The Mechanism of Action Of The Ophthalmic Preservative Purite™ and a Comparison To Other Preservatives.

By

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ABSTRACT

The use of preservatives in ophthalmic pharmaceuticals is mandatory to inhibit the growth of micro-organisms whilst on the shelf and during use. Traditional preservatives used in this application, over the long-term, are reported to cause serious patient reactions limiting their use. The novel oxy-chloro compound Purite™ is a newer generation ocular preservative that overcomes some of the patient related problems. Although the major constituent of Purite™ is sodium chlorite, very little is known about the exact mechanism for the activity of chlorite solutions as preservatives and the reasons for the differential toxicity between micro-organisms and ocular tissue.

Electrospray mass spectrometry and NMR were used to investigate the types of reactions Purite™ was capable of undergoing in the presence of biological molecules *in vitro*. When added to preparations of phospholipids, at concentrations shown to be toxic to microbial cells, Purite™ was found to be a very mild oxidant of phospholipid vesicles generating very low levels of lipid hydroperoxides and no lipid chlorohydrins. However, compared to other oxidants, Purite™ was found to rapidly oxidise the reduced form of glutathione *in vitro*.

Experiments on mammalian and microbial cells showed that the resistance could not be correlated to native membrane phospholipid profiles. Also, there was no membrane phospholipid oxidation observed in organisms treated with toxic doses of Purite™. The differential resistance of test organisms to Purite™ related to their native levels of the antioxidant glutathione. Further research showed close correlation between cellular resistance and the ability of cells to maintain their cytosolic glutathione with increasing Purite™ doses.

Anti-oxidant inhibitors were used to deplete specific resistance pathways in each test organism. The loss of resistance to Purite™ in specific organisms was found to correlate well not only to glutathione depletion but also catalase depletion.

This study shows that Purite™ preferentially oxidises cellular glutathione to induce oxidative stress and resistance depends upon the anti-oxidant repertoire of each organism.

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Chapter 1:

Introduction.

Antimicrobial Detergents and Preservatives.

In the 1970's the U.S. Food and Drug Administration and the US and world-wide Pharmacopoeia issued mandates that all multi-dose ophthalmic preparations were to contain a preservative to maintain a non-hazardous level of contamination. The preservatives in ophthalmic solutions have decreased the incidence of ocular infection. They have provided a level of antimicrobial activity in the bottle and limited bacterial, mycotic and ameoboid ocular infections caused by contaminated solutions (Noecker, 2001). Preservatives also prolong the shelf life of the formulation by preventing biodegradation and maintaining drug potency (Tripathi *et al.*, 1992).

Since the 1960's the development and popularity of contact lenses has increased the use of non-prescribed 'over-the counter' contact lens wash solutions and general availability of other ocular treatments for dry eye. At the same time there was growing use of ocular drugs for glaucoma in the ageing population. Chronic use of these multi-dose solutions led to increased reports of patient reactions and bacterial contamination of the solution during use (Hovarding and Sjursen, 1982).

Today, ocular solutions and medications are often composed of unique mixtures of the active drug, possibly a drug delivery system to preferentially transport the drug to its site of action, a preservative, viscosity-increasing agents, buffers and stabilisers, and a vehicle by which all the above ingredients are "carried". Of these, it is the preservative which is most often considered the culprit in damaging the corneal epithelium leading to disruption of the glycocalyx, when drops are used beyond the recommended dosing. This sequence leaves the epithelium unable to keep the tear film in place and can lead to ocular surface disease. However, research on this speculation has been scarce. Preservative associated toxic and inflammatory changes of the ocular surface are now considered a major patient complication reported by clinicians (Debbasch *et al.*, 2000; Abelson and Washburn, 2002).

Purite™: a Preservative and Oxidant.

The objective of this research investigation was to understand some of the mechanisms of action of novel ocular preservative formulations containing Purite™. Purite™ is a trademark of Allergan Corporation. It is a stabilized oxy-chloro complex purchased from Bio-Cide International, Inc (Norman, Oklahoma. USA). Reviews in ophthalmology journals have described Purite™ as a comparatively safe and effective preservative compared to other ocular formulations on the market (Noecker, 2001; Abelson and Washburn, 2002).

Many chemical preservatives were developed initially as detergents and disinfectants. Generally, disinfectants are used to kill micro-organisms, but not bacterial or fungal spores. In contrast, preservatives are added to foods, pharmaceutical/ healthcare products and cosmetics to limit microbial growth during shelf-life and use (Russell, 1991).

Stock Purite™ solution is defined as having sufficient oxy-chloro complex to generate ~2% w/v chlorine dioxide. The reaction stoichiometry indicates that five molecules of sodium chlorite, in the presence of acid, will generate four molecules of chlorine dioxide. Therefore, a maximum yield of 80% can be achieved. Purite™ 2% stock solution has a pH range of 8.2-8.7. This helps maintain the stability of the oxy-chloro complex. The main oxy-chloro compound in Purite™ is sodium chlorite. The 2% stock solution consists of sodium chlorite ($NaClO_2$ 3.35% w/v), sodium chlorate ($NaClO_3$ 0.04% w/v), trace amounts of dissolved chlorine dioxide (ClO_2), sodium chloride (NaCl trace amounts), sodium carbonate/sodium hydroxide (to provide pH= 8.2-8.7), and ~97% water. Sodium chlorite has been described as a latent oxidant, with low reactivity and high water solubility. Chlorine dioxide is a very reactive strong oxidant, and is a free radical gas that is soluble in water. Purite™ can be converted to chlorine dioxide by acidification, exposure to light, metal catalysts, and certain oxidising agents.

Purite™ has shown a high margin of safety and low cytotoxicity compared to conventional ophthalmic preservatives such as benzalkonium chloride (BAK). Purite™ is formulated in the Refresh Tears, Saline-Purite™ and Alphagan-P finished products at 50ppm (0.005% w/v) to provide adequate preservative effectiveness over the shelf life of the product. These products are unique in their preservative formulation. Refresh

Tears is the market leader in over-the-counter dry eye treatments within the U.S.A. (Allergan, 2001). Alphagan-P has been well received by clinicians as a new formulation of an existing glaucoma drug with Purite™ replacing BAK showing promising results (Katz, 2002). Although the preservative mechanism of action is not fully elucidated, Purite™ has been described as a transient oxidative preservative (Noecker, 2001) due to its oxidation potential.

A Brief History.

In pre-historical times putrification of both food and wounds were common. The earliest forms of preservation were for food. This was accomplished by the simple drying of seeds and meat. Later salting, pickling, smoking, and sugaring were used to adjust the water activity in foods and, thus, preserve them from spoilage by microbes. However, at this time the concept of spontaneous generation of micro-organisms still existed. Thus, the existence of micro-organisms wasn't known.

Indeed, the Persians (circa. 450BC) knew that water stored in copper and silver vessels remained sweet; while in earthenware containers it rapidly became foul. Aristotle recommended to Alexander the Great that, to avoid illness, troops should boil water before drinking it. From these shrewd observations the beginnings of preservation and sterilisation techniques arose. However, it was not until Pasteur discovered that milk and wine souring was due to micro-organisms (Pasteur, 1866) that the fuller role of anti-microbial agents and sterilisation techniques became apparent.

As industrialisation took hold in the 18th and 19th centuries many scientific discoveries of novel antimicrobial, detergent, and disinfectant compounds occurred (Hugo, 1991). These helped in the mass manufacture of food, pharmaceutical and medicinal products free from viable microbes such as bacteria and fungi.

More relevantly, contamination of ophthalmic preparations used in contact lens and multi-use eye drop solutions has been recorded since their introduction (Ayliffe *et al.*, 1966; Savin, 1967; Templeton *et al.*, 1982). The Medicines Act now restricts these solutions to be of a sterile nature (Anon. 1968). Prolonged exposure to traditional ophthalmic solution preservatives has led to eye irritation (Norton *et al.*, 1974; Richardson *et al.*, 1979). Hence, their preservative formulation in this regard is rather more demanding.

Types & Characteristics of Chemical Preservatives Used In Ophthalmic Pharmaceuticals.

Sterilisation and preservative processes/agents may exhibit a chemical (e.g. glutaraldehyde) or physical (e.g. heat) mode of antimicrobial action, or a combination of both (Block, 1991). Today's definition of an anti-microbial agent is 'one which will quantitatively reduce or inhibit the growth of microorganisms (Black, 1996).

Disinfectants and preservatives must show anti-microbial activity in a variety of environments, even though the environment may modify its efficiency against specific species. The anti-microbial agents used in ophthalmic pharmaceuticals, and a few specifically related compounds including mercurials, coal tar extracts, halogen based compounds, perborates, peroxides, quaternary ammonium compounds and alcohols, will be reviewed in a historical context.

Mercury has been used since the 4th century. Arab physicians used mercuric salts as antiseptics. They became unpopular in the 1950's, due to growing environmental awareness. However, phenyl-mercuric salts and organo-mercurials, including Thimerosal, Figure 1.1, and phenylmercuric salts, continue to be licensed in sterile pharmaceutical formulations (Denyer, 1996).

Coal Tar Extracts including the phenols and cresols were discovered by Smith in 1836. Their potential was not fully appreciated until Lister used them to develop aseptic surgery (Lister, 1867). Phenol, and later halogenated derivatives, were the first properly characterized antimicrobial agents. Indeed, the Phenol Index (Rideal-Walker Test; Anon, 1934) was widely accepted for comparing new compounds. Some phenol derivatives continue to be used as ocular preservatives include methyl and propyl paraben. Their usual concentration of use is 0.1% and at this level their mode of action is to disrupt the microbial cell wall and the cell membrane causing cell lysis.

Chlorine has never been used as a preservative alone, but chlorine water (Semmelweis, 1801) was used as a disinfectant, and this led to other chlorine based preservatives. Hypochlorites were discovered in 1744 in Javel, France. Highly reactive chlorine was 'fixed' by bubbling it through a pot-ash solution. This was initially sold as a bleaching agent, then as a disinfectant. By 1836 the London Pharmacopoeia contained a preparation that was essentially calcium hypochlorite (CaOCl). Hypochlorites are now

used as pharmaceutical disinfectants and the chemical structure can be seen in Figure 1.1.

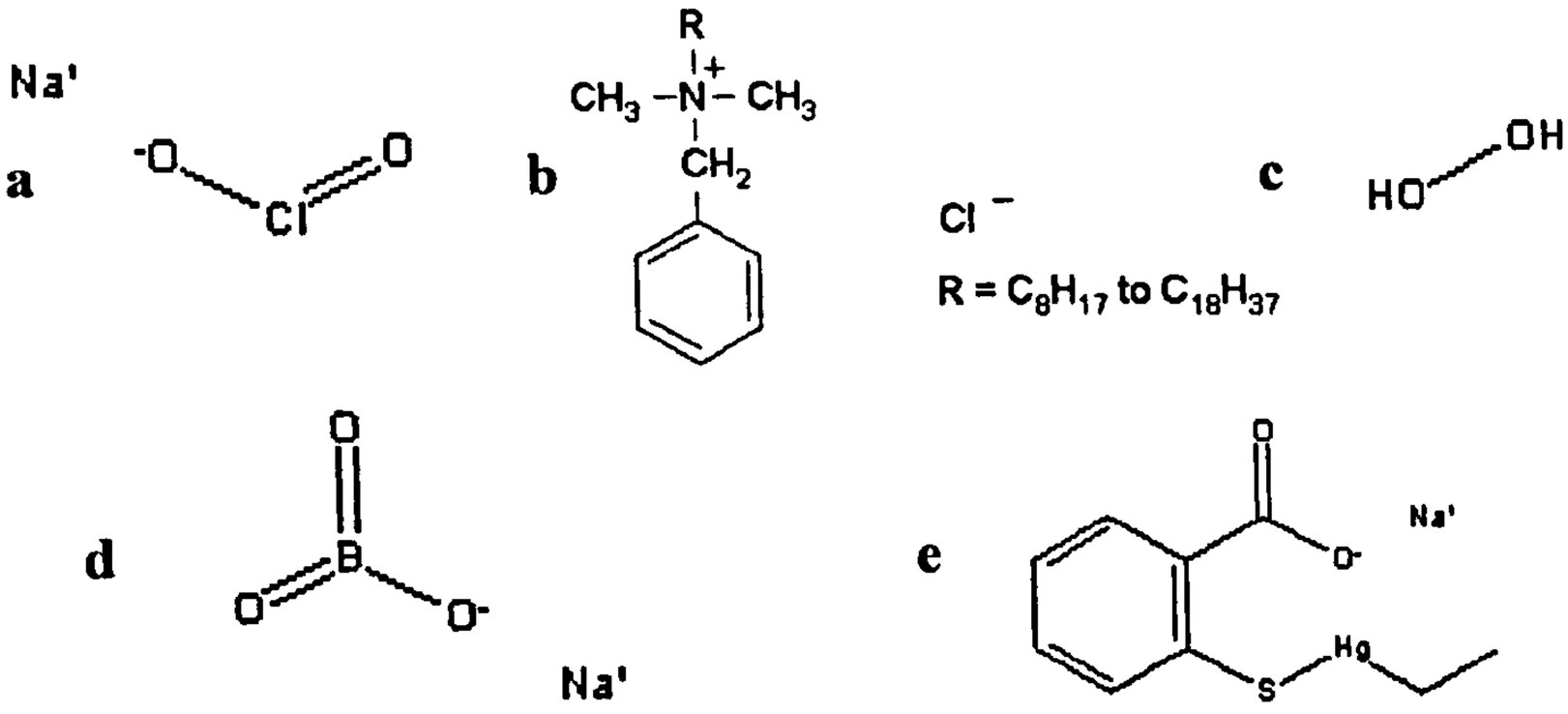


Figure 1.1 Chemical Structure of a:Purite ™ b:Benzalkonium chloride c:Hydrogen peroxide, d:Sodium perborate and e:Thimerosal (organo-mercurial).

N-chloro compounds were developed by Dakin (1915a,b,c) and are different from the chlorinated aryl-compounds. Chlorite compounds have been used since 1944 primarily used as biocides (Masschelein, 1979). Sodium chlorite, $NaClO_2$, (Purite ™) is recently licensed as an ophthalmic-pharmaceutical preservative and is shown above in Figure 1.1. It has been observed to differ from the other halogen based preservatives by showing positive differential toxicity between microbial and ocular tissue related complications. Other uses of sodium chlorite include the treatment of certain drinking water and food preparations to lower the microbial load.

Chlorinated aryl compounds are used commonly as disinfectants in medical and pharmaceutical laboratories. Dakin's solution, toluene-4 sulpho sodiochloramide, is still used as a disinfectant.

Iodide solutions are formed in alcohol and water as potassium iodides. These compounds were first used clinically by Davies (1839) and were subsequently evaluated by Koch and Pasteur. Iodinated compounds were later developed by Shelanski in 1949 as topical anti-microbial agents. These are reported to have a better patient acceptability.

The first use of chlorhexidine as a disinfectant, in 1916, led to its popularity in industrial cleaning (Davies *et al.*, 1954). Chlorohexidine is an amidine compound that

has also been used at preservative doses of 0.005-0.01% to inhibit potassium transmembrane transport.

Quaternary ammonium compounds (QACs) are positively charged derivatives of ammonium ions. The QAC benzalkonium chloride (BAK) has detergent, disinfectant and preservative properties. It has been used since the 1960's in the majority of preserved multi-use ophthalmic products at 0.02-0.005% (Denyer, 1996; Prisella *et al.* 2000). Alkyl triethanol ammonium chloride, benzethonium chloride, cetrimide (cetyltrimethylammonium bromide), dymed and polyquad are also examples of quaternary ammonium compounds that are used in ophthalmic preparations. The newer generation QAC polyquaternium-1 (Polyquad) is a polymeric quaternary ammonium antimicrobial preservative. It can be found in both contact lens solutions and the artificial tear product Tears Naturale II (Alcon). Polyquad has been proven to have less of an effect on corneal epithelial cells than BAK (Pham and Huff, 2000). One rabbit study showed that Polyquad decreases the uptake of fluorescence dye into the cornea compared to BAK and only causes superficial epithelial damage compared to BAK.

Perborates are strong oxidising agents which function as anti-microbial agents. As one of the first oxidative preservatives to be developed, they destroy numerous types of bacteria and can destroy *Aspergillus niger*, a fungus that is difficult to kill. When sodium perborate is combined with water it is converted to hydrogen peroxide, an effective antimicrobial agent (Chibret, 1997). Genteal (Novartis Ophthalmics), an ocular lubricant, is currently the only product containing a sodium perborate preservative system. Hydrogen peroxide (H_2O_2) has been used as an anti-microbial agent and ophthalmic disinfectant for many years since its introduction in 1887 (Duliba, 1995; Hugo, 1991).

Chlorobutanol is an alcohol-based preservative. It works by disorganising the lipid structure of the cell membrane, which increases permeability of the cell and leads to cell lysis. Chlorobutanol at 0.5% has broad-spectrum antimicrobial action. It has been found to be safe in rabbit cornea cells, even at 100 times the concentrations found in commercial products (Van Ooteghem, 1995). Other alcohol-based preservatives include benzyl and phenylethyl alcohol and phenoxyethanol.

Dilutions of disodium edetate EDTA in the region of 0.4% are used in preparations as a chelating agent. As a chelating agent, it is active upon divalent cations

and so can help remove calcium carbonate or calcium phosphate deposits from the ocular surface tissue, and is, therefore, useful for irrigation in ocular emergencies. Lower concentrations, typically less than 0.1%, are used as a preservative to chelate microbial membrane magnesium and act as a mild bacteriostatic agent in combination with BAK.

One simple way of grouping ophthalmic pharmaceutical preservatives is by their general chemical and cellular route of action (Denyer, 1996). The compounds reviewed above can be classified by having an oxidative or non-oxidative chemical basis/route of cellular interaction. These are shown in Table 1.1.

Oxidative Based Agents		Non-oxidative Based Agents
Hydrogen Peroxide Sodium perborate Purite ™		Mercurials QACs Alcohols Chlorhexidine EDTA

Table 1.1. Preservatives grouped by chemical activity.

Physical Chemistry of Antimicrobial Preservatives.

The factors that may alter a preservative's anti-microbial activity include preservative kinetics, pH, organic matter, incompatabilities, and the type of cells being treated.

The kinetics, or dynamics, of the anti-microbial process were examined by Kronig and Paul in 1897. These studies led to the introduction of the dilution coefficient, η, which is a measure of the effect of changes in concentration upon cell death rate. It is more precisely defined as:

$$\eta = \frac{(\log t_1 - \log t_2)}{(\log C_2 - \log C_1)}$$

Where t is kill time, and C is concentration (Russell, 1990).

It has been proposed that a relationship may exist between η and the type of interaction that occurs between a preservative and its target cell. Agents may be divided into three groups by their η value. Compounds that have $\eta < 2$, although widely different

in their mode of action, interact strongly on a chemical basis. Their η value may correspond to an order similar to that of a chemical reaction. Agents with $\eta > 4$ are thought to have a weakly physical interaction with the lipophilic components of the cell envelope, as chemical reactions of this order are unknown. Whereas, preservatives with an η between 2 and 4 appear to have the ability to act both in a physical and chemical manner (Hugo and Denyer, 1987).

A substance with a high η value, e.g. phenolics where $\eta=6$, show a marked increase in the time necessary to achieve a comparable cell-kill when dilute, if other conditions remain unchanged. In contrast, preservatives with a low η values, e.g. mercurials and QACs, cytotoxic kinetics, are less influenced by concentration.

An increase in temperature normally increases a preservative's antimicrobial activity (Russell, 1990). This is expressed in the equation:

$$\theta^{T2-T1}=k_2/k_1$$

θ = temperature co-efficient per $^{T2-T1}$°C rise.

k_2 and k_1 = rate constants for cell kill at temperature T^2 and T^1, respectively.

This property was commonly used in the preparation of eye drop products, with added preservatives, when sterilised or heated to 100°C (Russell, 1990). The dependence of reaction rate constants on temperature is given by the Arrhenius equation:

$$k = Ae^{-Ea/RT}$$

where: A is the Arrhenius constant,

e is the mathematical constant (2.718...)

E_a activation energy (in joules per mole)

R is the gas constant (from pV=nRT), and

T is the temperature (in Kelvin)

The Arrhenius equation was originally applied to calculate the effect of heat upon chemical and enzymatic reactions, but was later extended to complex processes such as microbial growth (Farrell and Rose, 1965). Reactions that are enzymatic give a linear 'Arrhenius plot' over a specific temperature range (30-40°C, Hagen, 1971). For bacterial growth above and below 30-40°C, linearity is lost and growth slows to a stop (Gounot, 1991). This is supported by the fact that a θ^{10} value for both chemical and

enzymatic reactions is 2-3. The θ^{10} varies dramatically for disinfectants (Hugo and Russell, 1992). Consequently, it has been suggested that trying to combine chemical and disinfection/preservation kinetics has no foundation. This may, in part, be due to the effect temperature change has, at specific thresholds, upon the other factors influencing antimicrobial activity.

The effect of pH can be two-fold; it can affect the preservative molecule and/or affect the microbial cell surface. pH can also influence partitioning of a compound between the external solution and the microbial cell (Bean, 1967). Substances showing a change in activity with pH shift include phenols, hypochlorites, iodine compounds, QACs, chlorhexidine, and glutaraldehyde. Organic acids and phenols show decreased antimicrobial activity with increased dissociation at increased pH (de Navarre, 1962; Wedderburn, 1964). The antimicrobial activity of hypochlorites also decreases with increasing pH (Bean, 1967; Dychdala, 1968; Truman, 1971), since the active bactericidal factor is undissociated hypochlorous acid (Lee and Reimann, 1971). As discussed previously, the effect of pH is clearly noted in Purite™ chemistry. At pH's <8.7, $NaClO_2$ is converted to $HClO_2$ and by pH 7 ClO_2 forms naturally. In contrast, activity of QACs rises as pH rises due to their increased cellular uptake by microbes (Sykes, 1965).

