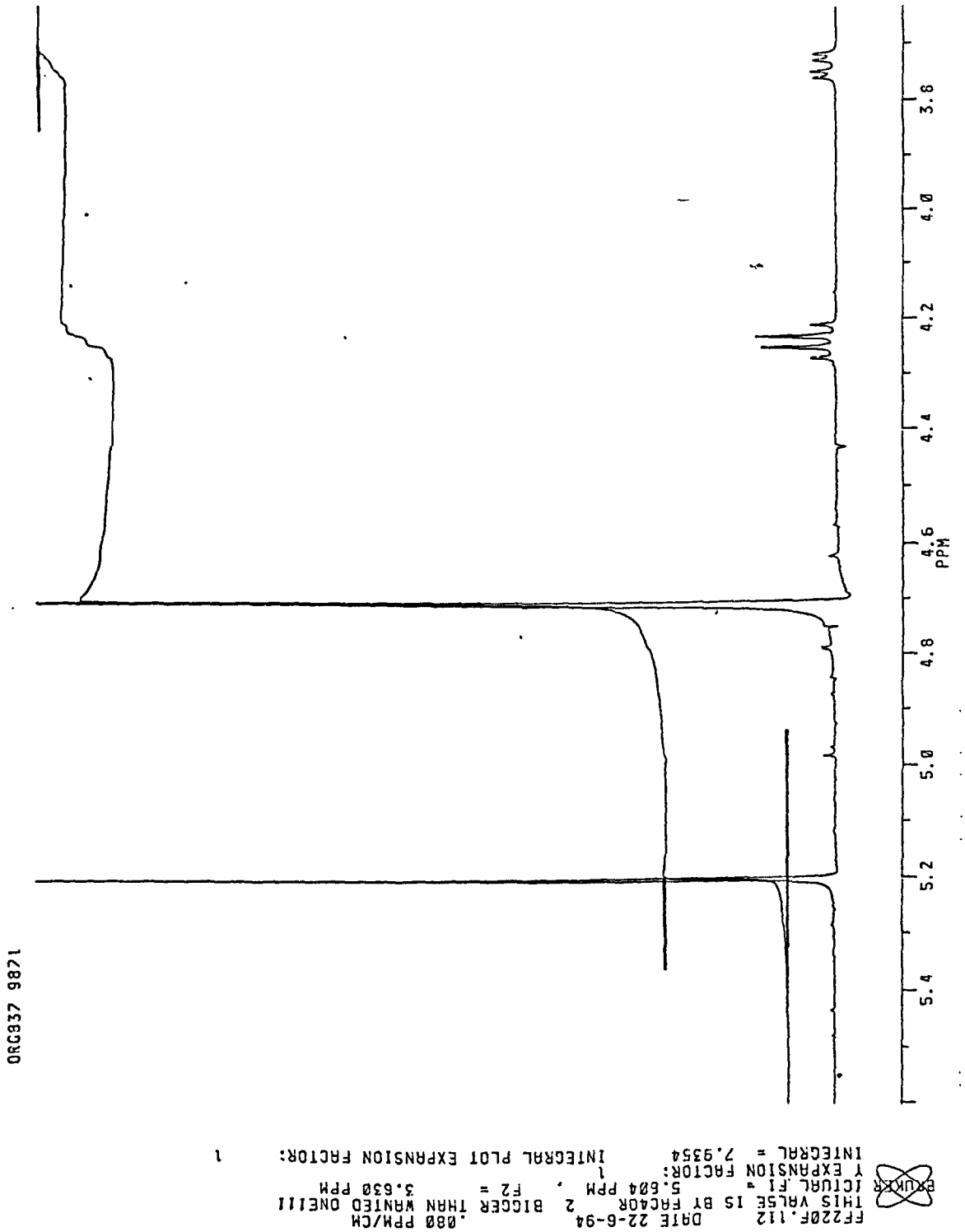
Fig. 52. NMR spectrum for calculation of weight percent product for C11 propyl FAES