The presence of organic matter, e.g. serum, dirt, blood, etc., may reduce the effectiveness of preservatives. This may arise either as a result of a chemical reaction between the compound and the organic matter, thus, leaving a small antimicrobial concentration for attacking micro-organisms, or because of a blocking effect which protects the organisms from attack (Sykes, 1965). Organic matter decreases the efficacy of hypochlorites against bacteria (Truman, 1971), *Mycobacterium* (Croshaw, 1971), bacterial spores (Russell and Ditchett, 1973) and viruses (Morris and Barlow, 1971). Basically, pharmaceuticals require adequate formulation design and manufacture to stringent quality assurance parameters to enable the preservatives in the formulation to act in a consistent and predictable manner during normal use. One such approach would be to use a combination of disinfectant/preservative agent and a detergent. In 1967, Clegg indicated that hypochlorite at pH 11 gave an enhanced disinfectant effect. As stated above, hypochlorites are more active oxidants at an acid pH, but they achieve a more rapid removal of a bacterial film at pH 11 through saponification of

polysaccarides, glycoproteins and other constiteunts of the outermost glycocalyx. Clegg (1967) claims the high pH alone was responsible for the enhanced killing observed, but it may be that upon absorption of HOCl into the bacterial cytosol that the pH was lowered, to a physiological pH where hypochlorites are more oxidative, and the combined effects enhanced bacterial biofilm killing.

Macromolecular polymers, such as polyethylene glycol 4000, gelatin, and non-ionic surfactants, may be incompatable or interact with preservatives. This has been noted to reduce the preservative activity of QACs and benzoates (Deluca and Kostenbauder 1960). In the presence of such polymers a higher concentration of preservative is required to inhibit the growth of a given microbe. This concentration is defined as the Minimum Inhibitory Concentration (MIC) for the preservative. In some circumstances preservative action may be enhanced by surfactants (Elworthy, 1967; Richards, 1972). Soluble surfactants have a polar character and an affinity for water. Preservative balance between lipid and aqueous phases, due to the drug formulation can potentiate antimicrobial activity of specific agents by determining how much preservative is available in each phase and how readily it with cross lipid membranes.

The concentration of preservative in the aqueous phase may be obtained from the equation:

$$C_w = C(\phi)$$

Where C_w is the preservative concentration (in water);

C is the total preservative concentration and

ϕ is the water/oil ratio.

Cellular Aspects.

Gram-positive bacteria, Gram-negative bacteria, bacterial spores, viruses and fungi show considerable differences in their structure and biochemical composition. As expected, this is reflected in the heterogeneous sensitivities of prokayotic and eukaryotic microbes to the various preservatives in use. All of these microbial groups contain potentially pathogenic organisms. Gram-positive bacteria have for some years caused concern in hospitals because of their drug resistance, e.g. multi-drug resistant *Staphylococcus aureus. Staphylococcus aureus* and *Staphylococcus epidermidis* contamination of eye dropper bottles has been reported (Geyer *et al.*, 1995). Gram-negative bacteria including *Pseudomonas* species and *Serratia marcescens* frequently

contaminate multi-use ocular solutions (Schein *et al.*, 1992). However, they appear less sensitive to preservatives than Gram-positive bacteria (Lawrence, 1969; Baird-Parker, 1971). This may result from the considerable differences found in the cell envelopes of these two groups of bacteria (see Chapter 2). Preservatives are generally non-sporicidal, although they should inhibit cell growth upon bacterial or fungal spore germination (Russell, 1971). The fungi found to contaminate the solutions of interest include *Candida albicans* and *Aspergillus niger* (Geyer *et al.*, 1995; Schein *et al.*, 1992).

D'Arcy (1971) researched anti-fungal agents extensively. The conclusion of his work was that compounds with anti-bacterial activity generally exhibit some anti-fungal activity. Potent anti-fungal activity has been observed by the cationic QAC benzalkonium chloride at preservative concentrations (Furrer *et al.*, 2002). Certain fatty acids have also been shown to have significant anti-fungal activity (Knapp and Melly, 1986).

The Eye.

Assuming that, during normal use, preservative agents in ophthalmic formulations will also be exposed to the ocular surface, it is important to understand the normal anatomy and physiology of the relevant tissues, and any potential toxic effect preservatives may have. Due to their lack of microbial specificity, current ophthalmic preservatives can potentially cause ocular damage. This risk may be enhanced due to the ocular dysfunction/injury they are attempting to treat. Damage may be initially upon the front of the eye and then the back. These concerns support the need for a better understanding of the mechanisms of action of preservative compounds.

The Ocular Surface.

Thoft and Friend (1977) coined the term 'ocular surface' to emphasize the potential interdependence of the stratified non-keratinizing epithelium of the cornea and conjunctiva. Initially there was an anatomical or histological classification. Research later led to a functional relationship between these two adjacent cell populations of the eye, as seen in Figure 1.4.

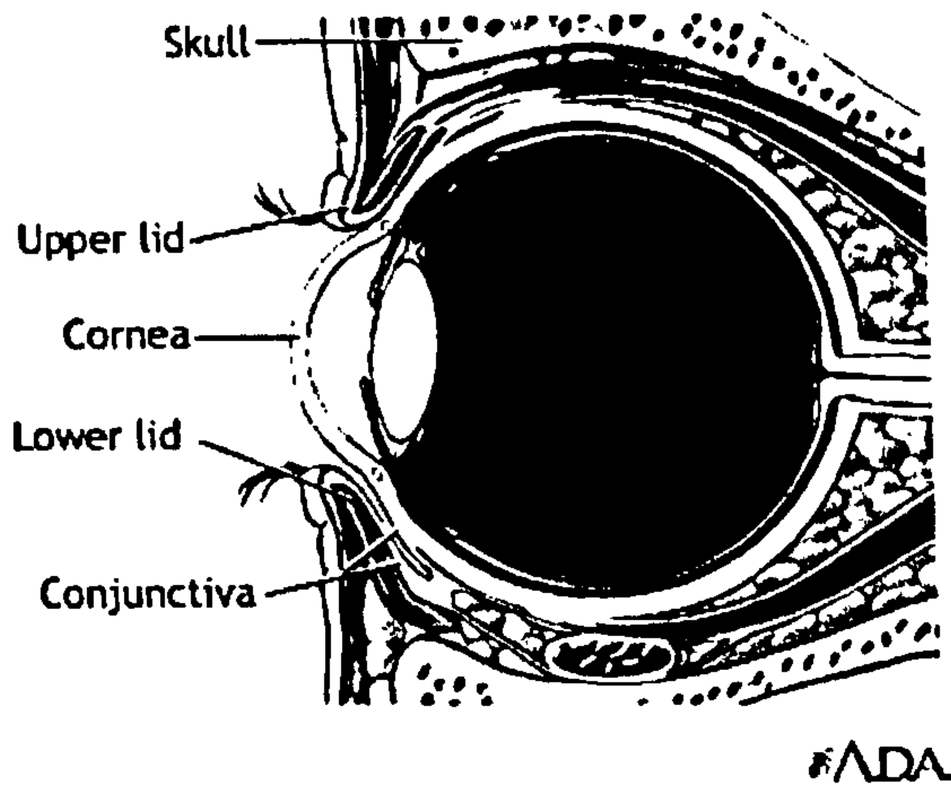


Figure 1.4. Overview of ocular anatomy (National Library of Medicine)

The corneal epithelium is made up of 5-6 cell layers. Surface cells are normally sloughed off into the tear film at a rapid turnover of 4-6 days (Hanna *et al.*, 1960). Non-keratinized squamous stratified epithelial cells normally populate these tissues. These characteristics play an important role in maintaining a smooth optical surface which is vital in excluding microorganisms from the cornea. Tears undergo four processes: production by the lacrimal gland, distribution by blinking, evaporation from the ocular surface and drainage through the nasolacrimal duct. Abnormalities in any of these steps can cause dry eye. There are two kinds of tear production, basic and reflex, which can be distinguished from each other by the Schirmer test with nasal stimulation (Matarasso, 2002). Reflex tearing is important because it supplies such essential components as epidermal growth factor and vitamin A. Deficiency of these may cause squamous metaplasia. There is no reflex tearing in Sjogren's syndrome because of destruction of the lacrimal gland. In cases of diminished or absent reflex tearing, topical autologous serum is the treatment of choice. Even when there is adequate tear production, insufficient distribution, such as occurs with the decreased blinking associated with the use of visual display units (VDU), may cause dry eye. Any process or activity that suppresses blinking interferes with tear distribution. Tear evaporation increases under certain conditions and in some diseases. When the exposed ocular surface area is increased, such as in VDU work, tear evaporation increases. Meibomian gland dysfunction (MGD) also causes increased tear evaporation by altering the quality of the

oily layer in tears. Tear evaporation can be suppressed by using a humidifier or wearing protective eyeglasses. The tear clearance rate is measured by fluorescein dye dilution in the conjunctiva.

At the periphery of the cornea (the limbus) the stratification thickens to approximately 10 cell layers. From here stem cells migrate along the basal epithelial layer and mature as they move anteriorly toward the surface. The limbus is vascular, while the corneal zone 1mm beyond this is truly avascular in non-pathological states (Allansmith, 1984). This cellular arrangement is vital to the maintainance of vision, both in healthy eyes and those with a surface injury associated with contact lens use (Bruce & Brennan, 1990).

The conjunctival epithelium consists of 2 or more layers of stratified columnar cells with numerous goblet cells. It is well established that stem cells of conjunctival epithelium are capable of re-populating the corneal epithelial surface following traumatic injury. Conjunctival cells have many important roles to facilitate ocular surface function. When the tear clearance is low, inflammatory cytokines or preservatives accumulate in the conjunctival sac, resulting in ocular surface diseases. Frequent use of artificial tears is the key treatment. Upon chemical injury these functions are compromised and this affects mucous production and limbal vascular supply.

The corneal surface regenerates by centripetal movement of cells from the peripheral cornea, limbus or conjunctiva into the visual axis. This continues, at an increased rate to the normal replacement of corneal epithelium, after trauma. Irrespective of the original source of the epithelium, if it is intact it prevents the development of corneal ulceration and keratitis. The rate of epithelial migration, after injury, may be slowed by persistant inflammation. Clinically, it is well established that after severe or chronic injury, resurfacing of the cornea occurs with conjunctival epithelium. Delayed re-epithelialization, superficial and deep stromal neo-vasularisation, and the persistence of conjunctival goblet cells within the corneal epithelium are possible. All pathologies lead to impaired function of the ocular surface, which may cause impaired vision.

Studies have been reported to show that preservative concentrations below the effective anti-microbial concentration cause little or no ocular cell damage using

0.005% BAK (Ingram *et al.*, in press), 0.005% Thimerersal (Browne *et al.*, 1975), 0.04% Parabens (Dormans and van Logten, 1982) and 0.001% Chlorhexidine (Burnstein, 1980). However, tissue cytotoxicity was reported in all of these studies in ocular tissues treated with preservative concentrations, well above the MIC, found to be nearer the preservative formulation levels.

More specifically, the most commonly used preservative, BAK, has been investigated by many groups and shown to cause a wide range of responses in the eye. At the cellular level BAK has been found to generate the loss of microvilli and integrity across the corneal cell membrane, swelling, lifting of the cell borders and cellular desquamation (Green, 1992; Tonjum, 1975). BAK has also been shown to decrease the conjunctiva cell viability (Barkman *et al.*, 1969) and tear film stability (Burnstein, 1985). Allergic responses have been reported with patients using thiomersal (Anon, 1972) phenylmercuric nitrate, BAK (Chiambareta *at al.*, 1997) chlorhexidine, EDTA, polyquat and sorbic acid preservative formulations.

The deleterious effects of most preservative formulations have been found to become magnified when combined with EDTA (Kilp and Brewitt, 1984; Collin and Carroll, 1986).

The Chemistry of Oxidation

This section describes the reactions that an oxidative preservative may undergo to exercise its effects. The term oxidative relates to oxidation, and hence involves oxygen. A free radical is a species capable of independent existence that contains one or more unpaired electrons. Free radicals can be formed by homolytic cleavage of a covalent bond, by addition of an electron, or by abstraction of an electron. Oxygen tends to occur as the dimer (O_2), which has 2 unpaired electrons. However, having 2 restricts its reactivity compared to other oxygen containing molecules. Superoxide is oxygen plus an electron (now one unpaired). The reactions of molecular oxygen (O_2) were researched by Harber and Willistatter (1931). They identified that it can accept up to 4 electrons to give products in the following order: Oxygen (O_2), Superoxide ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH.) and water (H_2O) (Harber and Willistatter, 1931; Fridovich, 1978). Thus, not all intermediates of oxygen are free radicals. This depends on how many electrons have been added. The other

intermediates are called Reactive Oxygen Species (ROS). These are also relatively reactive and damaging to bio-molecules. These are shown in Table 1.2.

Reactive Species (Oxidants)	Free radicals
Hydrogen peroxide (H_2O_2)	Superoxide ($O_2^{-\cdot}$)
Singlet oxygen ($1O_2$)	Triplet oxygen ($3O_2$)
?Purite™ ($NaClO_2$)?	Hydroxyl radical (OH.)
Hypochlorous acid (HOCl)	Chlorine dioxide (ClO_2)
Sulphur dioxide (SO_2)	

Table 1.2 Grouping of intermediates of oxygen and other species.

Chemical Interconversion Reactions

All the species detailed in Table 1.2 may produce superoxide, hydrogen peroxide or hydroxyl radicals by being inter-converted in various chemical reactions, often involving the presence of transition metals. The most common of these reactions is the Fenton reaction (below) (Halliwell and Gutteridge, 1985). The -O-O- bond in hydrogen peroxide is not very strong, and is susceptible to homolytic cleavage to give the hydroxyl radical; this is accelerated by either Fe^{2+} or Cu^{+}.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^- \qquad \text{(Fenton reaction)}$$

Iron can redox cycle in combination with superoxide:

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$

The total reaction is effectively an Fe^{2+}-catalysed reaction between hydrogen peroxide and superoxide:

$$O_2^- + H_2O_2 \rightarrow OH\bullet + OH^- + O_2 \qquad \text{(Haber-Weiss reaction)}$$

Superoxide can also dismutate to hydrogen peroxide and oxygen in a reaction that is catalysed by superoxide dismutase. These intermediates are highly interchangeable in biological systems.

$$2\,O_2^- + 2\,H^+ \rightarrow H_2O_2 + O_2$$

The potential for damage is also dependent on the solubility of the compounds. Hydrogen peroxide and oxygen are both very soluble in organic, lipophilic solvents, and will accumulate in phospholipid membranes. Oxidants and free radicals can attack most biomolecules, but it is the macromolecules including proteins, DNA and lipids that are the most susceptible and induce the most significant damage to cells.

Oxidation of Proteins

Proteins consist of chains of amino acids linked together by peptide bonds. Both the peptide linkages and the amino acid side-chains are at risk from oxidation. Cleavage of the peptide bonds will lead to scission of the protein backbone. This may not be critical to the structure of the protein, as globular proteins are largely held in their tertiary structure by the interactions of internal hydrophobic residues. However, oxidation may destabilise the protein if it occurs close to these hydrophobic residues. Moreover, the secondary structural elements are no longer constrained by Phi and Psi angles either side of the peptide bond, and thus, the secondary structure may be disrupted. A number of side-chains in the protein can readily be oxidised. As the side-chains determine both the tertiary structure and the function of the protein, this is likely to have a major impact on the protein and the cell containing it.

Cysteine is an amino acid which has side-chains containing a thiol (-SH) group that can be oxidised to a disulfide (-S-S-). This is important as disulfide bridges are used to link different parts of the polypeptide chain, or separate polypeptide chains. Cysteine oxidation can, therefore, result in cross-linking and aggregation of proteins. Dilley and Pirie (1984) researched this specific reaction in the ageing of the eye and implicated it in forming premature cataracts. Aggregation of a number of protein molecules results in precipitation. These proteins are unlikely to be functional.

Tryptophan has an aromatic side-chain containing nitrogen and several double bonds. The exact mechanism of tryptophan oxidation is not known. It probably involves the loss of the conjugated carbon ring, as it is established that tryptophan fluorescence is lost on oxidation. Hydroxylation may be one possible mechanism.

Oxidation of proteins as described above can have various effects. Inactivation of proteins, especially of the enzyme's mitochondrial redox catalytic centre, is common and may involve oxidation of cysteine residues. This has been researched using the newer proteomic analysis tools evaluating thiol interaction with a novel labelling compound (4-iodobutyl)triphenylphosphonium (Lin, *et al.*, 2002). Following more extensive oxidation the protein may denature, leading to precipitation. Crosslinking of sidechains will also cause aggregation of the protein. All these oxidative modifications involve the loss of catalytic or structural function, and can affect the operation of the cell or tissue.

The chemical modifications described above result from free radical attack, such as by hydroxyl radical or hydrogen peroxide. If other oxidants are present different modifications may occur. HOCl is a reactive oxidant that tends to perform electrophilic attack, adding across double bonds that researchers can easily detect by NMR (Arnold, *et al.*, 1995). Fu *et al.* (2002) have show that HOCl reacts with amine groups to give chloramines and lysine. This is a major target of HOCl in low density lipoproteins. It also oxidises cysteine, tryptophan, methionine, and can chlorinate tyrosine.

Oxidation of DNA

Preventing damage to, and alteration of, genetic material is important in all organisms. In eukaryotic cells, DNA is packaged in chromatin and compartmentalised in the nucleus. This may offer some protection against oxidative damage, compared to prokaryotic systems where the DNA exists loose in the cytosol (Demopoulos, 1973). Nucleic acids are susceptible to oxidation. It is known that damage is continually occurring and being repaired. DNA consists of a sugar-phosphate backbone with purine and pyrimidine bases attached to the deoxyribose. Both the backbone and the bases are susceptible to oxidative attack.

Free radical damage to the ribose ring under aerobic conditions causes backbone fragmentation. The exact mechanism depends on which hydrogen is abstracted on the

ribose ring. This can cause cleavage of the ribose-phosphate bond or of the ribose ring itself. The damage to DNA described can have various effects, all potentially deleterious. The effects can be twofold, primarily with truncation or mutation of the transcribed protein, and secondary due to errors perpetuated during DNA replication.

Chain scission can cause loss of genetic material and errors in replication. Modifications of the bases can cause mismatching, although DNA repair systems exist which excise altered bases (e.g 8-oxoguanine) and replace them. However, the conversion of cytosine to uracil is less likely to be detected, and will cause point mutations in the protein, possibly leading to dysfunction.

Oxidation of Cell Membrane Lipids

Lipids are integral and essential components of all cell membranes. They form the barrier which limits diffusion of substances into and out of the cell. Damage to the lipid part of the cell's plasma membrane alters the membrane integrity and, therefore, the viability of the cell. There are many different types of lipid, but phospholipids are commonly found in the membrane. These consist of a glycerol with 2 fatty acids (-acyl chains) and 1 polar head group (e.g. phosphatidylcholine, inositol, ethanolamine) joined via a phosphate group. Sphingolipids consist of an amine alcohol that contains a $(CH_2)_{12}$ chain (sphingosine), with another fatty acid joined via an amide, and a phosphatidylcholine headgroup. Cholesterol is a multi-cyclic sterol compound that lowers the membrane lipid phase transition phenomenon. Both phospholipids and sphingomyelins have fatty acids attached. These fatty acids may be saturated (no double bonds) or unsaturated (1 or more double bonds). Poly-unsaturated fatty acids (PUFA) are much more susceptible to oxidative attack. Examples of PUFAs are arachidonic acid (C20:4), linoleic acid (C18:2) and linolenic acid (C18:3).

One type of oxidative attack which may occur is lipid peroxidation. Lipid peroxidation can be defined as the 'oxidative deterioration of polyunsaturated lipids (Halliwell and Gutteridge, 1985). Lipid hydroperoxides are the major initial products of peroxidation (MacMillan, 1995). These are formed by a free radical process, consisting of three main events; initiation, a propagation cycle and termination. Within the cornea, lipid hydroperoxides are believed to play a role in ageing and cataract formation

(Gutteridge and Halliwell, 1994). Cellular susceptibility to lipid peroxidation *in vitro* is dependent upon the degree of poly-unsaturation of cellular lipids (Wagner *et al.*, 1994). Secondary peroxidation products include the cytotoxic breakdown products malondialdehyde and 4-hydroxynonenal (Negro *et al.*, 1981).

Peroxidation of lipids is initiated by the abstraction of hydrogen from the methylene group of a fatty acyl chain by a hydroxyl radical (Slater, 1984), $OH^{\bullet}$, or the protonated form of the superoxide anion, $HO_2^{\bullet}$ (reaction 1, Figure 1.5).

1	$L\text{-}H + X^{\bullet} \longrightarrow L^{\bullet} + X\text{-}H$		**Initiation**
2	$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$		
3	$LOO^{\bullet} + L\text{-}H \longrightarrow L^{\bullet} + LOOH$		**Propagation cycle**
4	$LOO^{\bullet} + LOO^{\bullet} \longrightarrow$	crosslinked fatty acyl chains	
5	$LOO^{\bullet} + H \longrightarrow LOOH$		**Termination**

Figure 1.5. Schematic outline of lipid peroxidation reactions.
Note: L denotes the lipid, H denotes hydrogen and
X denotes the initiating species.

Polyunsaturated fatty acids (PUFAs) with carbon-carbon double bonds (C=C) separated by methylene groups ($-CH_2-$), are particularly prone to hydrogen atom abstraction. Protons of bisallylic methylene groups have the weakest C-H bonds (75-80 kcal mol^{-1}), and so abstraction of these protons is energetically favoured (Koppenol, 1990). As such, these fatty acyl chains have a greater oxidisability. Linoleic acid (18:2) and arachidonic acid (20:4) are examples of highly oxidisable PUFAs, containing one and three bis-allylic methylene groups respectively (Wagner *et al.*, 1994) which can be removed to leave a carbon-centred radical. Following the carbon-centred radical formation, the radical is stabilised by molecular rearrangement to give a conjugated diene. Once formed, dienes can react further by a number of different pathways. Collision with another carbon radical causing cross-linking of the fatty acyl chains can occur, whereby the two unpaired electrons form a covalent bond (reaction 4). Reaction

with membrane proteins is also possible. However, the most common reaction of carbon-centred radicals is combination with oxygen, generating a peroxyl radical, $LOO^{\cdot}$ (reactions 2 and 3).

This is the chain-carrying radical of the propagation cycle. It is reactive enough to attack adjacent fatty acyl chains, causing further hydrogen abstraction and carbon-centred radical formation, and hence propagating the radical reaction (Halliwell, 1991). One $OH^{\cdot}$ radical is capable of causing the peroxidation of many hundreds of fatty acyl chains. Peroxyl radicals are capable of attacking a double bond on the same chain. This generates cyclic peroxide radicals, which can abstract hydrogen atoms and also generate isoprostanes (Nourooz-Zadeh *at al.*, 1997). Termination of the reaction can occur when a hydrogen atom reacts with the peroxyl radical, resulting in a lipid hydroperoxide, LOOH (reaction 5). To generate this reaction a chain breaking antioxidant is required.

In contrast to the free radicals which cause peroxidation, HOCl oxidises lipid by electrophilic attack, a 2-electron process that involves addition across double bonds. Unlike peroxidation, it doesn't require bis-allylic hydrogens, and so monounsaturated lipids are also possible sites of oxidative attack. This process results in the formation of lipid-chlorohydrins.

A membrane is a bilayer of phospholipids, with a hydrophobic core and polar headgroups on the outside. Oxidation of the fatty acyl chains results in the introduction of bulky polar atoms (oxygen or chlorine) into the hydrophobic region, thus disrupting the hydrocarbon chain packing. This leads to physical disruption of the bilayer and loss of membrane integrity.

Peroxide induced oxidative stress has been shown to cause corneal cell membrane blebbing (Tripathi, *et al.*, 1992). When the membrane is damaged, small blebs form on the surface and bud off. This probably results from the inability of the bilayer to fit together normally. This process also occurs in apoptosis, and is a method for reducing the size of the cell to help in its removal following programmed cell death.

Aims.

The overall aim of this project was to elucidate the differential mechanisms by which Purite™ acts as an effective antimicrobial agent for use in ocular solutions, while being less damaging than other popular preservatives in its effects on the eye. In understanding the differences in anti-microbial effect from its toxic effect an improved Purite™ formulation may be possible.

Purite™ is an oxy-chloro compound with structural similarity to sodium hypochlorite (NaOCl), the major component of bleach, which is commonly used as a disinfectant. The acidic form of hypochlorite, HOCl, is known to react readily with unsaturated fatty acids and antioxidants, in addition to a variety of other biological molecules. This study was, therefore, directed towards determining the effects of Purite™ on unsaturated fatty acids of phospholipids and a number of antioxidants, both in simple chemical model systems and in cells. The effects of commercially available oxidants and preservatives, some of which have established effects, were compared with Purite™.

Most cell membranes contain unsaturated phospholipids with essential structural and functional roles. Owing to the double bond(s) present in the fatty acyl chains, these are much more susceptible to oxidative damage than saturated lipids. Lipid peroxidation results from free radical attack and tends to occur preferentially on lipids containing two or more double bonds whereas HOCl commonly attacks unsaturated lipids by an electrophilic addition reaction (Jerlich *et al.*, 1998). Therefore, monounsaturated lipids are also susceptible. Spickett *et al* (1998, 2001) have shown in a number of previous studies that such oxidative modifications to phospholipids can be monitored in a sensitive and informative way using electrospray mass spectrometry (ESMS) detection. It is important in the understanding of the preservative effect of Purite™, to establish whether it can react with phospholipids and other biological molecules, and if so, whether this is by a radical or electrophilic mechanism. As one of our objectives, this end-point could be investigated by incubating Purite™ and other preservatives first with synthetic phospholipid vesicles as a model system described by Spickett *et al.* (1998), and then with a range of cell types. Our aim was to evaluate the effects of preservatives and control oxidants on the lipids and other biological molecules assessed by ESMS and nuclear magnetic resonance detection.

As antioxidants are present in all aerobic cells and are vital for protecting against damage by free radicals and oxidants. A common antioxidant, which is of central importance in mammalian cells, is the tripeptide glutathione (γ-glutamyl cysteinylglycine). Ascorbic acid (vitamin C) is another key antioxidant that occurs in plants, some bacteria and most mammalian cells. Ascorbic acid can be found in the aqueous humour at levels significantly above that in the plasma. It can be regenerated using glutathione following oxidation. Oxidized glutathione itself can be regenerated enzymatically using NADPH in what is known as the glutathione redox cycle. Glutathione levels are known to be important in protecting bacteria, fungi and mammalian cells from damage by peroxides and free radicals, while ascorbic acid has been shown to be effective in detoxifying HOCl. Determining the native levels of the antioxidants in test organisms, and their levels after treatment with the Purite™ and other preservatives, was another aim of this project. Furthermore, studies using inhibitors of the specific antioxidant pathways to determine their importance in Purite™ resistance/detoxification will generate new insights into the mechanism of Purite™ action.

For investigation of the mechanisms of action of Purite™, a variety of cell types were chosen to represent the eye and micro-organisms, which were thought likely to present a problem either through contamination of the solutions or infection. These were Rabbit Corneal Epithelial cell (RCE), Human Conjunctival Epithelial cell (WKD), *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans*, and *Alternaria* spp.

Preservative treatments used during this investigation were Purite™ (a novel preservative), BAK and hydrogen peroxide H_2O_2, preservatives known to cause pathologies of the eye (Bruce and Brennan, 1990; Riley and Wilson, 1993). Other treatments used as control oxidants were NaOCl (an oxidant known to cause chlorohydrins (Jerlich *et al.* 1998), $H_2O_2 + Fe^{2+}$, and *tert*-butylhydroperoxide (t-BHP) + Fe^{2+}(oxidants known to cause hydroperoxides (Spickett *et al.*, 2001)).

Chapter 2:

The interaction of preservatives with biological molecules *in vitro*.

Introduction

To investigate the potential mechanisms of anti-microbial action and/or resistance to Purite™ (sodium chlorite/$NaClO_2$) in comparison to other ocular preservatives, it was sensible to start with simple, *in vitro*, model systems. Thus, identifying changes that can arise; then correlating these *in vitro* changes to those seen *in vivo* in subsequent cell-based experiments. In this chapter the chemical changes that Purite™ was able to induce in biological molecules were investigated and these were compared to the changes created by control oxidants (hypochlorous acid (HOCl) and tert-butyl hydrogen peroxide (t-BHP)) and both oxidative (hydrogen peroxide (H_2O_2)) and detergent (benzalkonium chloride (BAK)) ocular preservatives. Biological molecules known to be highly susceptible to modification by oxidants were selected. Purite™ is an oxychloro compound with structural similarity to sodium hypochlorite (NaOCl), the major component of bleach, which is commonly used as a disinfectant. The acidic form of hypochlorite, HOCl, is known to react readily with a variety of other biological molecules including unsaturated fatty acids and antioxidants. This study was, therefore, directed towards determining the effects of Purite™ on unsaturated fatty acids of phospholipids and a number of antioxidants, both in model systems and in cells. The effects of other oxidants and preservatives, some of which have established effects, were also studied for comparison with Purite™.

Most cell membranes contain unsaturated phospholipids, which have essential structural and functional roles. The double bond(s) present in the fatty acyl chains make them much more susceptible to oxidative damage than saturated lipids. Lipid peroxidation results from free radical attack and tends to occur preferentially on lipids containing two or more double bonds. In contrast, HOCl commonly attacks by an electrophilic addition reaction. Therefore, monounsaturated lipids are also susceptible. Spickett *et al.* (1998, 2001) have shown in a number of previous studies

that such oxidative modifications to phospholipids can be monitored in a sensitive and informative way using electrospray mass spectrometry (ESMS). It is important in the understanding of the preservative effect of Purite™ to establish whether Purite™ can react with bio-molecules, and if so whether this is by a radical or electrophilic mechanism.

In particular, we were interested in its effects on cell membranes and intracellular antioxidants. Phosphatidylcholine vesicles were used as a model for membrane lipid damage. The aromatic amino acid tryptophan is known to be highly susceptible to modification by hypochlorite (Winterbourn and Brennan, 1997). Its oxidation was monitored to determine the possible interactions with aromatic amino acid residues in proteins. In addition, glutathione was investigated to determine what oxidative modifications occurred. These experiments allow the study of biological molecules involved in oxidative stress, in controlled reaction mixtures and compositions, within the simple in-vitro model systems. Analysis of the reactions by electrospray ionization mass-spectrometry (ESMS) and proton NMR can provide evidence of chemical changes arising in test and control samples. This was investigated by incubating Purite™ and other preservatives first with bio-molecules in model systems described below.

Biological Molecules and Oxidative Stress

As a rational for selecting biological molecules to assess, there are three main cellular sites for preservatives to exert their antimicrobial effect upon the organisms of interest. Firstly, at the membrane/envelope, second, in the cytosol, and third, upon the DNA. These may be considered individually as primary, secondary and tertiary sites of potential interaction.

Primary Interaction of Preservatives at the Cell Surface.

In order to be active, all anti-microbial agents must be capable of reaching their biochemical targets. These are commonly located within the cell envelope and cytosol, rather than being on the immediate outer surface of the cell. All agents must, therefore, move into the outer layers of the cell in order to reach anti-microbial levels

at a primary target. Differences in the cell surface between various species may affect the ability of a preservative to reach its site of action.

Bacteria have a different cell envelope structure compared to eukaryotic yeast and fungi. The bacterial cell envelope composition can also be differentiated by its ability to Gram-stain; so that species are divided into Gram positive or Gram negative. The major structural differences between Gram-positive and Gram-negative bacteria are shown in Figure 2.1.

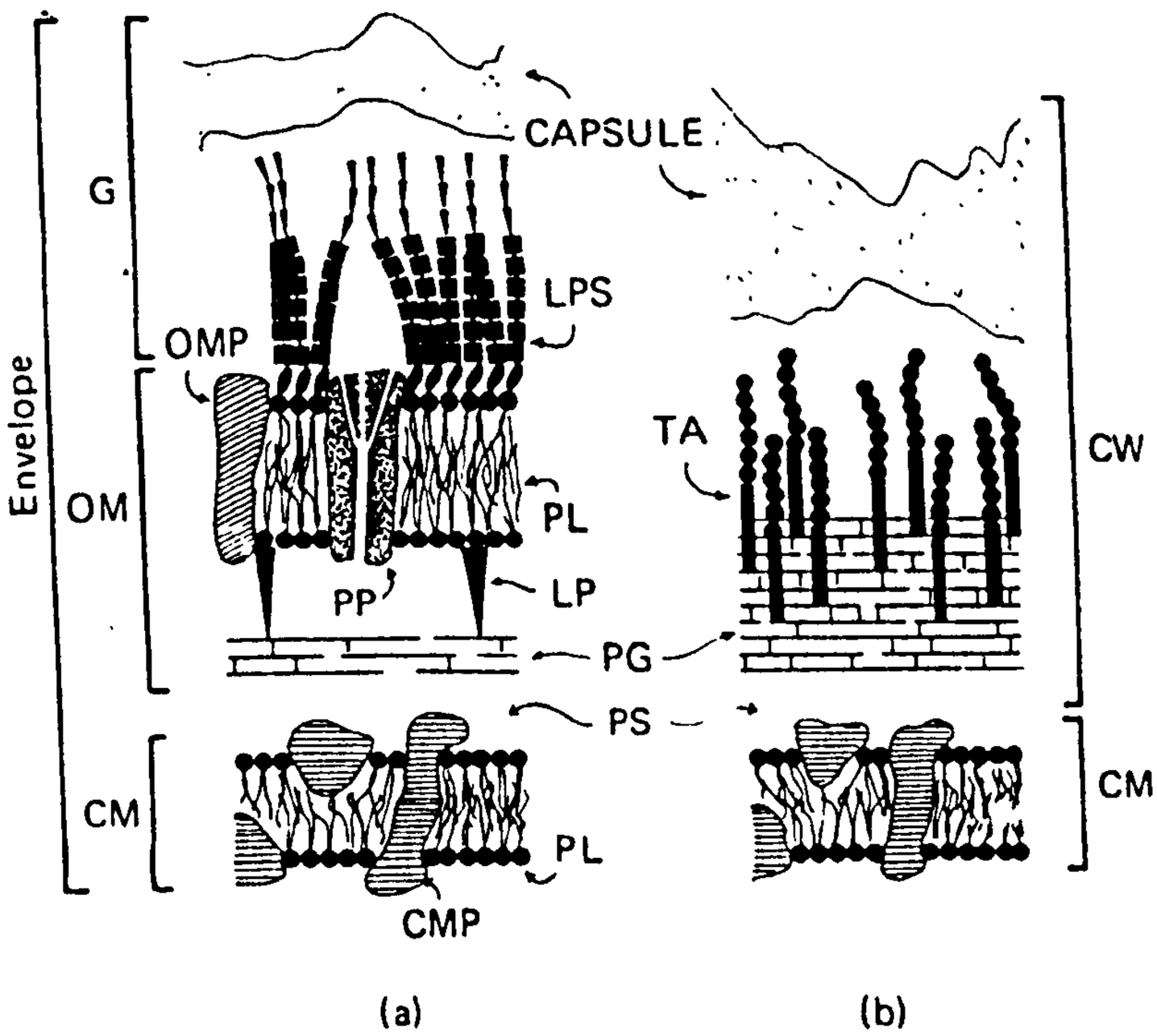


Figure 2.1. Diagrammatic representation of the (a) Gram-negative and (b) Gram-positive cell envelopes: CW= cell wall; PS= periplasm; CM= cytoplasmic membrane; OM= outer membrane; CMP= cytoplasmic membrane protein; G= glycocalyx; OMP= outer-membrane protein; LPS= lipopolysaccharide; LP= lipoprotein; PL= phospholipid; PP= porin protein, TA= techoic acid; PG= peptidoglycan. (Ref: Gilbert & Das, 1996).

Gram-negative bacteria generally have higher total phospholipid content and a much reduced mucopeptide capsule due to their cell wall when compared to Gram-positive bacteria (Russell, 1969). Prokaryotic bacteria have cytochromes, succinic dehydrogenases, and permeases embedded across the cell membrane (Nikaido and

Vaara, 1985), as they lack cytoplasmic organelles/mitochondria. This potentially places many sensitive reduction/oxidation biochemical pathways in close proximity to antimicrobial agents. However, in comparison with Gram-positive and eukaryotic cells, Gram-negative bacteria have an outer-membrane which creates a layer impervious to many hydrophobic agents. This is, in part, due to its structure since the inner leaflet of the inner-membrane is richer in phospholipids while the outer is richer in close packed lipopolysaccharide (Gilbert and Das, 1996). It has also been observed that the level of bacterial membrane porosity decreases as peptidoglycan cross-linkage increases.

All cells have cytoplasmic membranes with structural composition of the fluid mosaic model (Singer & Nicholson, 1972). Additional to this eukaryotic microbes, i.e., yeasts and moulds (fungi), have an external, chitin-rich, cell wall (Bartnicki & Garcia, 1968). Mammalian cells do not have a cell wall stucture.

The primary site of bacterial cell/preservative interaction is a stage for both oxidant and permeabilization mechanisms of action. It has long been accepted that cells whose membrane proteins and phospholipids have been oxidised show a marked loss of membrane fluidity and permeability (Sinclair *et al.*, 1990). Further to this, toxic reactive by-products, such as malonic-dialdehyde or 4-hydroxynonenal are formed (Kappus, 1987; Halliwell, 1991). Preservative inhibition of membrane proteins and enzymes involved in cellular bio-energetics and ATPase Ca^{2+} and K^{+} pumps amplifies cellular permeability changes (Lambert, 1984). Free radical inhibition of enzymes may result in rapid loss of bacterial cell viability. QACs have been implicated in damaging the outer membrane of Gram-negative bacteria, thus promoting their own uptake (Hancock, 1984).

Secondary Interaction of Preservatives.

Cytoplasmic and organellular enzymes (eukaryotes only), and proteins involved in microbial cellular metabolism and active transport can be disrupted further by both oxidising and non-oxidising preservatives (Aldrich *et al.*, 1981; Matheson *et al.*, 1979). BAK is a rapid inhibitor of lactate oxidase (Knox *et al.*, 1949). Mercurials and oxidants interact with thiol/sulphydryl groups of proteins, enzymes and antioxidant molecules to form mercaptides and thiolsulphonates

(Winterbourne and Brennan, 1997). Inactivation of these vital pathways leads to rapid increases in cellular permeability, loss of electrolyte balance and viability.

Tertiary Interaction of Preservatives.

The final point of potential interaction of a preservative is by covalent interaction with DNA (Prutz, 1988). Studies in eukaryotic cells have shown that chemicals acting in this way cause initial damage, which may result in mutations. Ultimately, loss of viability is due to sporadic damage to the genetic base code and DNA fragmentation upon replication (Spector *et al.,* 1989). Preservatives forming covalent bonds with DNA act over longer time periods in comparison to those acting at primary and secondary sites. The specific relationship between DNA fragmentation/chemical alteration of purine and pyrimidine bases and overall cell damage is not clear (Von Sonntag, 1987; Halliwell and Aruoma, 1991).

Oxidative Pathways and Oxidative Stress.

All aerobically respiring cells generate oxidation products and free radicals similar to those generated by the oxidant preservatives. Oxidative stress has been defined as a disturbance in the oxidant/antioxidant balance in cells in favour of the oxidant state. This state may be due to accumulation of normal oxidative products of cellular metabolism, or could result from the action of toxic chemicals. Reactions causing the cellular depletion of the antioxidant glutathione result in oxidative stress. In eukaryotic cells one of the major protective mechanisms which protects cells from subsequent free radical damage is the glutathione redox cycle. This cycle is not clearly apparent in bacteria (Penninckx *et al.,* 1993). The protective biochemical pathways, utilized in eukaryotic and bacterial cells against oxidant generated, free radical attack, afford cellular protection from oxidative damage.

Oxygen acting as a terminal 4-electron acceptor is eventually converted to water using cytosolic catalase, superoxide dismutase, thiol-redox enzymes and mitochondrial cytochrome C oxidative phosphorylation enzymes (Porter, 1984). Many of the biochemical pathways are membrane bound. Prokaryotic cells do not possess mitchondria and, thus, the only membrane site available is the cytoplasmic

membrane. In excess these reactive oxygen species and other free radicals can then interact covalently to create hydroperoxides and chlorohydrins in native lipids/phospholipids (Mason *et al.*, 1980; Hazen *et al.*, 1996). Further toxic oxygen species are generated auto-catalytically, by reactions causing altered glutathione redox balance or other thiol-based anti-oxidant concentrations. Reactive oxygen species can also give modified sulphydryl groups on native proteins/enzymes and DNA. Sensitivity of *Escherichia coli* to HOCl is associated with changes in systemic glutathione (GSH), glucose-6-phosphate-dehydrogenase, SOD and DNA alterations (Dukan *et al.*, 1999). From these researchers observations, systematic levels of cell damage result in cellular death. Ultimately this may result in alteration of membrane permeability, or lipid/phospholipid oxidation products reacting with respiratory proteins and DNA.

Antioxidant Mechanisms and Resistance.

Both eukaryotic and prokaryotic microorganisms may possess an array of biological molecules to prevent and resist antimicrobial agents exerting their effects. The array of mechanisms varies from species to species. Within eukaryotic cells, enzymatic protection against native oxidants, as previously discussed, are often given by cytosolic catalase, superoxide dismutase, and the glutathione redox cycle. Many of these enzymes have transition metals as co-factors.

Antioxidants are present in all aerobic cells and are vital for protecting against damage by free radicals and oxidants. A common antioxidant, which is of central importance in mammalian cells, is the tripeptide glutathione (γ-glutamyl cysteinylglycine). Oxidized glutathione itself can be regenerated enzymatically using NADPH in what is known as the glutathione redox cycle. The pathways of glutathione reduction and oxidation to detoxify reactive oxygen species are shown in Figure 2.2. Chemical analysis of GSH/GSSG oxido/reduction states have been investigated by nuclear magnetic resonance (Reglinski *et al.*, 1991). Glutathione levels may also be determined by HPLC (Newton *et al.*, 1981) and electrospray mass spectrometry (Carr and Winterbourn, 1997).

Glutathione levels are known to be important in protecting fungi and mammalian cells from damage by peroxides and free radicals, while ascorbic acid has been shown to be effective in detoxifying HOCl (Avery and Avery, 2001; Rose *et al.*, 1998).

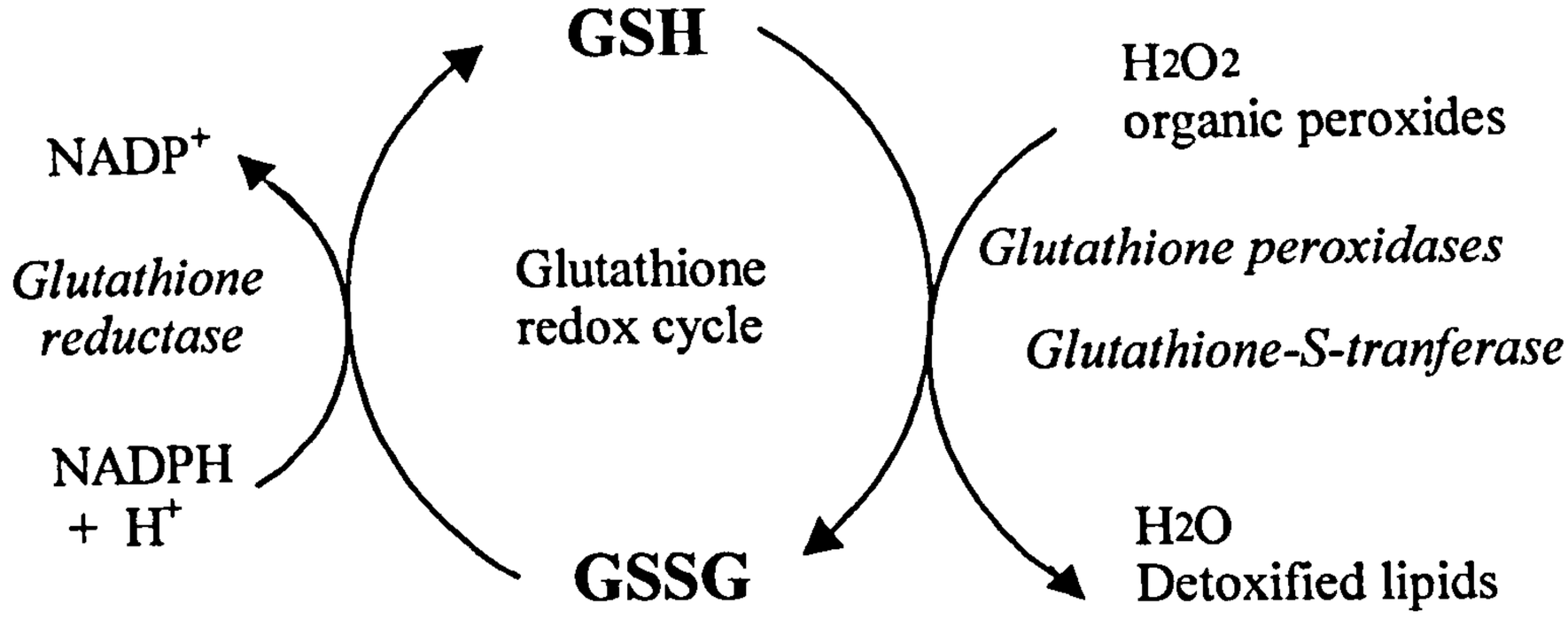


Figure 2.2. An Overview of Cysteine-Based Antioxidant Pathways in Eukaryotic Cells.