Weight sample : 0.0150g

Weight Trioxan in 1ml solvent : 0.00281g

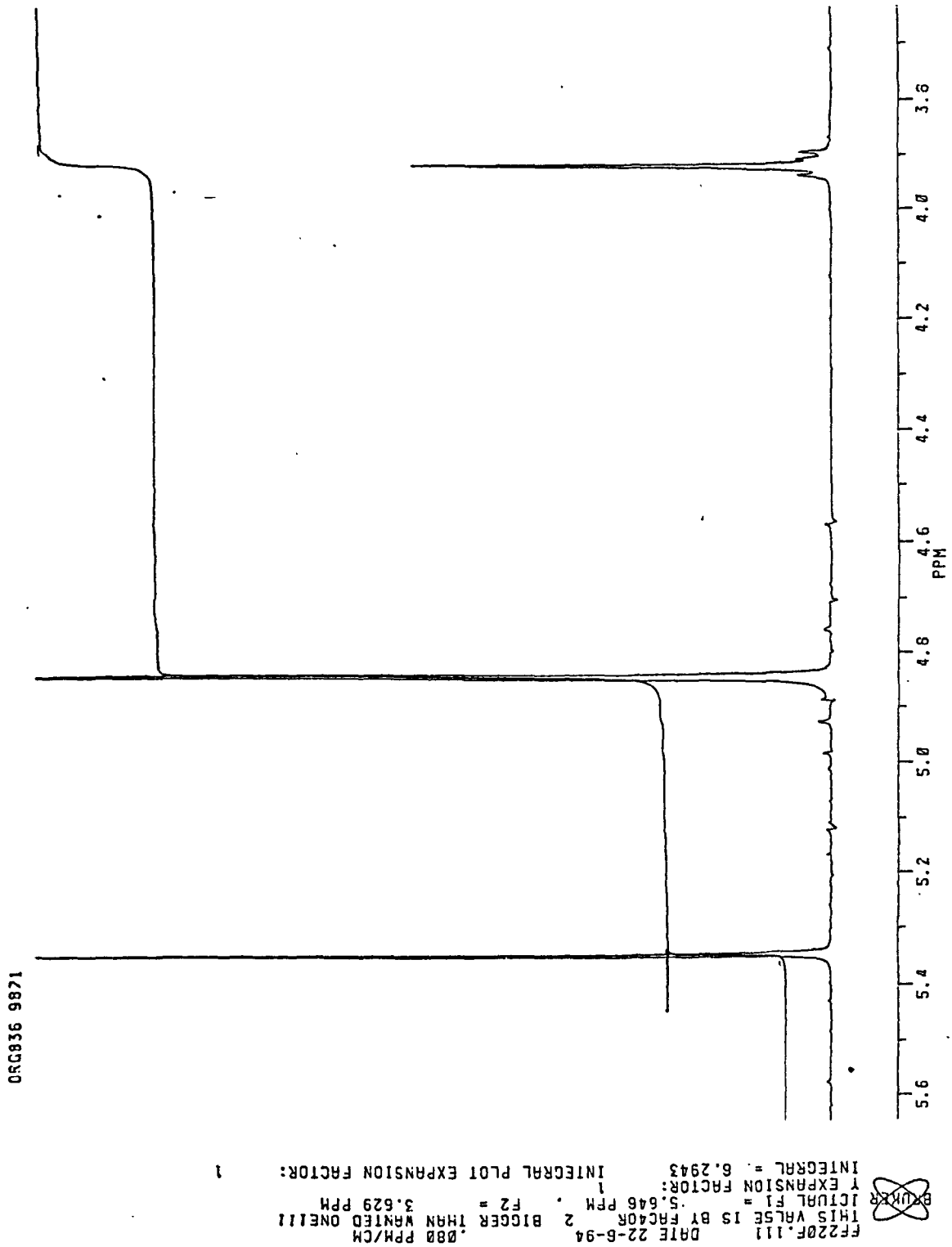
Fig. 53. NMR spectrum for calculation of weight percent product for C12 ethyl FAES