Selenium-dependent glutathione peroxidase (Gutteridge & Halliwell, 1994) is of particular importance in controlling the level of hydrogen peroxide in the cytosol. As controlled production of this enzyme is observed under normal conditions, it could be expected that an increase in transcription will occur if a cell is placed under higher, but sub-lethal, oxidant/preservative stress (Ferguson, 1999).

Non-enzymatic free radical scavengers are a second mechanism for cellular protection. Antioxidant free radical scavengers offer direct protection from highly reactive hydroxyl species. Under varying conditions other molecules can act as free radical scavengers. These include glutathione, ascorbic acid, uric acid and cysteine. Elevated levels of the following factors are associated with increased resistance to many antimicrobial agents whose major route of action is oxidative change.

Ascorbic acid (vitamin C) is an essential, water soluble, dietary vitamin. Mammalian cells lacking L-gluconolactone oxidase cannot synthesise it. Cytosolic ascorbic acid, normally at 40-80μM, is a powerful reducing agent and antioxidant (Levine, 1986). It reacts with superoxide, peroxide and hydroxyl radicals to form

mono-dehydroascorbic acid radicals (Bates, 1981). Ascorbic acid may reduce iron, and thus potentiate the Fenton reaction under specific conditions. Ascorbic acid is regenerated upon interaction with glutathione via dehydroascorbate reductase.

α-Tocopherol (vitamin E) is a lipid soluble, free radical scavenger which gives more specific protection against lipid oxidation due to its compartmental location. It concentrates within the phospholipid bilayer (Lambert and Mourot, 1984). McCay (1985) demonstrated that α-tocopherol is a powerful scavenger of singlet oxygen, superoxide, and lipid peroxy radicals. Ascorbic acid acts as a reductant for α-tocopherol regeneration (Packer *et al.*, 1979).

Bacteria do not have the same repertoire of antioxidant mechanisms as the higher organisms. Some bacteria possess some of the non-enzymatic scavengers described above (Fahey *et al.*, 1998). These may play a vital role in resisting oxidant stress. GSH is not found in most Gram positive bacteria (Fahey *et al.*, 1978). In bacteria glutathione is thought to have sacrificial scavenger status (Cheesy *et al.*, 1996) as they often lack the enzymes to recycle it. These species often have other thiol containing compounds, eg CoASH, or cysteine based antioxidant pathways (Fahey and Newton, 1983). γ-glutamylcysteine appears to be protective only in the halobacteria (Newton and Javor, 1985). Mechanisms of enzymatic resistance to oxidation are highly specific to species and strains of prokayotic organisms (Misra, 1992; Loferer, *et al.*, 1994). A major taxonomic grouping of bacteria are those capable of catalase production. Catalase-positive bacteria should have an increased capability to resist elevated hydrogen peroxide exposure. Specific examples of plasmid encoded enzymatic resistance include organo-mercurial resistance which has been described in Gram positive and negative bacteria (Misra, 1992). An encoded lyase requires reduced nicotinamide adenosine dinucleotide phosphate (NADPH) and thiol residues to convert toxic Hg(II) to non-toxic Hg(0). A similar acquired resistance has been described for QACs (reviewed by Day and Russell, 1991). Altered phospholipid composition has also been associated with changes in preservative resistance (Pechey *et al.*, 1974; Tueber and Bader, 1976; Imai *et al.*, 1975; Ikeda *et al.*, 1984). Alteration of membrane hydrophobicity can lead to enhanced exclusion of antimicrobial agents, thus, allowing species to aquire

resistance. Microbial cell-cell interaction to form a glycocalyx-crosslinked biofilm can give added resistance to preservatives with weak detergent properties (Brown and Gilbert, 1993).

Detecting Oxidised Biological Molecules.

Electrospray mass spectrometry (ESMS) provides a sensitive method for analysing biological molecules, and also allows the observation of oxidative modification. The technique requires that the molecule of interest should be chargeable. Compounds can be observed in either positive ion or negative ion modes.

Lipids are esterified derivatives of glycerol (propan-1,2,3-triol), in which R_1 is usually a hydrophilic group ('headgroup') such as phosphorylcholine, and R_2 and R_3 are fatty acyl chains with varying degrees of unsaturation. Phospholipids have a headgroup containing a phosphate moiety, which is important in ESMS as it provides a counterion, usually H^+ or Na^+, and this affects the mass of the molecule. Membrane lipid composition is known to vary between species. This determines the fluidity of the membrane under the growth conditions of the organism. Common saturated and unsaturated fatty acids are given in Table 2.1.

Common Saturated Fatty Acids		**Common Unsaturated Fatty Acids**	
Name	Chain length: double bonds	Name	Chain length: double bonds
Lauric acid	12:0	Palmitoleic acid	16:1
Myristic acid	14:0	Oleic acid	18:1
Palmitic acid	16.0	Linoleic acid	18:2
Stearic acid	18:0	Linolenic acid	18:3
Arachidic acid	20:0	Arachidonic acid	20:4

TABLE 2.1. Overview of fatty acid terminology.

The cell membrane is the first site to encounter exogenously induced stress in the cell. Unsaturated lipids in particular are susceptible to oxidative stress, which causes the formation of a variety of initial oxidation products. Lipid hydroperoxides arise from the formation of hydroxides or chlorohydrins in the acyl chains by free radical attack, at double unsaturated sites, and electrophilic addition, at single

unsaturated sites. The specific oxidizing agent and the levels of acyl chain unsaturation will determine the products it is possible to generate. These chemical species can then react further to give chain shortened lipids, aldehydes and carboxylic acids. Initial lipid oxidation products can readily be observed by ESMS, due to the increase in molecular weight on addition of an oxygen or HOCl molecule to the fatty acyl chain. The pathway of hydroperoxidation in unsaturated fatty acyl chains of lipids is shown in Figure 2.3.

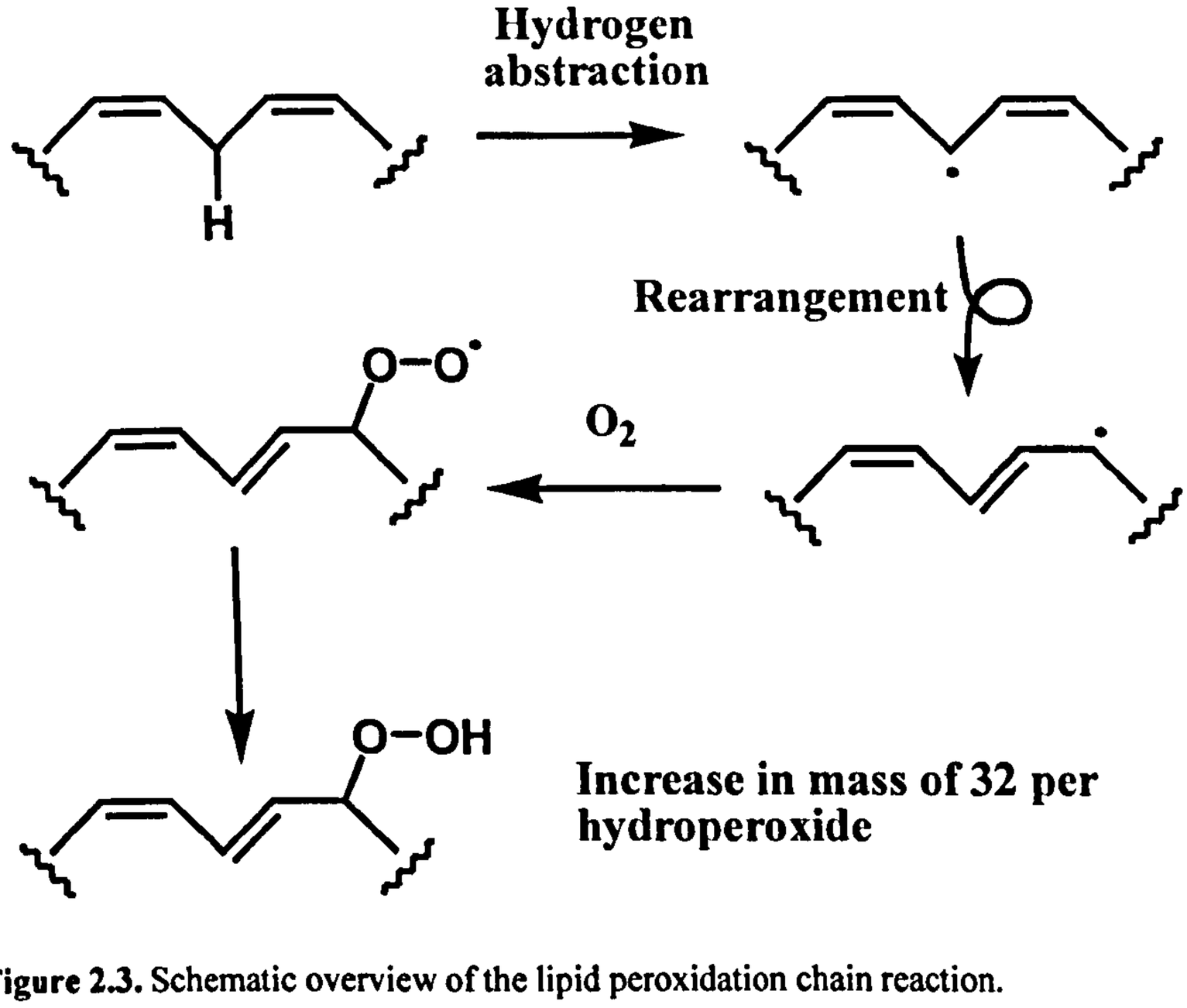


Figure 2.3. Schematic overview of the lipid peroxidation chain reaction.

The pathway of chlorohydrin formation of unsaturated fatty acyl chains of lipids is shown in Figure 2.4.

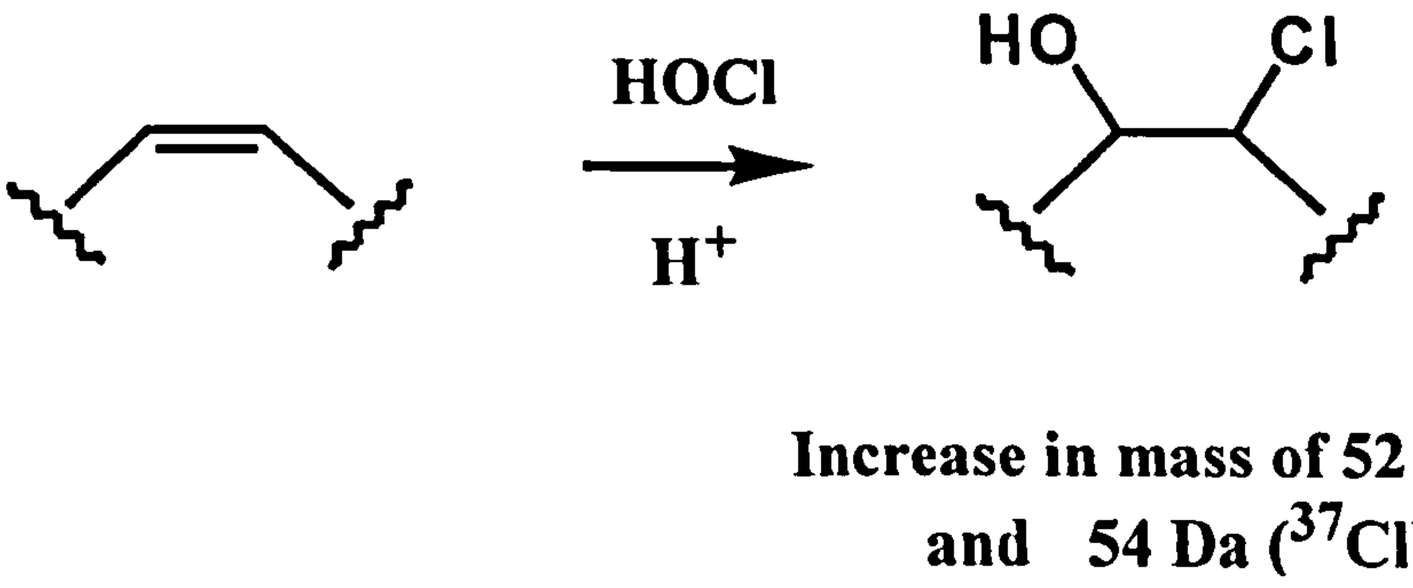


Figure 2.4. Schematic overview of the lipid chlorohydrination reaction by HOCl.

Phosphatidyl cholines (PC) and ethanolamines (PE) can be detected most effectively in positive ion mode (Spickett *et al.*, 1998). Phosphatidyl serines (PS), glycerols (PG) and PE are easily detected in negative ion mode (Watson *et al.*, 1998). In the simplest procedure, lipid samples are injected directly into the mass spectrometer and a spectrum of peaks at different mass-to-charge ratios (m/z), corresponding to different constituent lipids, is obtained. The lipids may occur in a protonated or sodiated form, depending on the counter ion present on the phosphate group. The sodiated form has a m/z 22 higher than the protonated form and ^{13}C isotope peaks at +1 m/z are observed for all lipids.

In biological samples from higher organisms, lipid peaks tend to occur in groups separated by 28 m/z units, corresponding to an increase of fatty acyl chain length by 2 methylenes. Within the groups, peaks separated by a decrease of 2 m/z units indicate increasing unsaturation of the chain. However, some bacteria have odd numbered fatty acyl chains, with increases of 14 m/z between groups (Black *et al.*, 1997).

LC-MS involves the coupling of HPLC to the ESMS, so that the sample moves first through an HPLC column (reverse phase in this study) to separate the lipids before they are detected by mass-to-charge ratio. All masses within the chosen mass range are monitored over the whole chromatogram. Hence, a chromatogram of total ion current (TIC) corresponding to all ionizable species within the mass range chosen eluting from the column, can be obtained. Similarly a reconstructed ion chromatogram (RIC) of any individual mass in the range can be drawn. This increases the amount of information which can be obtained from this method.

Acknowledgement has to be made to Natalie Homer, Rachel Smith and the Department of Chemistry, University of Strathclyde. In collaboration the analysis of glutathione and tryptophan oxidation using NMR and mass-spectrometry was performed.

Materials and Methods

Phospholipids were purchased from Sigma Chemical Co., Dorset, UK, as were BAK, tBHP, NaOCl, glutathione and tryptophan. Stabilized sodium chlorite (Purite™) was obtained from Biocide, Oklahoma, USA, and hydrogen peroxide was from Fisher, UK. D_2O was supplied by Goss Scientific Instruments, UK. All solvents and chemicals were of analytical grade or equivalent.

Treatment of phospholipid vesicles

Phospholipid vesicles were prepared from stock lipid solutions of 10mg/mL in chloroform, as described previously (Spickett *et al.*, 1998). The mixture contained 2:1:1(v/v) of 99% fresh egg yolk phosphatidylcholine: arachidonyl-stearoyl phosphatidylcholine: dipalmitoyl phosphatidylcholine. The vesicles were stored at -20°C until required.

Vesicles were treated with various oxidants and preservatives at the following concentrations: sodium chlorite (Purite™) at 0.005%, 0.03% [=3.5 mM], 0.2%, 0.4% (wt/v); benzalkonium chloride (BAK) at 0.005%, 0.2% (wt/v); NaOCl at 3.5 mM pH 7.2 (v/v); H_2O_2 + Fe^{2+} at 0.1 M + 1 mM (wt/v); and *tert*-butylhydroperoxide (tBHP) + Fe^{2+} at 0.25 M + 1 mM (wt/v). Treatments were freshly prepared for each experiment. The incubations were carried out at room temperature, and contained 10µL of stock phospholipid vesicles and 10µL of 10x final concentration of treatment in a final aqueous volume of 100µL. Both short (30-120 mins) and long (24 and 48 hours) timecourses of treatment were carried out. At each time point, 10µL of the incubation mixture was removed and mixed with 90µL of methanol for analysis by electrospray mass spectrometry (ESMS).

The effect of pH was investigated with both 0.4 % sodium chlorite and 3.5mM NaOCl treatments. The treatment stock was adjusted to a pH of 7.2, 8 or 8.9 prior to addition to the incubation, and the vesicles were treated for 48 hours at each of these pHs before analysing by ESMS.

Analysis of phospholipid vesicles by electrospray mass spectrometry.

Direct injection and LC-MS were performed in positive-ion mode on a VG Platform mass spectrometer (Micromass, Cheshire, UK). The source temperature was set to 75°C with a nebulizing gas flow of 20 L/h and a drying gas flow of 400 L/h. All data were acquired and manipulated using the MassLynx software.

The samples from the short time-courses were analysed by direct injection. Aliquots of 20μL of the methanol dilution were injected at a flow rate of 20μL/min in 9:1 methanol/water. Data was collected between 400-1000 m/z with a sweep time of 5 seconds. Spectra were combined from 15 consecutive scans. Summed spectra were mean smoothed twice with a window of 0.3 Da. Samples from the long timecourses were diluted a further 1 in 10 in 71:5:7 methanol/hexane/0.1M ammonium acetate for analysis by LC-MS, using a Shimadzu LC-10 with a flow rate of 100μL/min (71:5:7. (v/v) methanol/hexane/0.1M ammonium acetate). 20μL aliquots were injected onto a Luna C8 reverse phase HPLC column (5μm, 1mm i.d. x 150mm; Phenomenex, UK). The eluent was split 2:1 between the electrospray mass spectrometer and waste. Peak top data were collected between 500 and 1000 m/z with a sweep time of 2 s over chromatograms of 40 mins.

Analysis of glutathione oxidation.

Solutions of 12.5 mM reduced glutathione (GSH) were freshly prepared in phosphate-buffered saline in deuterated water (0.71g Na_2HPO_4, 0.195g NaH_2PO_4, and 0.45g NaCl in 50mL D_2O, pD 7.0; PBS-D_2O). 400μL aliquots of GSH solution were placed in a 5 mm NMR tube and the reaction was started by the addition of 100 μl of either hydrogen peroxide, NaOCl, BAK or sodium chlorite diluted in PBS-D_2O. A range of oxidant-GSH ratios were tested as specified in the results. The reaction was monitored over a timecourse of 30 mins, with spectra acquired every 5 mins. The effect of high concentrations of sodium chlorite on glutathione disulphide (GSSG) was also investigated.

The effect of the treatments on glutathione were analysed by ^{1}H-NMR on a Bruker AMX 400Hz spectrometer, operating at 400.13 MHz, and analysed using UNIXNMR software. Single 90° pulses were applied, corresponding to a pulse length of 12.5μs. A presaturation pulse of 55 dB was applied to the HDO resonance

during the relaxation delay, in order to suppress the water signal. The FIDs were the sum of 32 scans, containing 32K data points zero-filled to 64K. The analysis was performed at 300 K with the NMR tube spinning at 20 rpm. The spectra were referenced to the D_2O resonance at 4.8 ppm. The ratio of reduced to oxidized glutathione was determined from the integrals of the cysteinyl-β proton peaks as described previously (Reglinski *et al.*, 1991).

Analysis of tryptophan oxidation.

Tryptophan (1 mM) in 50:50 (v:v) water / acetonitrile containing 1% trifluoroacetic acid (TFA) was treated with either hydrogen peroxide, NaOCl, or sodium chlorite at concentrations of 1-10 mM. Immediately after addition of the oxidant to the reaction mixture, a 1/20 diluted sample was prepared, and a 10μL sample was analysed by LC-MS using the Luna C8 column described above. The eluent was split 1:1 between the electrospray mass spectrometer and a UV/visible detector monitoring at 254nm. A gradient from 50% acetonitrile plus TFA to 100% acetonitrile plus TFA was run over 24 mins. The source temperature was set to 75°C with a nebulizing gas flow of 20 L/h and a drying gas flow of 400 L/h, and data were collected from 100-400 m/z.

Results.

Analysis of phospholipid vesicles by electrospray mass spectrometry.

Figure 2.5 shows a typical direct injection positive ion ESMS spectrum of control vesicles, with peaks corresponding to a variety of phosphatidylcholines (PCs) which are labelled with their predominant fatty acyl composition.

ESMS is a soft ionisation technique that does not normally cause fragmentation (Spickett *et al.*, 1998, 2001; Jerlich *et al.*, 2000.). Therefore, each peak observed in the spectrum corresponds to an intact molecular ion. In the direct injection mode most of the lipids observed are in the sodiated, rather than protonated form.

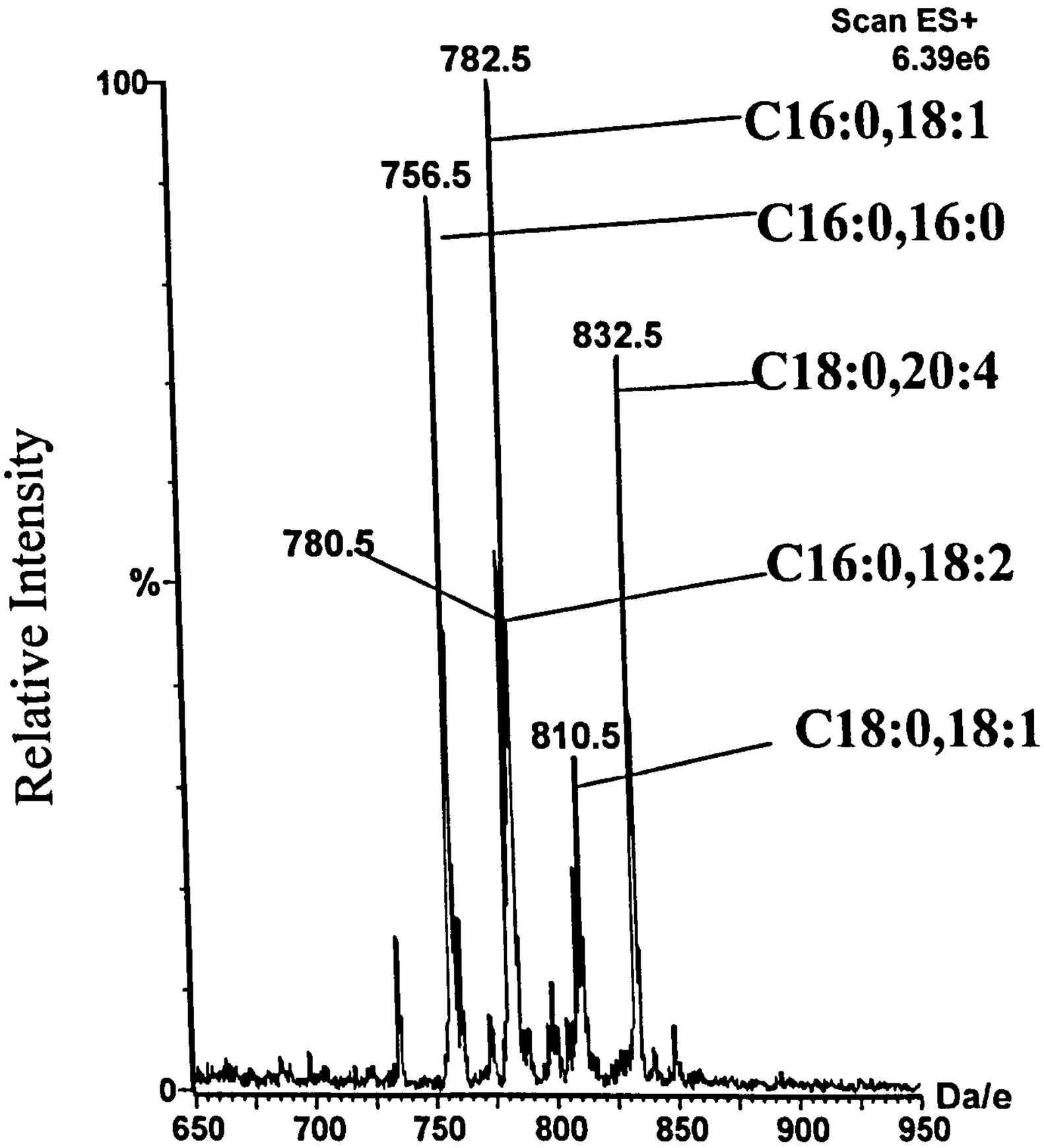


Figure 2.5. Direct Injection ESMS of Phospholipid vesicle control. A positive ion mass spectrum of a control phosphatidylcholine vesicle preparation directly infused into the mass spectrometer. A range of different phosphatidylcholines (PC) can be identified in the sodiated forms.
Note: Some of the major species are labelled with their R_1 : R_2 acyl-chain composition ratio. Each R-chain is identified by the number of carbon atons:number of double bonds. This is type of identification is possible in control vesicle studies as the phospholipids were of known acyl-chain compisition.

The effect of treating phospholipid vesicles with oxidants or preservatives was monitored initially by direct injection ESMS, and an example is shown in Figure 2.6. Treatment with 0.25 M t-BHP + Fe^{2+} or 3.5 mM NaOCl resulted in almost complete depletion of the polyunsaturated stearoyl-arachidonoyl PC at 832 m/z, whereas 0.4% Purite™, 0.02% BAK and 0.1M H_2O_2 + Fe^{2+} had very little effect. NaOCl also caused some depletion of other, less unsaturated PCs such as those at 780 and 782 m/z. The formation of some oxidised lipids was also be observed for some of the treatments. Lipid peroxidation corresponds to the addition of di-oxygen and can be monitored by the appearance of species at multiples of 32 m/z above the mass of the native lipid. The addition of HOCl across a double bond results in chlorohydrin formation with a corresponding mass increase of 52 m/z. A bis-hydroperoxide of stearoyl-arachidonoyl PC was observed at 896 m/z (+64) following treatment with t-BHP + Fe^{2+}. Treatment with NaOCl leads to the appearance of chlorohydrins at 834 m/z (from 782), 862 m/z (from 810) and 884 m/z (from 832). No oxidized phospholipids could be detected after 2 hours treatment with Purite™, BAK or H_2O_2.

Figure 2.7 summarises the data obtained from direct injection ESMS analysis for various native and oxidised phospholipids over a 2 hour treatment. The phospholipid levels were standardised against the fully saturated lipid dipalmitoyl PC at 756 m/z (PC16:0 16:0 shown as 100%), as this is not very susceptible to oxidative modification. There were no significant changes in PC levels in control-treated vesicles and Purite™-treated vesicles. BAK caused an apparent increase in the intensity of the peak at 810 m/z. This is probably due to the detection of protonated stearoyl-arachidonyl phosphatidyl-choline (SAPC), (PC18:0 20:4, 832Da-Na^+22Da = 810Da) additional to the sodiated stearoyl-oleoly phosphatidyl-choline (SOPC), PC18:0 18:1 (810Da). This is supported by the observation that the peak returned to 100% by 1.5-2 hours post treatment.

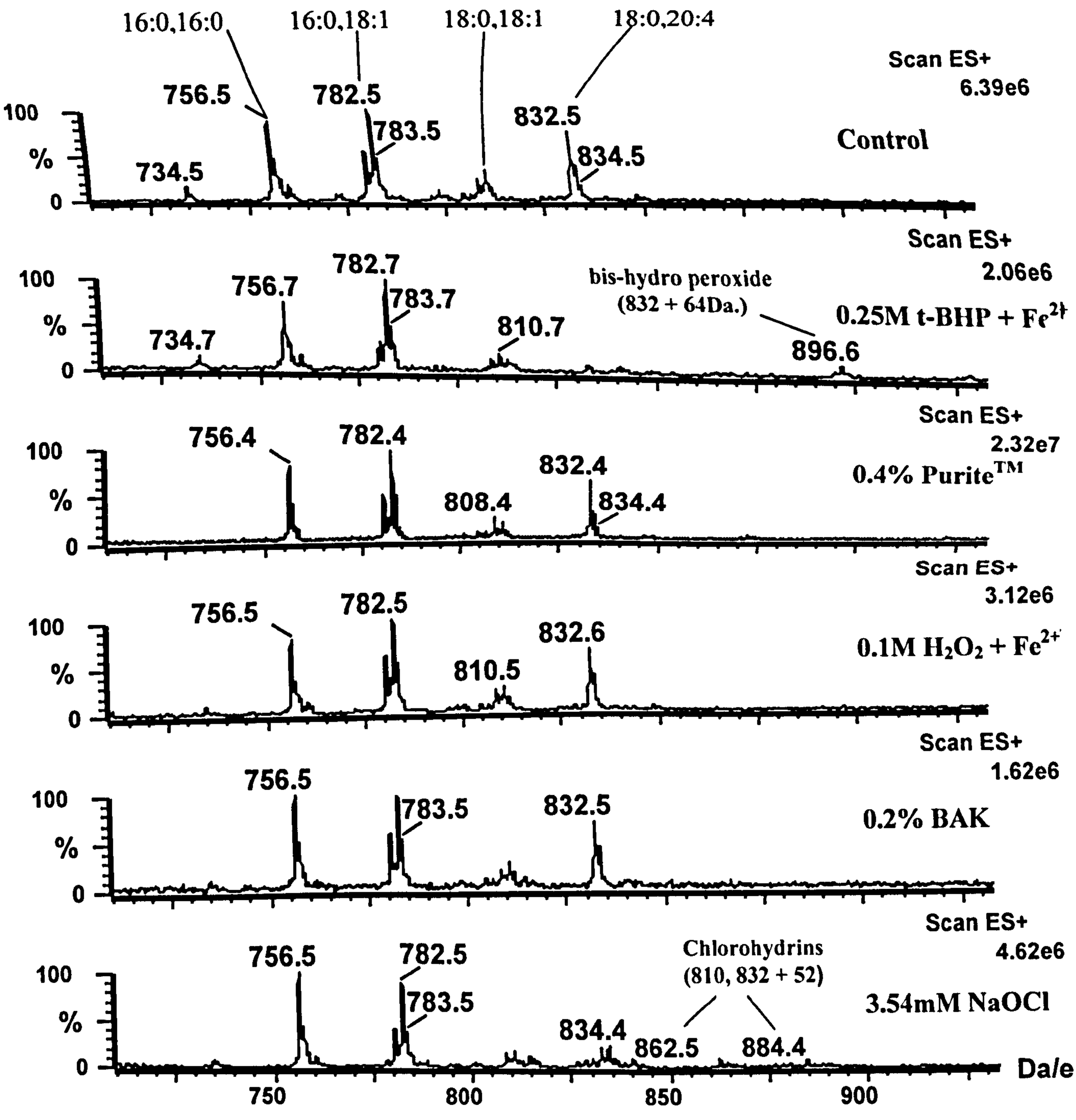
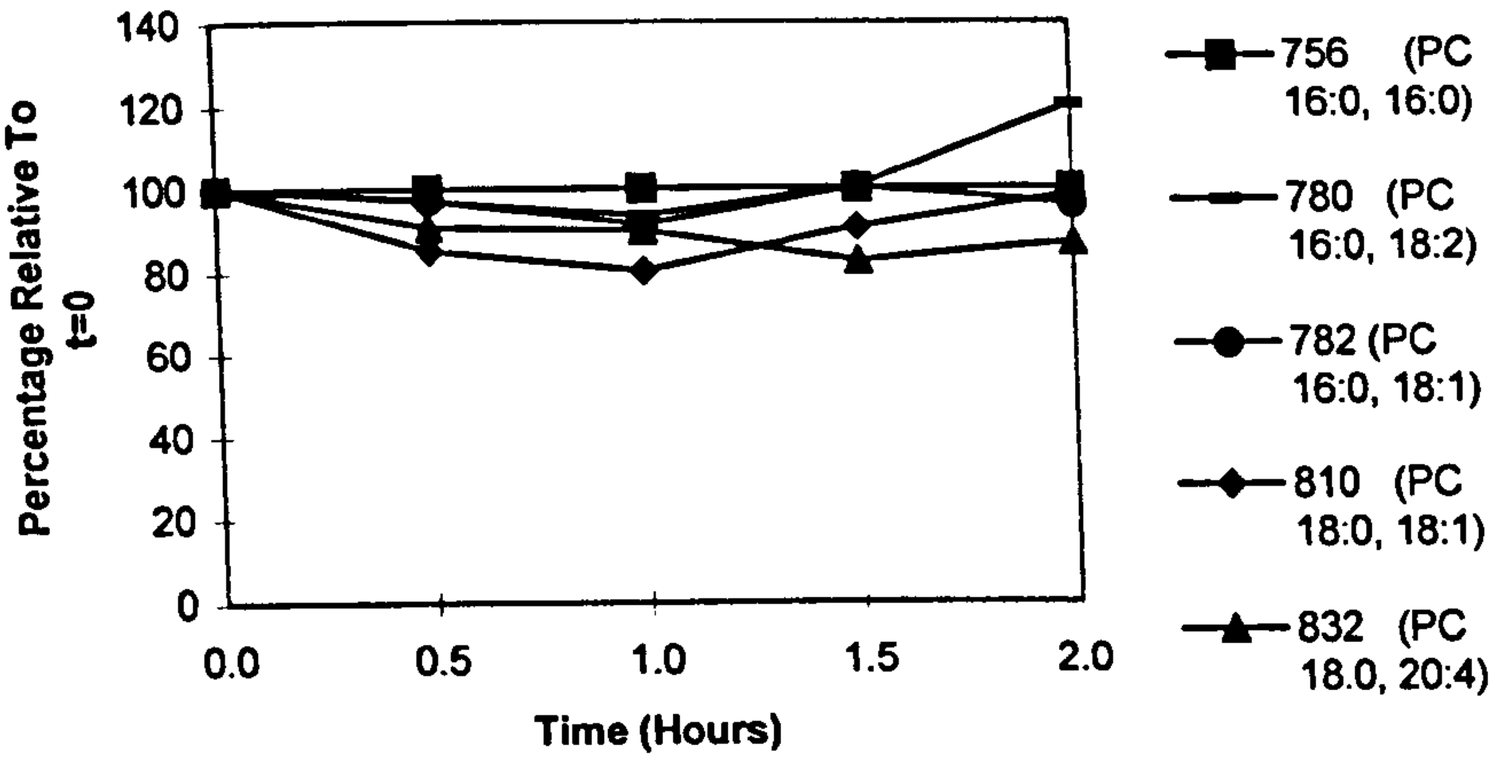
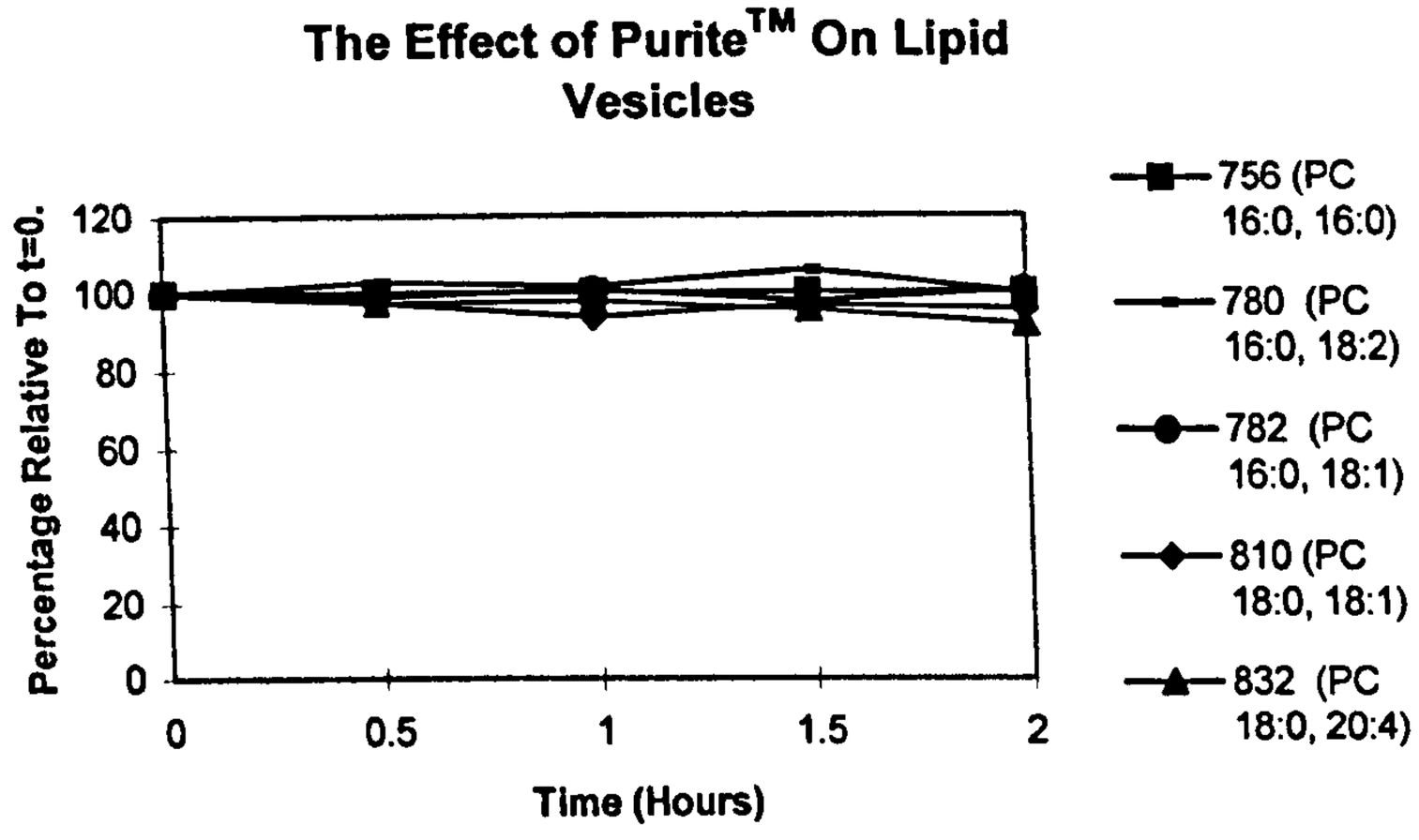


Figure 2.6. Effects of oxidants and preservatives on phospholipid vesicles. Direct injection ESMS of phospholipid vesicles treated for 120 minutes with an oxidant or preservative at concentrations shown.





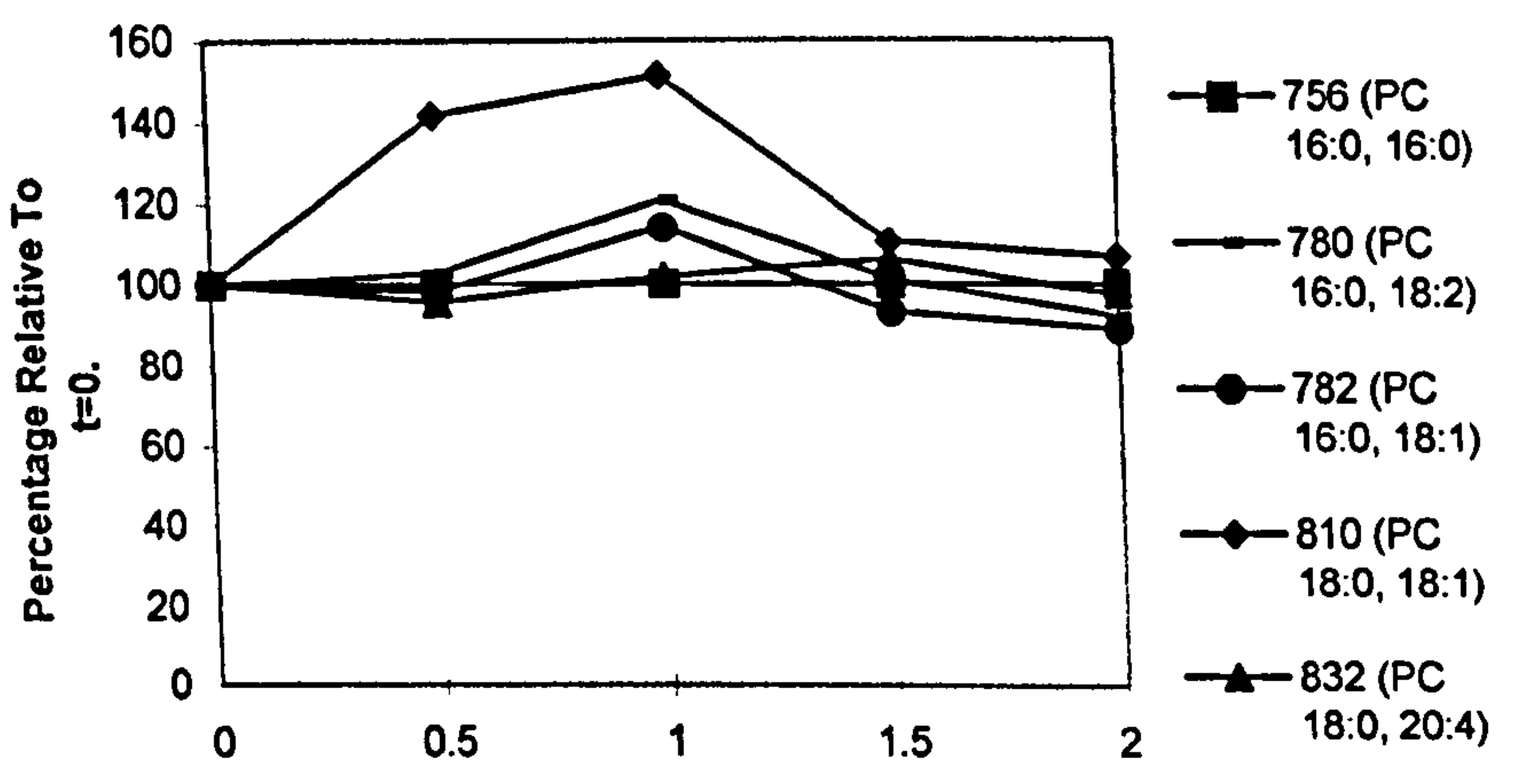


Figure 2.7. Relative changes in phospholipid vesicle components over time after treatment with oxidants and preservatives. Control and Purite™ and BAK treated vesicles compared to DPPC at 100%. (continued over)

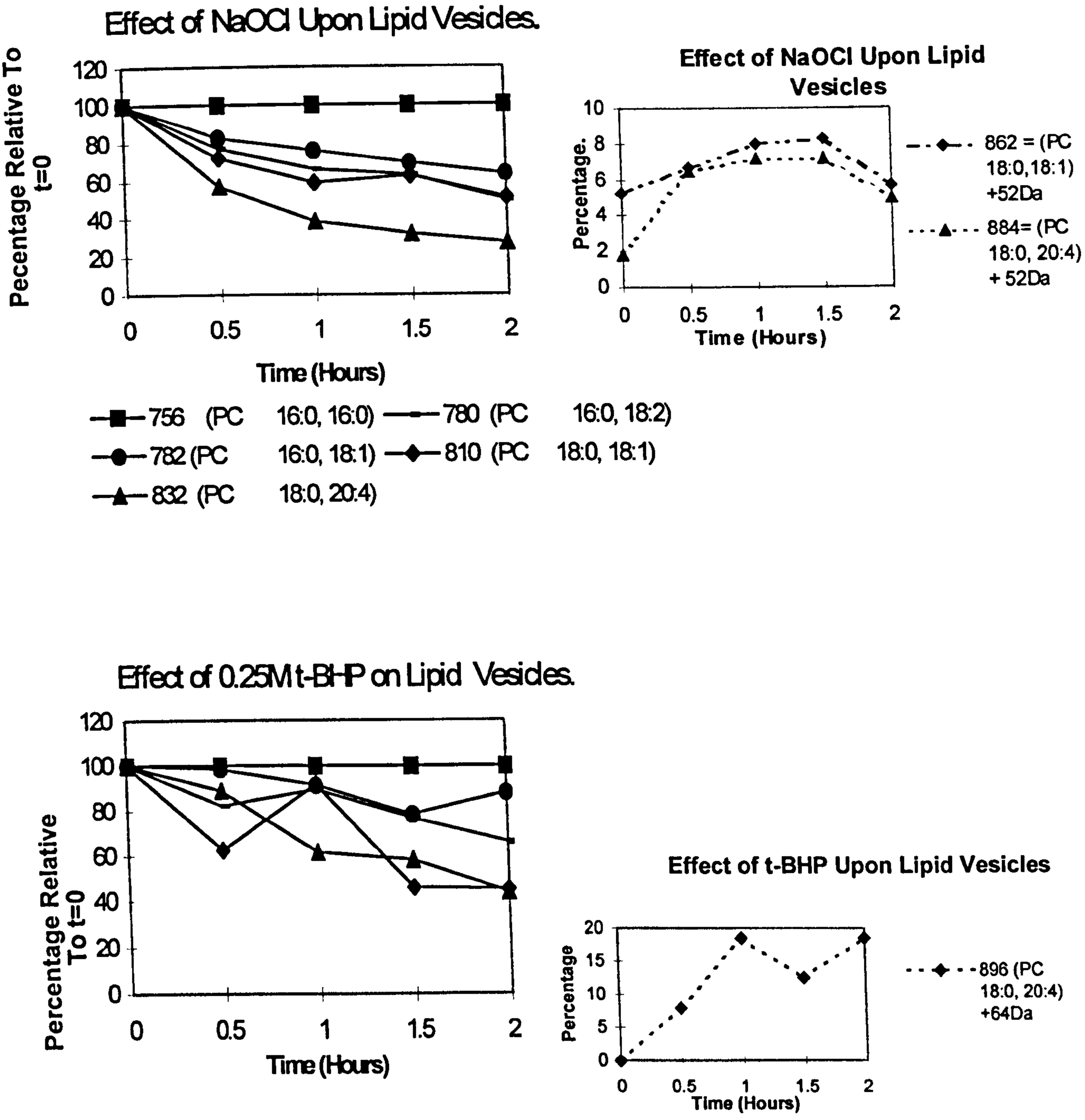


Figure 2.7 (cont.). Relative changes in phospholipid vesicle components over time after treatment with oxidants and preservatives. HOCl and t-BHP treated vesicles. Left hand panels show loss of unsaturated phospholipids. The right hand panels show the accumulation of chlorohydrins (+52 Da) in HOCl treated vesicles and a bishydroperoxide (+ 64Da) in t-BHP treated vesicles.