Weight sample : 0.0152g

Weight Trioxan in 1ml solvent : 0.00281g

Fig. 54. NMR spectrum for calculation of weight percent product for C13 methyl FAES



Weight sample : 0.0185g

Weight Trioxan in 1ml solvent : 0.00281g

Fig. 55. IR spectrum: fatty acid contaminant of SMMA

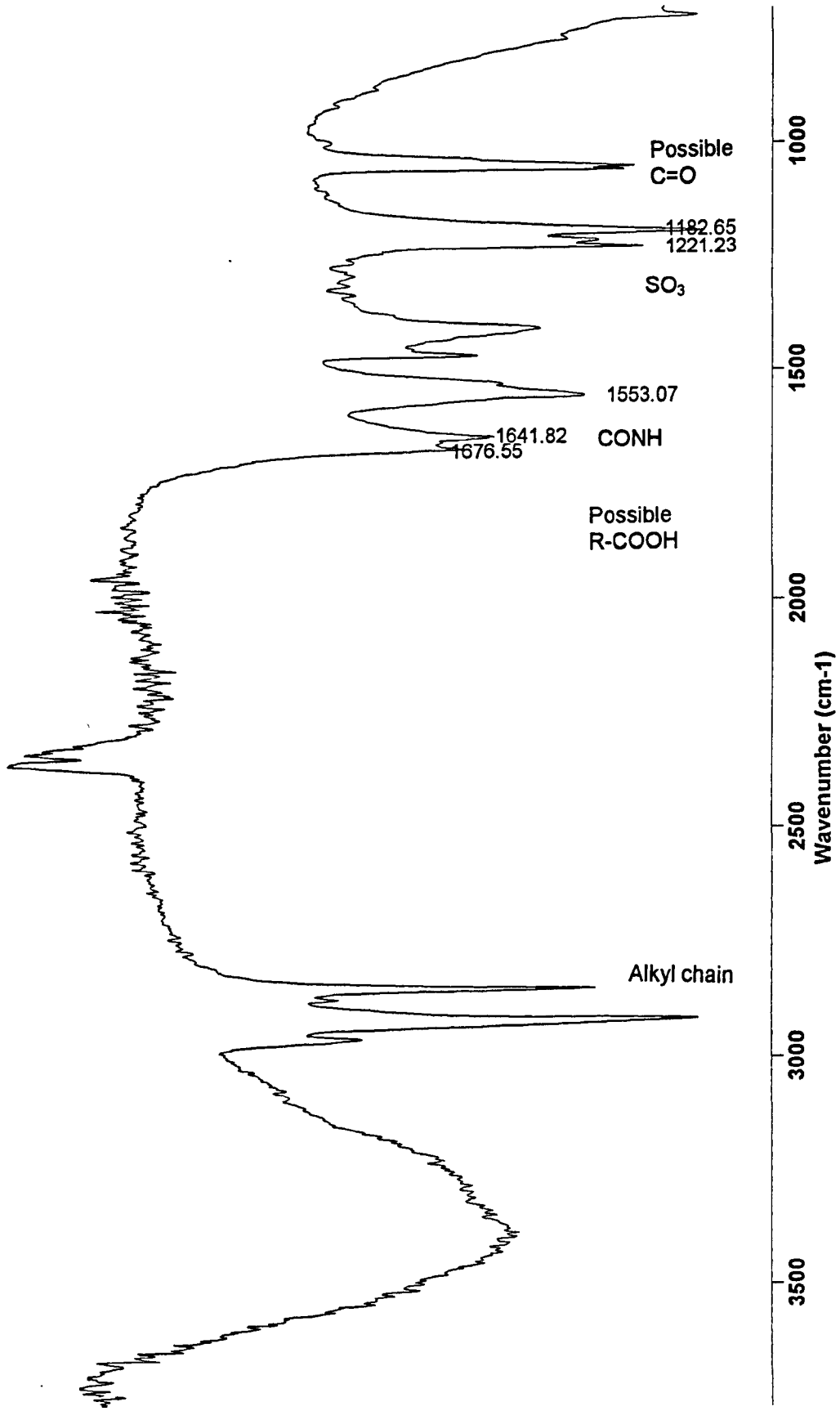