No other changes were observed with BAK. NaOCl treatment resulted in a steady decline of all the unsaturated PCs, with a concomitant and rapid appearance of chlorohydrins at 862 and 884 m/z. A decrease in the levels of most of the unsaturated PCs was observed after t-BHP treatment. The only significant oxidative product observed was the bis-hydroperoxide of stearoyl-arachidonoyl PC at 896 m/z.

LC-MS provides a more sensitive and informative method for detecting the appearance of oxidised phospholipid species, since these elute earlier on reverse phase HPLC, due to the decrease in hydrophobicity compared to the native form of the lipid. This approach also allows the separation of different species of phospholipids with the same mass, prior to their detection by ESMS.

Figure 2.8 shows the results of the separation of lipids from a control vesicle preparation. The bottom trace shows the total ion current chromatogram (TIC, the sum of all ions within the mass range passing into the detector) obtained from the lipid vesicles. It can be seen that there are 4 major species which contribute to the chromatogram. These are demonstrated more clearly in the reconstructed ion chromatograms (RIC) at specific mass-to-charge ratios. The column elution time of PCs is influenced by both the chain length and degree of unsaturation of the constituent fatty acid chains. Longer elution times are associated with phospholipids containing longer and more saturated acyl chains. The elution time in minutes is indicated above each peak in the chromatograms. In LC-MS the majority of the phospholipids occur in the protonated form. In contrast, direct injection ESMS of lipids detects most species in the sodiated form. The difference in protonated mass of HLPC-ESMS, compared to the direct injection ESMS, spectra of -22 Da is due to de-salting upon interaction with the HPLC column (Jerlich *et al.*, 2000).

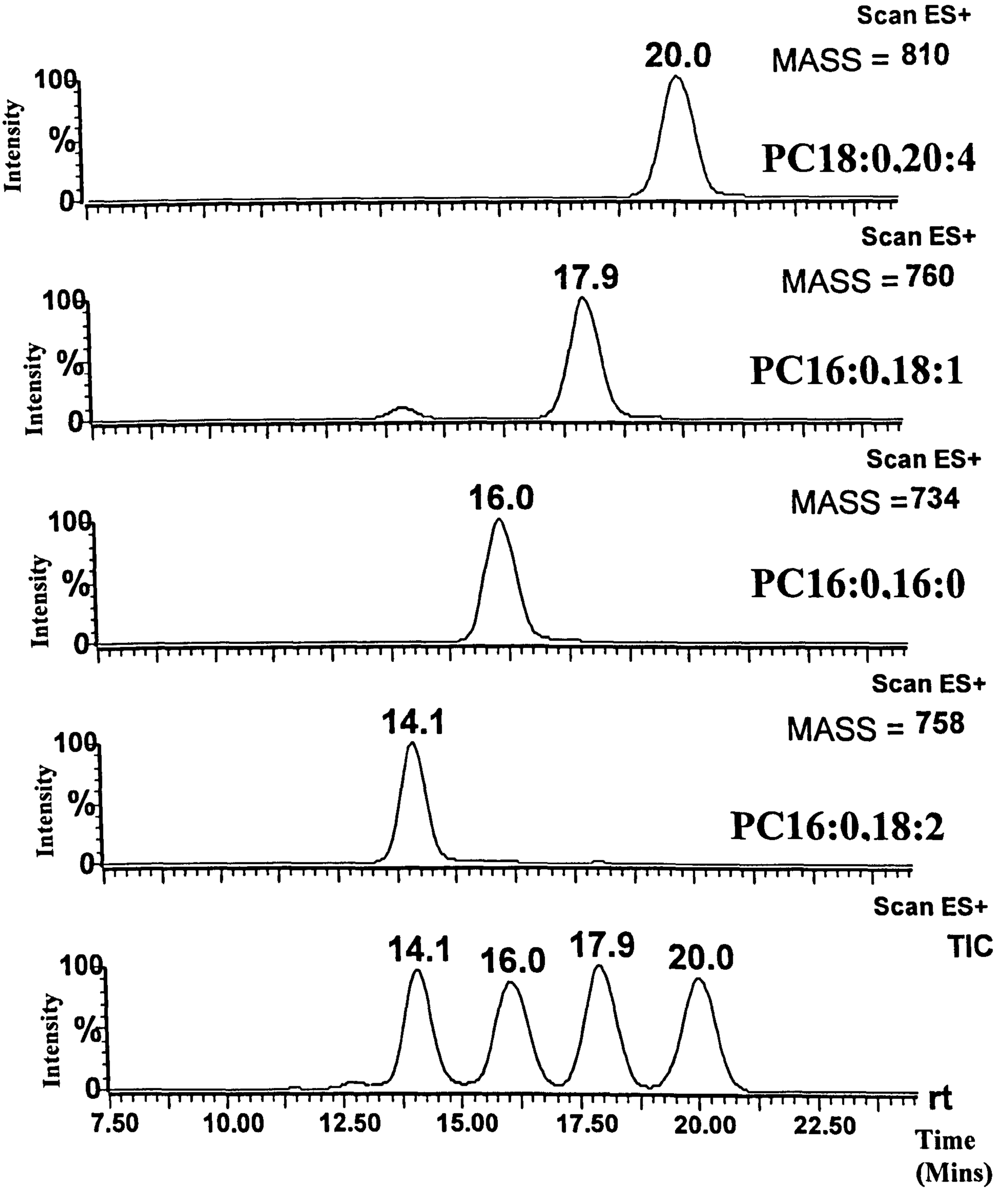


Figure 2.8. LC-MS Chromatograms of Lipid Vesicles. The bottom trace is the total ion current (TIC) chromatogram, (700-1000Da). Above are reconstructed ion chromatograms (RIC) of individual masses, showing the main components of the vesicle mixture.

LC-MS was used to investigate the possible oxidation of vesicles by Purite™ in more detail. Figure 2.9 shows the effect of longer treatments (48 hours) on the TIC of vesicles, compared to the control treatment at time 0. Small changes in the retention times are due to slight differences in the timing of the acquisitions and changes in the solvent over time due to evaporation and environmental/thermal changes. Even in the control sample by 48 hours post-incubation some oxidation of the unsaturated PCs has occurred. This was indicated by a decrease in 758 m/z signal intensity eluting at approximately 13.8 mins and the 810 m/z signal at 19.5 mins. In addition there was an increase in intensity of species eluting early in the chromatogram, corresponding to oxidatively modified lipids. Treatment with Purite™ for 48 hours resulted in a greater depletion of native lipids compared to the control values. There was also evidence of oxidation products in the early part of the chromatogram. These effects were relatively small compared to those induced both in the control and by NaOCl (48 hours post-treatment) and t-BHP (3 hours post-treatment). In these cases, the majority of native PCs had disappeared and the largest peaks in the chromatogram were due to oxidized PCs. The significantly faster reaction with t-BHP could be due to its higher concentration (70 times higher than NaOCl), and the presence of iron, which would facilitate the Fenton reaction (Sinclair *et al.*, 1990) and enhance free radical formation. This does not alter the type of reaction t-BHP has with native lipids, i.e. hydro-peroxidation, only the amount of hydro-peroxides produced. In contrast, BAK had little effect on the lipid profile at 48 hours, and the apparent decrease in 758 m/z observed is probably non-specific.

The nature of the oxidized PCs can be seen from Figure 2.9 (right), which shows spectra corresponding to the early regions of the chromatograms (approx. 3-9 mins). The spectra are normalised by vertically linking the y-axis, designated as 100%, within each sample. Thus, the amount of signal, generated by specific species in the early chromatogram region, can be compared to the other treatments indicating the relative amounts of product present.

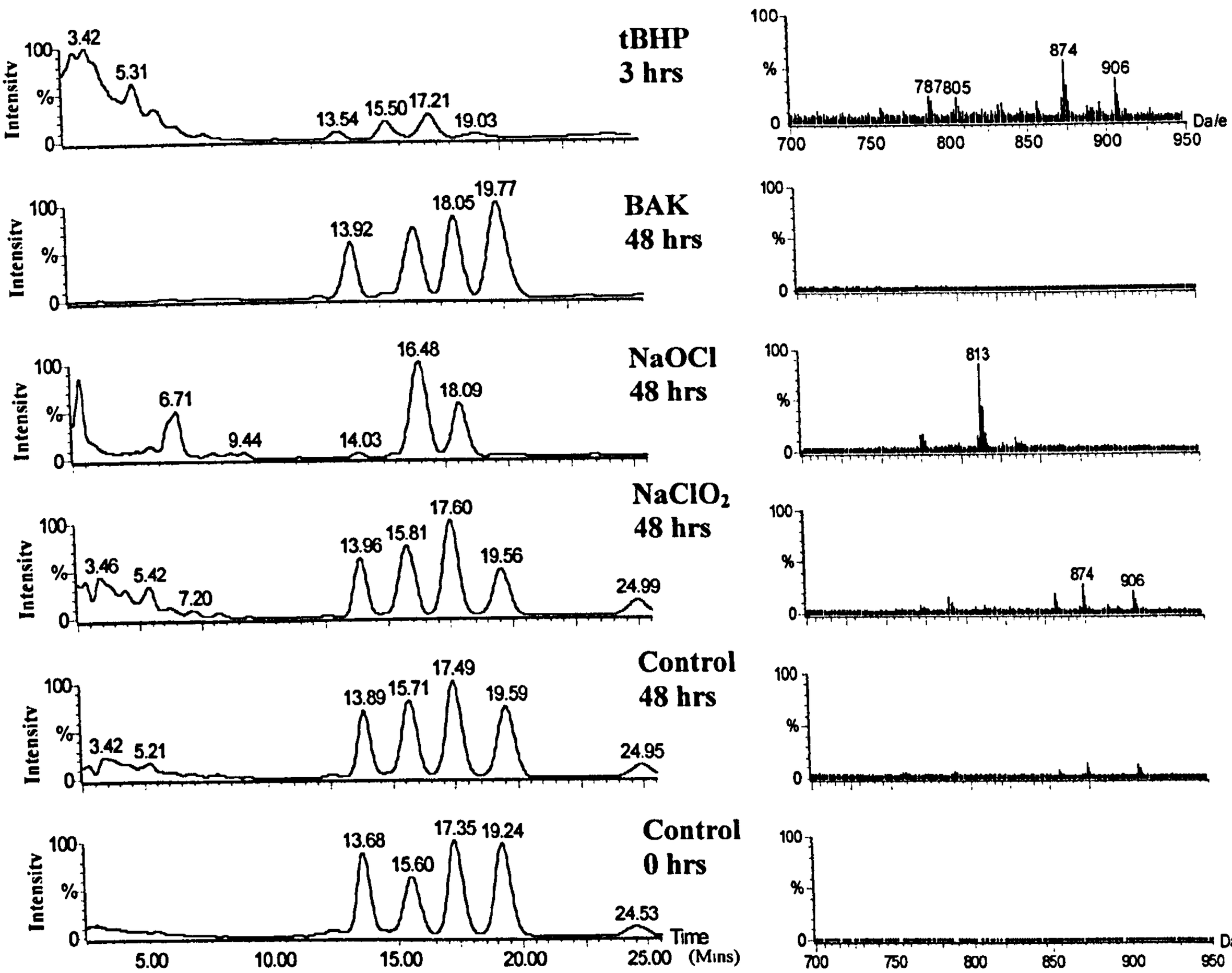


Figure 3.9 **LC-MS Chromatograms and Combined Spectra of Treated Lipid Vesicles.** The left traces are the total ion current (TIC) chromatograms, (700-1000Da). To the right, combined spectra from the early region of the corresponding chromatogram showing the modified components of the vesicle mixture.

As observed by previous researchers, NaOCl treatment leads to the formation of lipid-chlorohydrins (Jerlich *et al.*, 2000). The most stable product at this time is the chlorohydrin of monounsaturated palmitoyloleoyl PC at 812 m/z. t-BHP treatment generated monohydroperoxides from palmitoyl-linoleoyl PC apparent at 790 m/z, and bis- and tris-hydroperoxides of stearoyl-arachidonoyl PC (874 and 906 m/z). Bis- and tris-hydroperoxides of stearoyl-arachidonoyl PC were also formed

following Purite™ treatment, at only slightly higher levels than the control, indicating that this preservative may be capable of a free radical mechanism of attack. Despite the presence of chlorine in Purite™, no chlorinated lipid products of any kind were observed.

The relative level of Purite™-derived lipid peroxidation was noted to increase as the pH decreased towards neutral. This can be seen in Figure 2.10. Vertical linking of the x-axis between reconstucted ion-chromatograms (RICs) of products demonstrated that, compared to controls, systematic increases in bis- and tris-peroxides generated after treatment with the same concentration of Purite™ at lower pH's. Purite™ is normally formulated to neutral pH 7.2-7.4. Protonation of Purite™ is known to result in the generation of chlorine dioxide. This is a highly reactive oxidant, free radical gas which is soluble in water. No chlorine based oxidation products were detected with Purite™ treatments at any pH over 48 hours (n=3). This observation may be supported by the claims made by Allergan, and reported in the literature, that Purite™ ultimately forms NaCl and H_2O (D'Arienzo 2002)

Effect of pH on Lipid Peroxidation by Purite and NaOCl

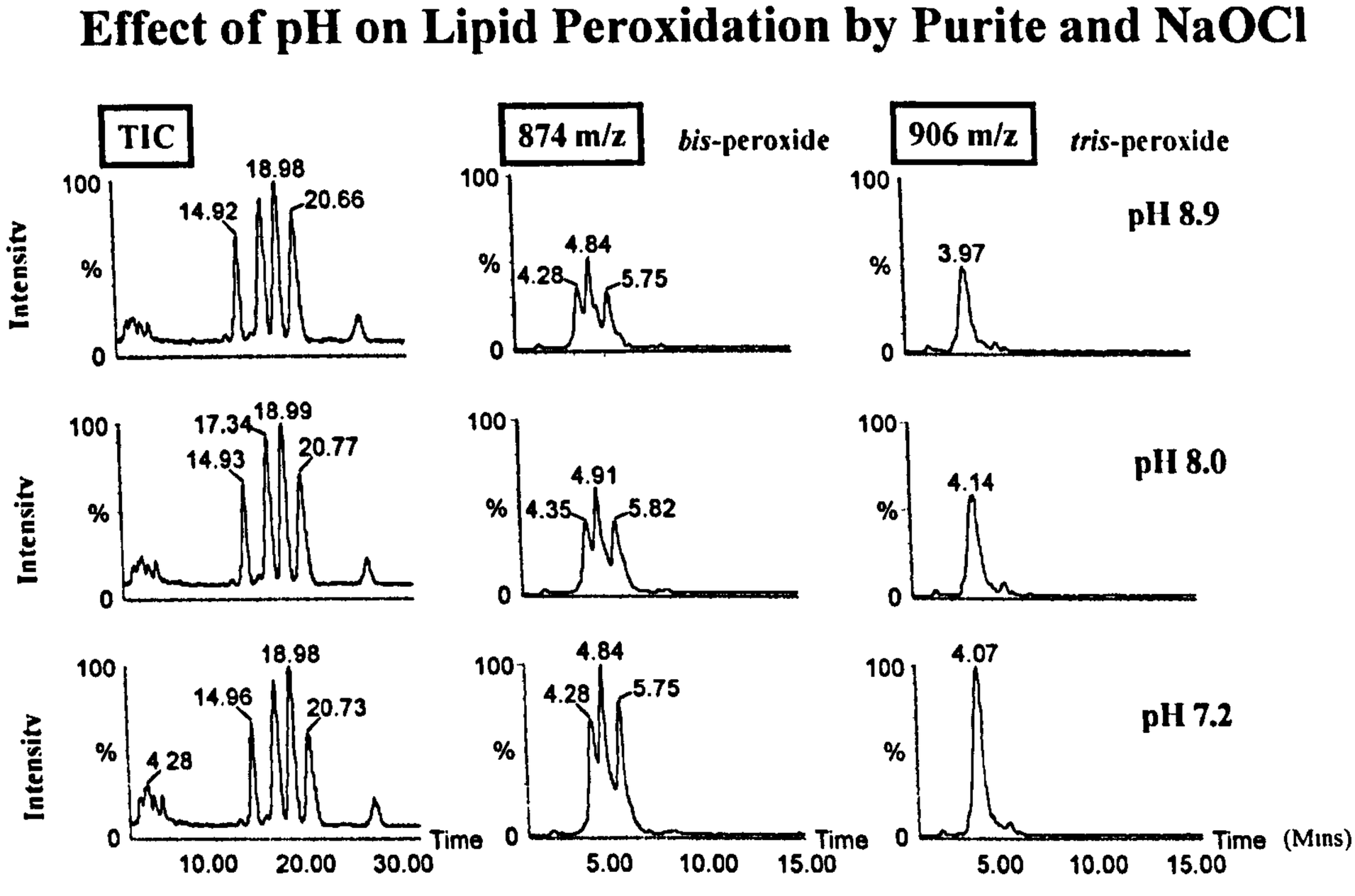


Figure 2.10. The effect of pH on oxidation by sodium chlorite. The total ion chromatograms (TICs) and chromatograms of specific masses are shown for phosphatidylcholine vesicles treated with 0.4 % sodium chlorite for 48 hours at pHs 8.8, 8.0 and 7.2. The specific masses shown are 874 m/z (bis-peroxide of SAPC) and 906 m/z (tris-peroxide of SAPC). The chromatograms are vertically linked within each panel to allow comparison of the intensities. The intensities of the two right hand panels are also linked. Peroxidised (e.g. m/z 856, 874 and 906) lipids can be identified in this way.

Lowering the pH also enhanced NaOCl breakdown of native lipids and increased the early chromatograph species over 48 hours (n=3). This can be seen in Figure 2.11. These changes resulted in increased differences in the breakdown products of m/z 524 and 638, but chlorohydrination products appeared to remain static within the early chromatograph region. This interpretation is supported by the observation that native PC peaks were found to decrease with pH in Figure 2.11.

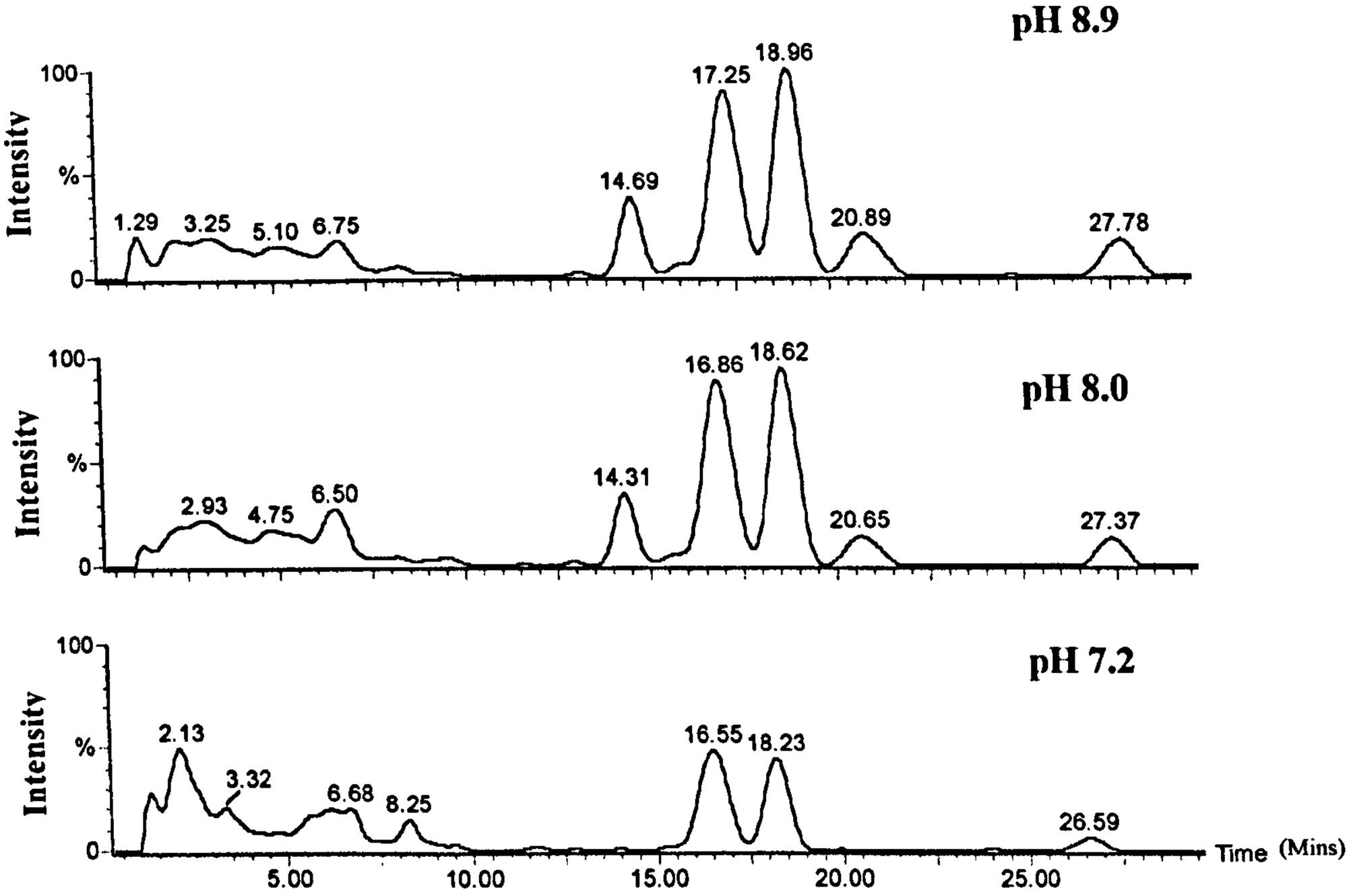


Figure 2.11. NaOCl treated vesicle chromatograms at pH 8.9, 8.0 and 7.2.

The total ion chromatograms (TICs) and chromatograms are shown for phosphatidylcholine vesicles treated with 3.5mM NaOCl for 48 hours at pHs 8.8, 8.0 and 7.2. The chromatograms are vertically linked within each panel to allow comparison of the intensities. The intensities of chlorohydrinated (e.g. m/z 810, 812 and 840) lipids can be identified in this way.

Analysis of glutathione oxidation.

^{1}H-NMR was used to monitor the interaction of sodium chlorite with the tripeptide antioxidant glutathione, and hydrogen peroxide. NaOCl and BAK were also investigated to provide a comparison. The extent of oxidation of the glutathione was

measured from the intregrals of the β-cysteinyl protons (g2) of GSH and GSSG (Figure 2.12) as described previously (Reglinski *et al.*, 1991). This method allows total glutathione to be monitored by the relative distance of the g1 proton from the others as well as the rapid and simultaneous monitoring of both the oxidized and reduced forms of glutathione.

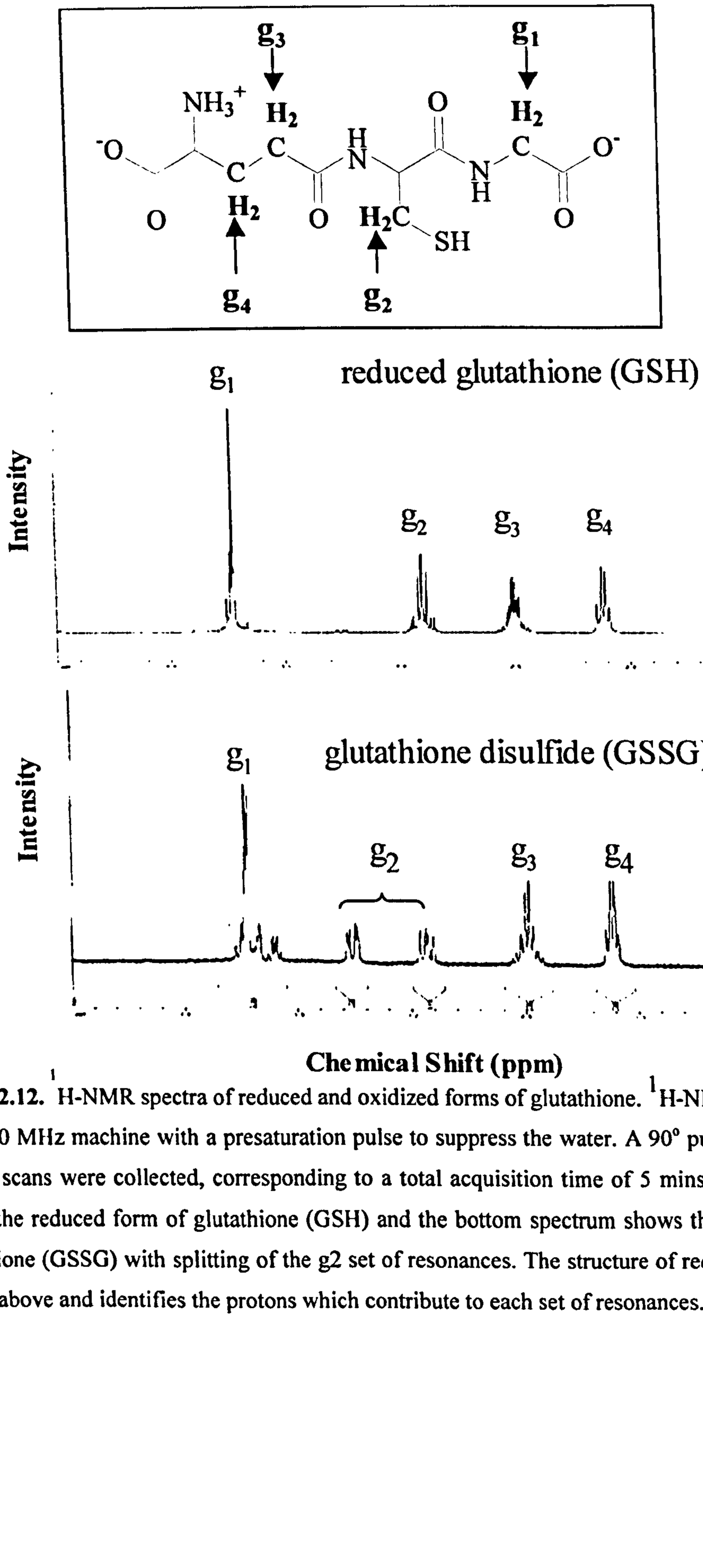


Figure 2.12. ^{1}H-NMR spectra of reduced and oxidized forms of glutathione. ^{1}H-NMR was carried out on a 400 MHz machine with a presaturation pulse to suppress the water. A 90° pulse angle was used and 32 scans were collected, corresponding to a total acquisition time of 5 mins. The top spectrum shows the reduced form of glutathione (GSH) and the bottom spectrum shows the disulfide form of glutathione (GSSG) with splitting of the g2 set of resonances. The structure of reduced glutathione is shown above and identifies the protons which contribute to each set of resonances.

Reduced glutathione was treated with a range of molar ratios of sodium chlorite, hydrogen peroxide and NaOCl, and ^{1}H-NMR spectra were acquired over time courses of 30 mins. Figure 2.13a shows the effect of increasing molar ratios of oxidant:GSH on glutathione oxidation after 5 minutes incubation. It can be seen that sodium chlorite was highly effective at oxidizing GSH and caused complete oxidation of glutathione at an oxidant:GSH ratio of 1:4. NaOCl reacted less efficiently with glutathione and showed a much smaller stoichiometry, and hydrogen peroxide was found to be the least effective oxidant.

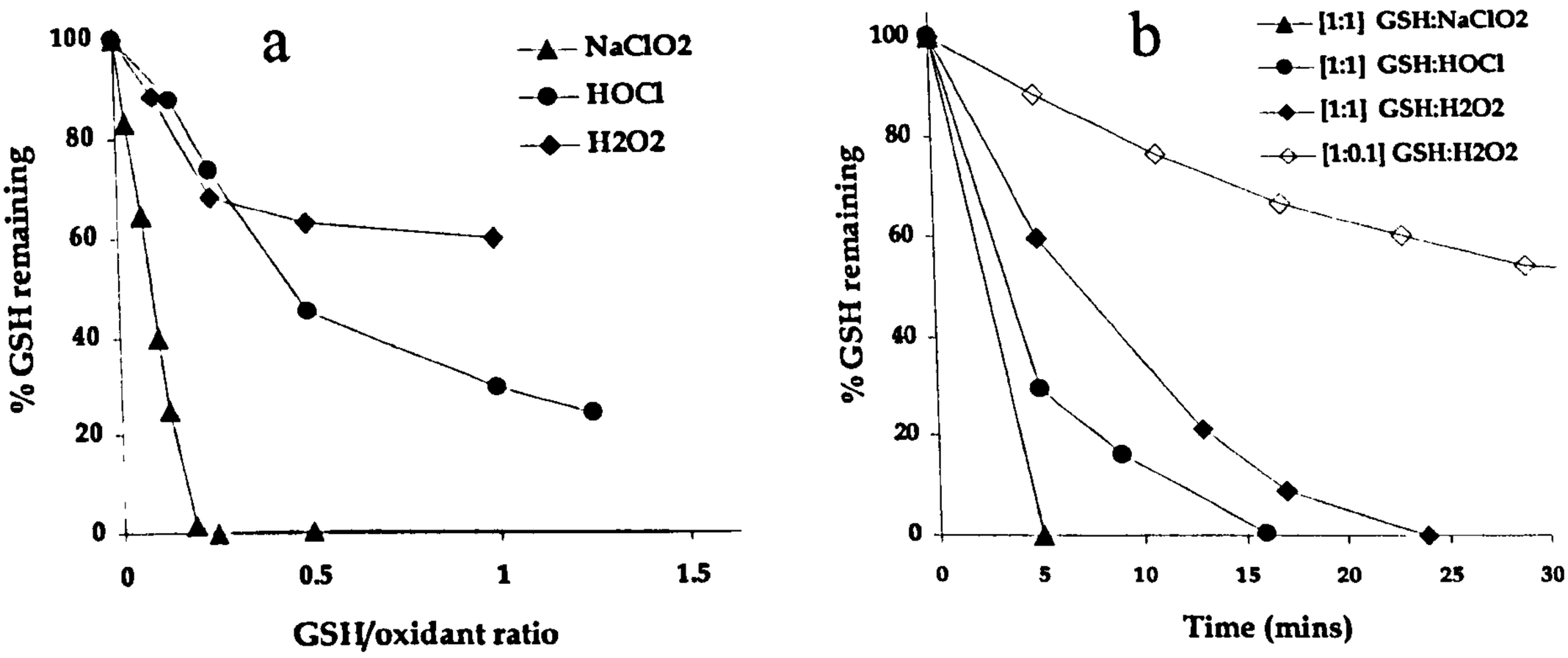


Figure 2.13. Glutathione oxidation by sodium chlorite, hypochlorite and hydrogen peroxide. The ratio of GSH to GSSG was determined from the relative sizes of the disulfide g_2 resonance and thiol g_2 resonance observed by ^{1}H-NMR. 10 mM glutathione was incubated with the oxidants at various GSH:oxidant molar ratios. a) shows the oxidation of GSH after 5 mins incubation with sodium chlorite, hypochlorite and hydrogen peroxide over a range of GSH:oxidant ratios (n=3). b) shows the oxidation of GSH over a timecourse of 30 mins by sodium chlorite, NaOCl and hydrogen peroxide at the molar ratios indicated in the legend (n=3).

As the stoichiometries for the reaction of NaOCl and hydrogen peroxide with GSH are both 1:2, it was concluded that the low effectiveness of these two oxidants might be due to slower kinetics of reaction. This was confirmed by the time course

experiments (Figure 2.13b). These showed that sodium chlorite caused very rapid oxidation of glutathione, with the reaction proceeding to completion within the time required to acquire the first spectrum. Whereas the NaOCl reaction reached completion in about 15 mins, and oxidation by hydrogen peroxide was even slower. Thus, sodium chlorite causes both very rapid and highly efficient oxidation of reduced glutathione, compared to the other oxidants tested. There was no evidence of the formation of higher oxidation products of the sulfur, such as sulfoxides or sulfones, when GSSG was treated with either H_2O_2 or sodium chlorite. Some depletion of the total glutathione pool was observed with NaOCl at high concentrations, in agreement with previous observations of further oxidation of GSSG by this oxidant (Riley, 1990). Comparable experiments were also carried out with BAK, and confirmed as expected that this preservative did not cause oxidation of GSH, even after 2 hours (data not shown).

Analysis of tryptophan oxidation.

The aromatic amino acid tryptophan was used as a model system to investigate the potential effects of these reagents on proteins. Tryptophan in aqueous solution at 1 mM was treated with 1-10 mM concentrations of sodium chlorite, hydrogen peroxide and NaOCl over timecourses up to 1 hour. The occurrence of oxidative modification was determined by LC-MS. Tryptophan (205 m/z) eluted at approximately 11.1 mins (Figure 2.14). Treatment with NaOCl at equimolar concentrations resulted in a decrease in the intensity of the tryptophan peak after 10 mins, with concomitant appearance of new species at 10.8 (146 m/z), 11.6 (144 m/z), and 16.8 min (178 and 180 m/z). The species with a mass of 144 m/z could result from the loss of the alpha-carbon fragment to give a formyl-indole derivative, but further analysis to confirm this identification was not carried out. By 40 mins treatment the native tryptophan had decreased further, the signal at 16.8 mins had undergone further modification, and the major species present eluted at 11.6 mins. In contrast, 1 mM sodium chlorite did not cause any detectable modifications of tryptophan after 40 mins of treatment (Figure 2.14) or after 1 hour. The same was found with H_2O_2 (data not shown). Treatment with much higher concentrations of these oxidants, up to 40 mM, also showed no evidence of tryptophan oxidation.

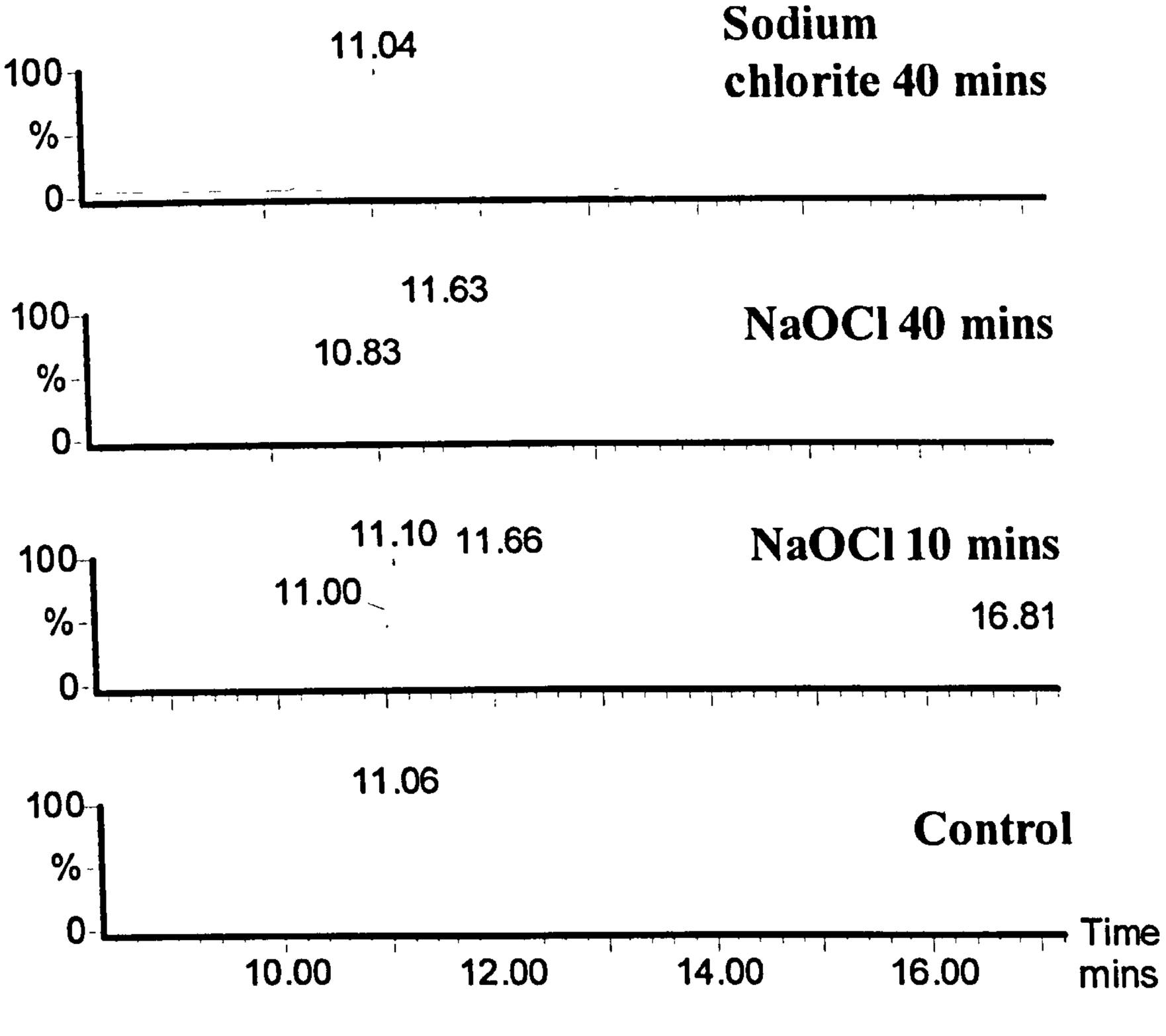


Figure 1.14. Effect of sodium chlorite and hypochlorite on tryptophan. The LC-MS chromatograms of 1 mM tryptophan treated with 1 mM sodium chlorite or hypochlorite for the time indicated on the chromatograms. The mass window used was 100-400 m/z, and a gradient was run from 50% acetonitrile plus TFA to 100% acetonitrile plus TFA over 24 mins. The vertical axes of the chromatograms are linked.

Discussion.

Phospholipid vesicle studies.

In these studies the effect of specific preservatives and oxidant treatments on biological molecules was determined. The depletion of native lipids and appearance of oxidised lipids was monitored either by direct injection electrospray mass spectrometry (ESMS) or by prior separation using HPLC interfaced with ESMS detection. Phosphatidylcholines (PCs) were identified by their mass-to-charge ratio. This method indicates the total number of carbons and double bonds in both fatty acyl chains, but does not give absolute confirmation of the individual chain composition. The limitation of ES-MS as a non-fragmentation technique does not cause great concern in this model system since the vesicle composition depends on the PC used in their formulation. Assignments of the predominant native lipid species are, therefore, based on the most common combinations of fatty acyl chains selected from stock PC's, and those PC's observed previously by the group to form the egg yolk mixed PC stock. These were determined by retention times relative to known phospholipids on a C_8 Luna HPLC column. This designation is not as definitive as gas chromatography MS. However, GCMS involves hydrolysis and trans-esterification prior to fragmentation analysis which could introduce confounding artifacts. ESMS is considerably simpler, more rapid, cheap and convenient given the scale and location of the analysis. One major limitation is that the use of a single quadropole MS detector did not detect natural breakdown products of treated lipids. Therefore, discrepancies in the loss of native lipids, without full appearance of oxidised products, is probably due to their subsequent breakdown being undetected.

Purite™, at comparable oxidising concentrations to NaOCl, did not cause significant damage to phosphatidylcholine vesicles over a 1-2 hour treatment period. Even over longer incubations (24-48 hours) Purite™ was much less damaging than the other oxidants tested. t-BHP was used at high concentrations and was used as a positive control to show that hydroperoxide generation in acyl-chains could be detected using this experimental system. Long incubations (24-48 hours) with Purite™ showed evidence of lipid hydro-peroxidation, without chlorohydrin

formation, indicating a free radical mechanism of attack. This is consistent with some of the known chemistry of ClO_2, a component of Purite™ breakdown. ClO_2 is thought to undergo 1-electron reduction reactions. Chlorine dioxide is a free radical and could be expected to cause hydrogen abstraction and, therefore, peroxidation.

The effect of pH on treatment also supports the ClO_2 pathway of reaction at lowered pH where the equilibrium of $NaClO_2$ is displaced to $HClO_2$, which, in higher concentrations, can form $ClO_2 + Cl^- + H^+ + H_2O$.

This observation agrees with some of the findings of Panasenko *et al.* (1997). They determined the levels of liposomal lipid peroxides by assaying for thiobarbituric acid-reactive substances and diene conjugates (see Figure 2.3) formed. This can be an indirect assay for peroxidation. They found that treatment of liposomes with 400μM $NaClO_2$ over 40 minutes at pH 7.2 generated lipid peroxides. This is clearly a lower level of sodium chlorite and shorter time but generated the same products.

Treatment with t-BHP and NaOCl results in the depletion of unsaturated lipids, and the appearance of corresponding hydroperoxide and chlorohydrin oxidised species. The NaOCl observation agrees with the findings of Panasenko *et al.* (1997). In addition, the formation of chlorohydrins, at native lipid m/z+52Da and hydroperoxides, at native lipid m/z+32Da agrees with Schaur *et al.* (1998), Spickett *et al.* (1998) and Jerlich *et al.* (2000). Although some researchers have detected lipid peroxidation products following hypochlorite treatment (Panasenko *et al.*, 1994) this has been suggested to depend on the presence of trace amounts of hydroperoxides or free radicals (Panasenko and Arnhold, 1999).