Fig. 56. NMR spectrum for calculation of weight percent product for SMMA

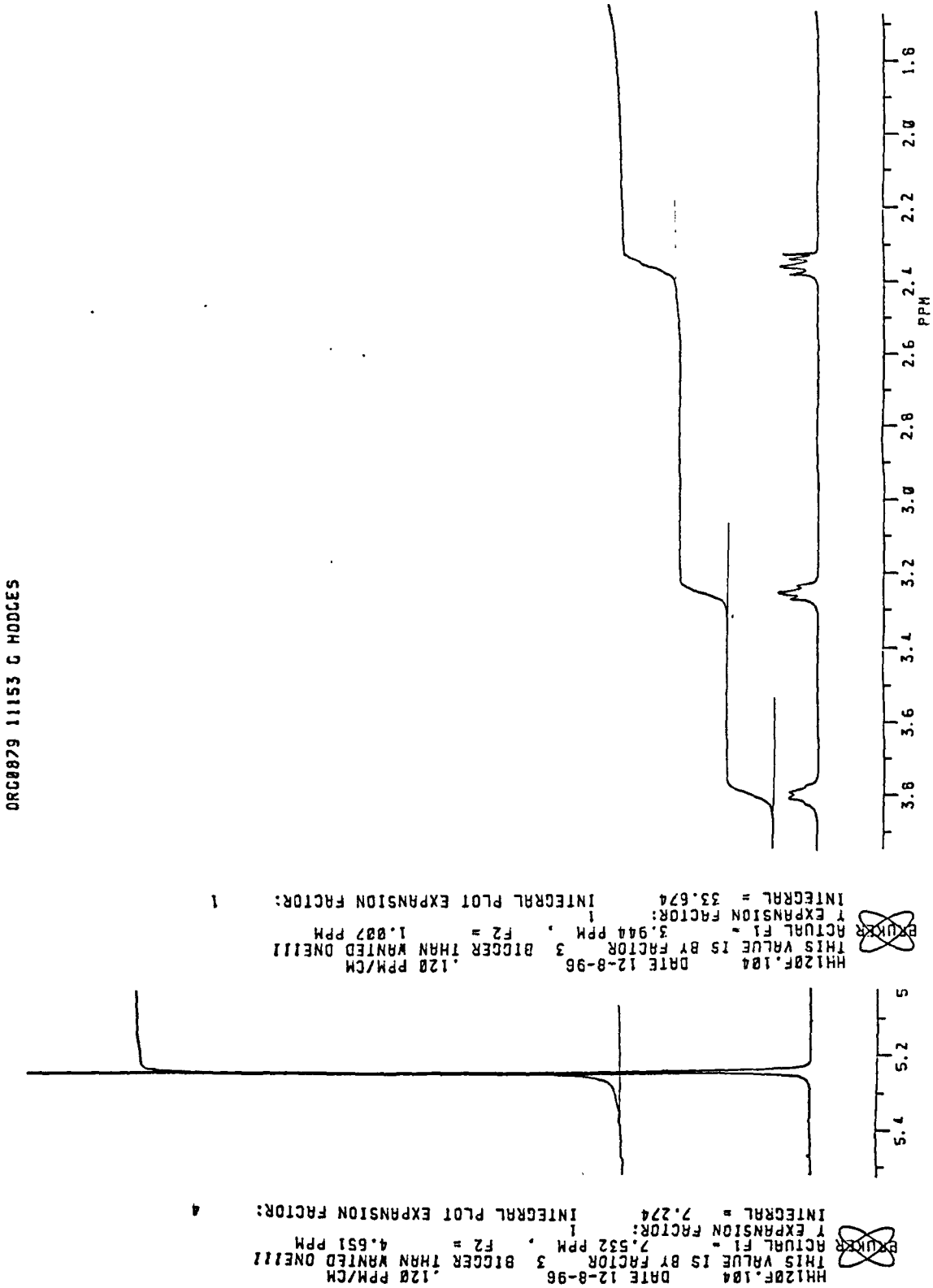


Fig. 57. NMR spectrum for calculation of weight percent product for SEMA

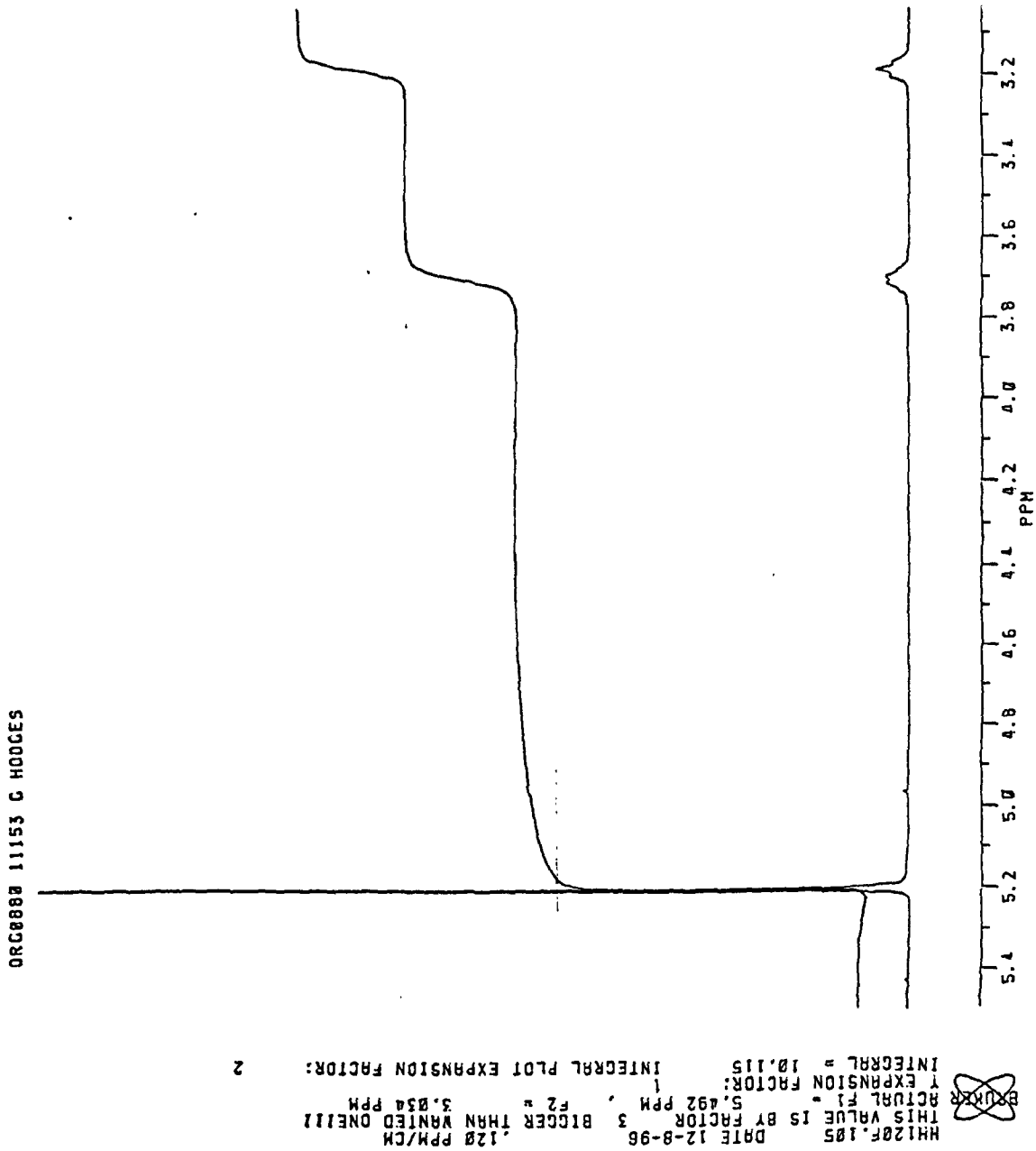


Fig. 58. IR spectrum: inorganics and parent diol in DSDS

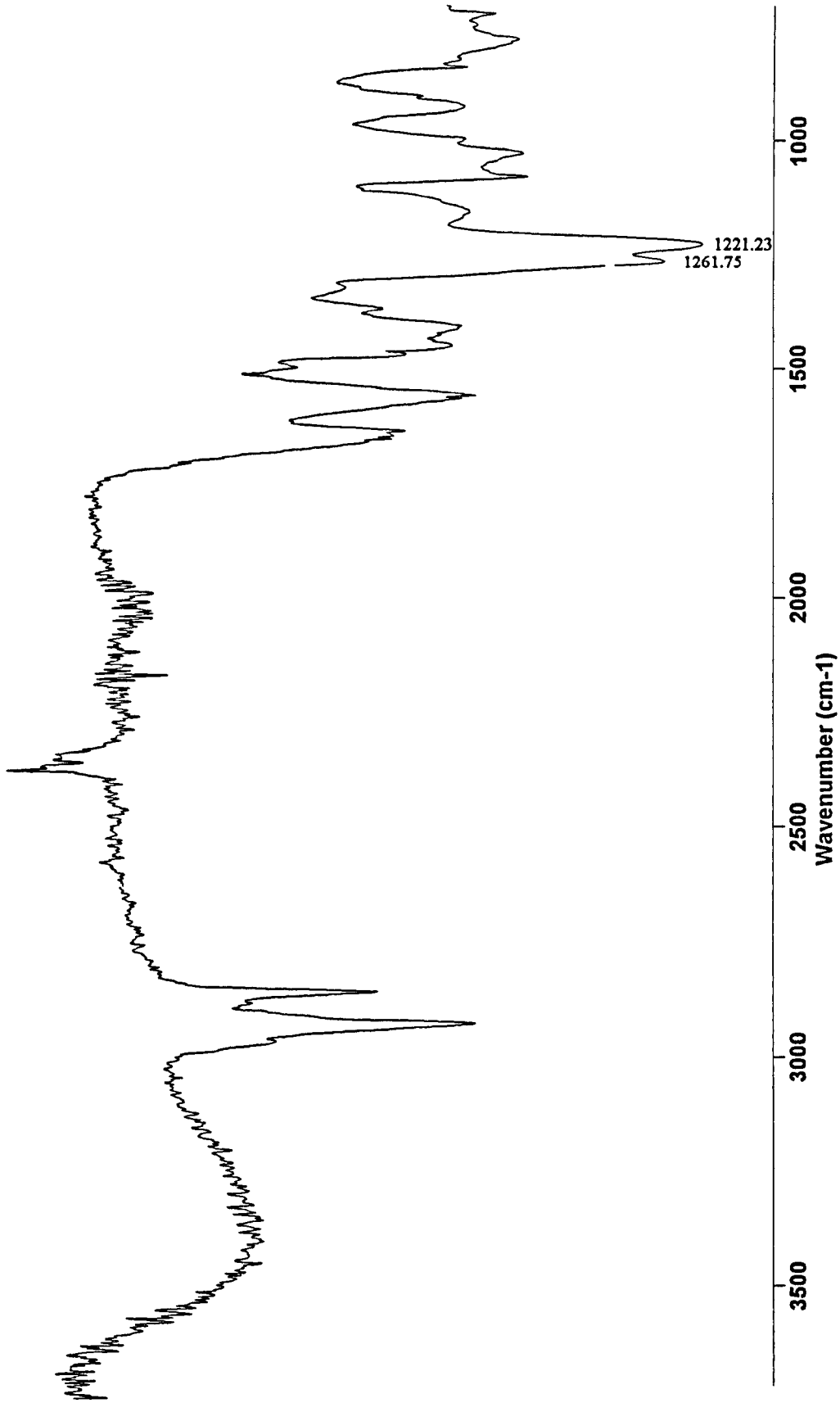


Fig. 59. MS spectrum for identification of DSDS

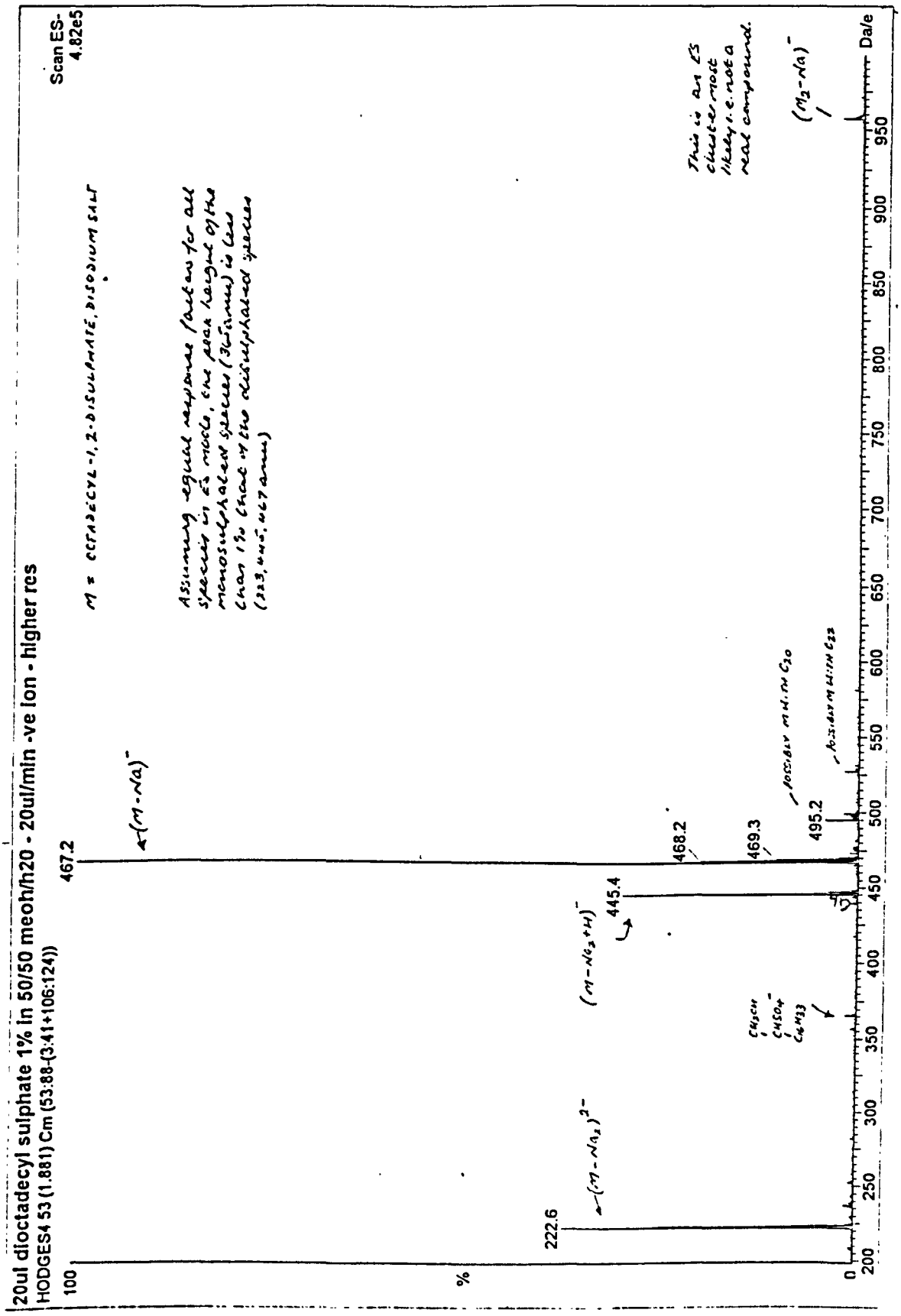


Fig. 60. NMR spectrum for calculation of weight percent product for DSDS