NaOCl depletion of native vesicle phospholipid peaks increased at lower pH. This agrees with the results of previous research that shows HOCl causing higher phospholipid oxidation when compared to NaOCl (Panasenko *et al.*, 1997). The lack of increase in chlorination product in this investigation may be due to the extended length of the experiment (48 hours) leading to product breakdown. This time was selected to allow comparison with the Purite™ results.

BAK did not cause specific oxidation of unsaturated lipids, but some non-specific native lipid breakdown was observed. This anti-microbial surfactant has not previously been investigated in the manner tested here. From the known chemistry,

BAK would not have a direct oxidative interaction with lipid vesicles in the model system.

Tryptophan studies.

With the aromatic amino acid tryptophan, a similar trend was observed. Even high concentrations of sodium chlorite gave no detectable modification of tryptophan, and the same was true of treatments with hydrogen peroxide. In contrast, NaOCl at relatively low concentrations caused loss of the native signal and appearance of a number of new species. This result agrees with previously published data on the modification of tryptophan residues in low-density lipoprotein. This has been measured by tryptophan fluorescence and is known to occur rapidly even at the micromolar concentrations of HOCl generated by myeloperoxidase (Jerlich *et al.*, 1998). Thus, it appears that the electrophilic oxidant is more efficient at attacking the indole ring system than the free radical oxidants.

Glutathione studies.

The results obtained when the reactions with the antioxidant glutathione were examined were somewhat different. Sodium chlorite was found to be highly effective at oxidizing glutathione to the disulfide form, and showed a much more rapid reaction than NaOCl or hydrogen peroxide. *In vivo*, the reaction of peroxides with glutathione would normally be catalysed by glutathione peroxidase. Hydrogen peroxide can additionally be detoxified by catalase, which is thought to be an important protective mechanism in the eye (Riley, 1990). The 1:4 stoichiometry observed suggests that during oxidation of glutathione by sodium chlorite, the chlorine is reduced completely to chloride in a 4-electron process, according to the following equation:

$$4\ GSH + NaClO_2 \rightarrow 2\ GSSG + 2\ H_2O + NaCl.$$

Although sodium chlorite converted GSH to GSSG very rapidly, no further oxidation of the sulphur was observed, unlike NaOCl which has been shown previously to be able to convert the thiol group of glutathione to sulfonamides and thiosulfonates (Winterbourn and Brennan, 1997). This may represent an important mechanism of toxicity of NaOCl, as it prevents the recycling of glutathione by the normal redox

pathways, leading to depletion of antioxidant defences. However, possibly incubation of the glutathione with higher concentrations of sodium chlorite over longer time-courses would have yielded further oxidation products.

The reaction of sodium chlorite with glutathione is consistent with a previous report that this compound can cause depletion of glutathione in the erythrocyte (Abdel-Rahman and Scatina, 1985). However, our results do not support the suggestion that damage to the lipid moiety of the membrane is responsible for the increased erythrocyte membrane fragility reported previously for 100 ppm sodium chlorite (Moore and Calabrese, 1980). It seems more likely that the primary cause of damage was depletion of glutathione, as this is an essential antioxidant in erythrocytes, and our study has shown that sodium chlorite was much more effective at oxidising glutathione than phospholipids. Although it is possible that depletion of glutathione may render the erythrocytes more susceptible to subsequent membrane damage either by the oxidant or by iron released from the haemoglobin.

Summary.

In conclusion, the observations presented here indicate that Purite™ does not have strong phospholipid oxidising activity in this anti-oxidant free model system. Purite™ is commonly used at 0.005%, at this level, as an oxidising preservative, its oxidative effects upon membrane phospholipids could be described as mild. Also, Purite™ behaves more like hydrogen peroxide in its reaction with tryptophan.

Purite™ is unlikely to interact by electrophilic addition of an oxy-chloro moiety, as observed with NaOCl, but appears to undergo free radical interactions to generate hydroperoxides. This is the known mechanism of t-BHP (Spickett *et al.*, 1998). However, the reaction of sodium chlorite/Purite™ with glutathione is much faster than that of peroxide, and involves a 4-electron process rather than a free radical reaction. This may reflect a fundamental difference in the nature of its lower reactivity with biological molecules containing unsaturated systems compared to thiol groups.

The concentrations of sodium chlorite required to cause peroxidative damage to phospholipids *in vitro* suggests that this is not likely to be an antimicrobial mechanism at the concentrations normally present in ocular drug formulations. The

reaction with aromatic amino acids are also unlikely to be an important mechanism of damage. On the other hand, the efficient reaction with glutathione suggests that this may well contribute to its preservative effect, by depleting antioxidant defences in micro-organisms where glutathione is an important protectant.

Chapter 3:

Effect of preservatives on mammalian cell and microorganism viability and antioxidant levels.

Introduction

Since the decisions by the FDA and International Pharmacopoeia to add preservatives to all multidose ophthalmic preparations, the potential benefits of inhibiting microbial contamination and drawbacks of patient reactions have been constantly reported (Abelson and Washburn 2002, Debbasch e*t al.*, 2000 and Noecker, 2001). Preservatives provide antimicrobial activity in the container during prolonged storage and during use. Research has shown that preservatives are necessary in multidose containers, because bacterial contamination frequently occurs with the use of the drug at least twice daily for one or two weeks (Schein *et al.*, 1992).

Most classical methods for testing anti-microbials have involved the use of liquid suspensions, treated with the compound under test, followed by a viable counting procedure to determine the survival levels, and end (extinction) point. The choice of test organisms is important to reflect fairly the antimicrobial challenge that a preservative would normally be exposed to. Microbes selected would best represent each of eukaryotic microbes, Gram-positive prokaryotes, and Gram-negative prokaryotes. The FDA uses the Preservative Efficiency Test (PET) as a minimum standard of preservative performance. In the PET, a standard concentration of common bacteria and fungi, such as *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger* are prepared and tested against each preservative. The inoculated tubes are incubated at 20 or 25°C for four weeks, with weekly examinations. After the completion of the PET, a preservative is considered effective if it reduces the bacterial concentration to 0.1 percent or less of the initial concentration after two weeks and keeps the concentration of yeasts and molds at or below their original concentration for the remaining two weeks (Schein, *et al.*, 1992).

The standard preservative test is a single bacterial inoculation of desired number and type of organism. Repeat inoculations, capacity tests, and mixed culture challenges may also be considered if they are relevant to the agent under investigation (Leak, 1983). Bacterial culture and inoculated sample holding conditions, time points of aliquot removal, mode of preservative neutralization, diluent and subsequent incubation conditions are all variables that have to be decided carefully (Russell, 1973). Early mammalian cell survival studies involved quantifying vitally stained cell/ preservative suspensions, e.g. cells in Trypan blue, using haemacytometers/counting chambers. Today, more sophisticated methods of evaluating cell viability have been developed. Tritiated-thymidine uptake studies utilise radio-labelled DNA pre-cursors and monitors isotope consumption to reflect cellular growth/viability during an experiment. In the same way, measuring cellular ATP by Luciferin/Luciferase luminescence has become popular as a method for assaying cellular cytotoxicity in high throughput screening and drug research.

The preservative compounds used in this application have been categorised as oxidants or detergents. The detergent preservative used in this investigation, benzalkonium chloride BAK, acts upon microorganisms by altering cell membrane permeability and releasing the cytoplasmic contents (Kreiner, 1979). Ocular toxicity occurs when the detergent preservatives affect eukaryotic cells. When preservatives are incorporated into mammalian cells, and are unable to be neutralised, cellular damage arises. Oxidative preservatives are usually smaller molecules that penetrate cell membranes and interfere with cellular function (Block, 1991). The stabilised oxychloro complex Purite*™* and the peroxide generating sodium perborate system are two examples of oxidative preservatives.

The antioxidants that have been investigated here are glutathione and ascorbic acid (vitamin C). Total glutathione measurement quantifies the total reduced (GSH) and oxidized (GSSG) forms in a cell. Measurement of GSH singularly can give an indication of the levels of oxidative stress if the ratio to GSSG is known. The total glutathione indicates the ability of a cell to cope with oxidative treatments (Sies, 1999). Hence, depletion of cellular glutathione leaves cells more susceptible to oxidative damage. There are a number of enzymatic and chromatographic assays for determination of the anti-oxidant glutathione (GSH) and glutathione disulfide

(GSSG) in biological samples. Modifications of the DNTB-GSSG reductase recycling assay for total glutathione are commonly used (Tietze, 1969; Griffith, 1980). Uncoupling this assay, by use of 2-vinylpyridine, is reported to assay for GSSG alone (Anderson and Meister, 1983). Glutathione levels may also be determined by HPLC (Newton *et al.*, 1981) and electrospray mass spectrometry (Carr and Winterbourn, 1997). Detailed chemical analysis of GSH/GSSG oxido/reduction states have been investigated by nuclear magnetic resonance (Reglinski *et al.*, 1991). Researchers have reported numerous methods for determining ascorbic acid and α-tocopherol levels. These include UV and fluoresence spectrophotometric assays coupled to HPLC (Wyss, 1985). Oxidation products have also been assayed indirectly by thiobituric acid reactive substances (TBA-RS) (Franke *et al.,* 1994), total peroxyl radical- trapping potential (TRAP) (Valkonen and Kuusi, 1997), and oxygen radical absorbance capacity (ORAC) (Cao, *et al.*, 1993) to mention a few.

The aim of these studies was to determine the cytotoxic effects that the panel of preservatives and control oxidants have upon the panel of cell types selected in Chapter 4 and to evaluate the levels of oxidative stress induced. Then to measure the concentrations present in untreated cells and investigate whether retaining viability upon preservative exposure correlated with higher intrinsic intracellular antioxidant concentrations. Then determine whether resistance to Purite™ or other preservatives correlated with the ability to maintain intracellular glutathione levels during stress. Thus, evaluating the relative toxicity of Purite™ and other preservatives to mammalian cells and micro-organisms, and subsequently, to relate differences in toxicity profiles to antioxidant status in a mechanistic approach. It is vital to determine that the levels of Purite™, and other treatments, used were cytotoxic to cells, otherwise it would not be possible to associate the specific effects, upon cellular phospholipids and antioxidants, with a specific loss of cellular viability.

Materials and Methods

Time Course Evaluation of Preservative and Oxidant Cytotoxicity

Mammalian Cell Stocks.

Rabbit corneal epithelial RCE (ECACC No. 95081046) cells were cultured in DMEM:Ham's F12 medium supplemented with 2mM glutamine, 5µg/mL insulin, 10ng/mL eidermal growth factor (EGF) and 15% foetal calf serum (Gibco BRL, UK) until confluent in a 5% CO_2 incubator at 37°C. Human conjunctival cells, WKD (ECACC No. 88021103), were cultured in Medium 199 (Hanks) supplemented with 2mM glutamine and 10% foetal calf serum (Gibco BRL, UK) until confluent in a 5% CO_2 incubator at 37°C.

Mammalian Cell Treatments.

Mammalian cells were sub-cultured into 24-well culture plates (Greiner, Gloucester UK) using the detaching agent Acutase (icT, California, USA) and allowed to adhere overnight in growth medium. Cells were seeded at 1.5mL per well of a $1x10^5$/mL stock. Cells were treated with various oxidants and preservatives at the following concentrations: sodium chlorite (Purite™) at 0.005% [=0.55mM], 0.03% [=3.5 mM] (w/v), benzalkonium chloride (BAK) at 0.005%, 0.2% (w/v); NaOCl at 3.5 mM pH 7.2 (w/v); and *tert*-butylhydroperoxide (tBHP) + Fe^{2+} at 0.25 M + 1 mM (w/v). Ten times final concentration cell treatments were freshly prepared for each experiment in sterile PBS and added to serum free culture medium. Control treatments had phosphate-buffered saline added in the place of a preservative or oxidant. Once adhered, wells were washed with sterile PBS and this wash was discarded. 1.5mL of the prepared preservatives or control oxidants in media, at their final concentration, were then added to each well of the culture plate. Treatments were incubated with the cells in a 5% CO_2 incubator at 37°C over a time course of 24 hours in the dark. Wells were examined at 1, 2, 3, 4, and 24 hours post-treatment. Loss of viability was determined by counting the number of non-adherent cells present in the medium. Counting after trypan blue staining (Sigma, Dorset UK) was carried out to assess the viability of floating and adherent cells.

Microbial Cell Stocks.

Stocks of the yeast, *Candida albicans* (Clinical islolate from Glasgow Royal Infirmary, UK), and bacteria *Pseudomonas aeruginosa* (NCTC 10781) and *Staphlococcus aureus* stocks were made on Protect storage beads (TSC Ltd, Lancashire, UK) and stored at –80°C for up to 3 years. Fungal stocks of *Alternaria spp.* (IMI389319 CABI Bioscience, UK), an isolate from Allergan Corp, California USA, were prepared by adding 0.5g/mL wet-weight cells in 50% YEPD broth with 50% Glycerol and stored at 5 °C

Microbial Cell Treatments.

Candida albicans and *Alternaria spp.* cultures were inoculated from stock organisms stored on Protect beads or from stock broth respectively. Cultures were grown aerobically for 18 hours in 250mL of YEPD broth in 1L shake flasks. The YEPD broth contained, 1% yeast extract (Difco, Michigan USA), 2% bacto peptone (Difco, Michigan USA) and 2% glucose (BDH, Dorset, UK) (all w/v). This was incubated at 37°C and 26°C for the two organisms respectively.

Bacterial cells were aerobically incubated in tryptone soya broth (TSB) (Oxoid, Hampshire, UK) at 37°C for 18 hours in 250mL of broth in 1L shake flasks after inoculation by a Protect storage bead (TSC Ltd, Lancashire, UK).

Bacteria and *C. albicans* were grown for 18 hours at 37°C in conical flasks in TSB and YEPD respectively. Cultures were centrifuged and washed twice in sterile PBS. Cultures were re-suspended at 10^7/mL in treatments using an adaption of the McFarland (1907) Standard turbidity technique.

Time-course treatments were incubated in smaller 25mL volumes in 250mL flasks. Experiments were carried out using the same concentration of treatments and time-course as the mammalian cell studies. At each time point serial dilutions of the samples were made in sterile water; followed by plating 100µL of cells onto TSB or YEPD agar. Microbial cell viability was determined from the number of colony forming units (CFU) after incubation at 37°C for 48 hours.

Alternaria spp. is a filamentous fungus, and cannot be cultured as readily as the other micro-organisms studied. It was not possible to assess the number of viable cells present by plating serial dilutions and counting the number of colonies formed,

as it does not form colonies. Hence, it was necessary to establish an alternative methodology for determining the effects of treatments on growth, and eventually conditions that allowed reproducible growth of *Alternaria* spp. were developed. *Alternaria* spp survival curves were performed by dry mass analysis. 0.2mL of *Alternaria* spp stock aliquots (0.5g/mL wet.wt cells in 50% YEPD 50% Glycerol stored at 5°C) were incubated for 18 hours at 26°C in 250mL conical flasks. Treatments, at the concentrations used for the other organisms, were prepared and added to the cultures. Control samples, of *Alternaria* and YEPD media only, were removed at 0, 2, 4, and 24hrs and vacuum filtered, using a magnetic disc holder (Pall Gelman, Hampshire UK), onto 47mm, 0.2μm, glass fibre filter discs (Pall Corp. Hampshire UK). Filters had been pre-dried at 50°C for 24 hours and pre-weighed. Wet filters were dried at 50°C for 24 hours and re-weighed. Dry cell mass was calculated. Treated samples were removed at 2, 4, and 24 hours; filtered the same as controls, dried, and dry cell weight calculated. All treatments were compared to their respective control for analysis.

Antioxidant Extraction.

All cells were cultured and treated following the same protocol used in cytotoxicity studies with two modifications. First, the volumes and cell numbers were scaled up to gain sufficient cells to extract the contents of interest. Micro-organisms were grown in 250mL of broth in a 2.5L flask and mammalian cells were grown in 10mL of media on 75cm^2 flasks. Second, the treatments were Purite^{TM} and NaOCl at 3.54 mM, BAK at 0.005% and t-BHP at 0.25M with 1mM Fe^{2+}. All cells were harvested into pre-weighed centrifuge tubes. Cells were centrifuged and washed twice in PBS. The final PBS wash was carefully removed to ensure only removal of all moisture and the cell pellet was weighed and recorded.

Mammalian cells contents were extracted by the addition of 5% trichloroacetic acid (TCA) (w/v) to precipitate cellular protein, followed by freeze-thawing and centrifugation to remove the cell debris. Microbial glutathione was extracted by re-suspending the cells in 1.5mL PBS and pressing the cells in a cell membrane disrupter (Constant Systems) at 20 kPa, followed by protein precipitation by adding 0.25mL 20% TCA (w/v) to 0.75mL pressed cell solution.

Antioxidant Analysis.

Glutathione.

Total glutathione was determined spectrophotometrically using the enzyme kinetic DTNB-recycling assay of Tietze (1969). The reactions occurring in the assay were:

2 GSH + DTNB → GSSG + TNB

GSSG + NADPH + H^+ → 2 GSH + NADP

GSH was being oxidised by DTNB (5,5'-dithiobis-2-nitrobenzoic acid) to give GSSG (oxidised glutathione) with the formation of TNB (5-thio-2-nitrobenzoic acid), and GSSG was being reduced to GSH by glutathione reductase and NADPH. The rate of TNB formation is read at 412nm and the slope of the reaction line was proportional to the sum of GSH and GSSG, i.e. total glutathione, present.

Glutathione (reduced form standard), glutathione reductase, DTNB (5,5'-dithiobis-2-nitrobenzoic acid), and NADPH (nicotinamide adenine di-nulceotide phosphate-reduced form) were obtained from Sigma Chemical Co., USA. The

following were obtained from BDH Laboratories, UK: Na_2HPO_4, NaH_2PO_4, EDTA, $(NH_4)_2SO_4$, and TCA. 125 mM NaPi (sodium inorganic phosphate) buffer contained 100 mM Na_2HPO_4, 25 mM NaH_2PO_4, and 8 mM EDTA. The pH was adjusted to pH 7.4. NaPi buffer was stored at room temperature (20-25°C), but was used at 30°C in the assay. The following solutions were prepared and used on the day the samples were to be assayed: 1.735 mg/ml NADPH (reduced form) in NaPi buffer, 6 mM DTNB in NaPi buffer, 340 U/ml glutathione reductase in 3.5 M ammonium sulphate $(NH_4)_2SO_4$, and 10 mM GSH standard in 5 % trichloroacetic acid (TCA) (w/v). The 10 mM standard solution was used to prepare 10 µM, 20 µM, 40 µM, 60 µM, 80 µM, and 100 µM GSH standard solutions in 5% TCA. All solutions were stored in the dark, on ice.

770 µl of NaPi buffer (warmed at 30°C), 100 µl of 6 mM DTNB, and 100 µl of 1.735 mg/ml NADPH were dispensed into an UV grade cuvette (Fisher, UK). The cuvette was warmed at 30°C in the spectrophotometer compartment. 25 µl of 2% TCA (blank), standard, or sample was added to the reagents and the cuvette was inverted gently to mix thoroughly. 5 µl of 340 U/ml glutathione reductase was added with mixing to initiate the assay reaction. The formation of TNB was followed continuously at 412 nm for a total of 2.5 absorbance units, with the linear portion of the curve usually occurring between 1 and 2 absorbance units. The amount of glutathione in the sample was determined from the standard curve in which the glutathione concentration was plotted against the rate of change in absorbance at 412 nm.

Ascorbate.

The intracellular anti-oxidant ascorbic acid concentration was measured by the extraction of cytosolic contents from all cell types and native ascorbate levels determined using a bipyridyl assay. Cell extracts were mixed with bipyridyl in the presence of Fe^{3+} by a scaled down adapted protocol of Kampfenkel *et al.* (1994). After incubation of the mixture at 42°C for 40 minutes the colour change produced by the presence of ascorbate was measured at 525nm in a spectrophotometer. Absorbance results were compared to an ascorbate standard curve to determine the concentration. Antioxidant concentrations are expressed in micromoles per g wet-weight of cells extracted.

Results

Time Course Evaluation of Preservatives and Oxidants Cytotoxicity.

The differential toxicity of microbes and mammalian cells to a panel of preservatives and positive control oxidants has been evaluated over a 24 hour time course in this chapter. A number of methods have been utilised to generate cytotoxicity responses in the different cell types tested. For this reason each organism type, with their specific viability assay will be evaluated.

Mammalian Cell Treatments.

Mammalian cells, RCE and WKD, were treated and viability assayed by Trypan blue exclusion and counting viable and non-viable cell numbers on a haemocytometer. Two Purite™ concentrations were used in cell survival studies: 0.032% (for comparison with 3.5mM NaOCl) and 0.005% (for comparison with BAK). The toxicity of the preservatives to mammalian cells is shown in Figure 3.1, which plots the number of nonviable cells against the treatment time.

Within treated RCE cells BAK was found to be the most damaging. It is possible that an over-estimation of dead RCE cells arose due to a single cell lysis generating numerous countable cell fragments. After only 1hour, post-treatment, BAK caused de-adherence. NaOCl and t-BHP + Fe^{2+} were also found to cause rapid loss of viability and cell lysis. Purite™ at 3.5 mM had a similar effect to NaOCl, but was less toxic, especially after 24 hours. t-BHP was actually more toxic than it appears from the graph, as at 24 hours few floating cells remain intact and therefore 'countable'. The lower Purite™ concentration had very little effect over the first few hours, but was slightly more toxic than the control by 24 hours post-treatment.

The WKD cell line appeared to be very susceptible to treatment with BAK. The apparent low toxicity was due to complete cell lysis, also observed in RCE cells, and that few cells remained countable after 24 hours with BAK treatment. 3.5 mM Purite™ was very toxic by 24 hours post-treatment, but its effects were less severe over the first 4 hours. 0.005% Purite™ was no more toxic than the control treatment at the early time points. t-BHP + Fe^{2+} was noticeably more toxic early in the incubation, but did not appear to be severely damaging at 24 hours. NaOCl had very little effect on the cells.

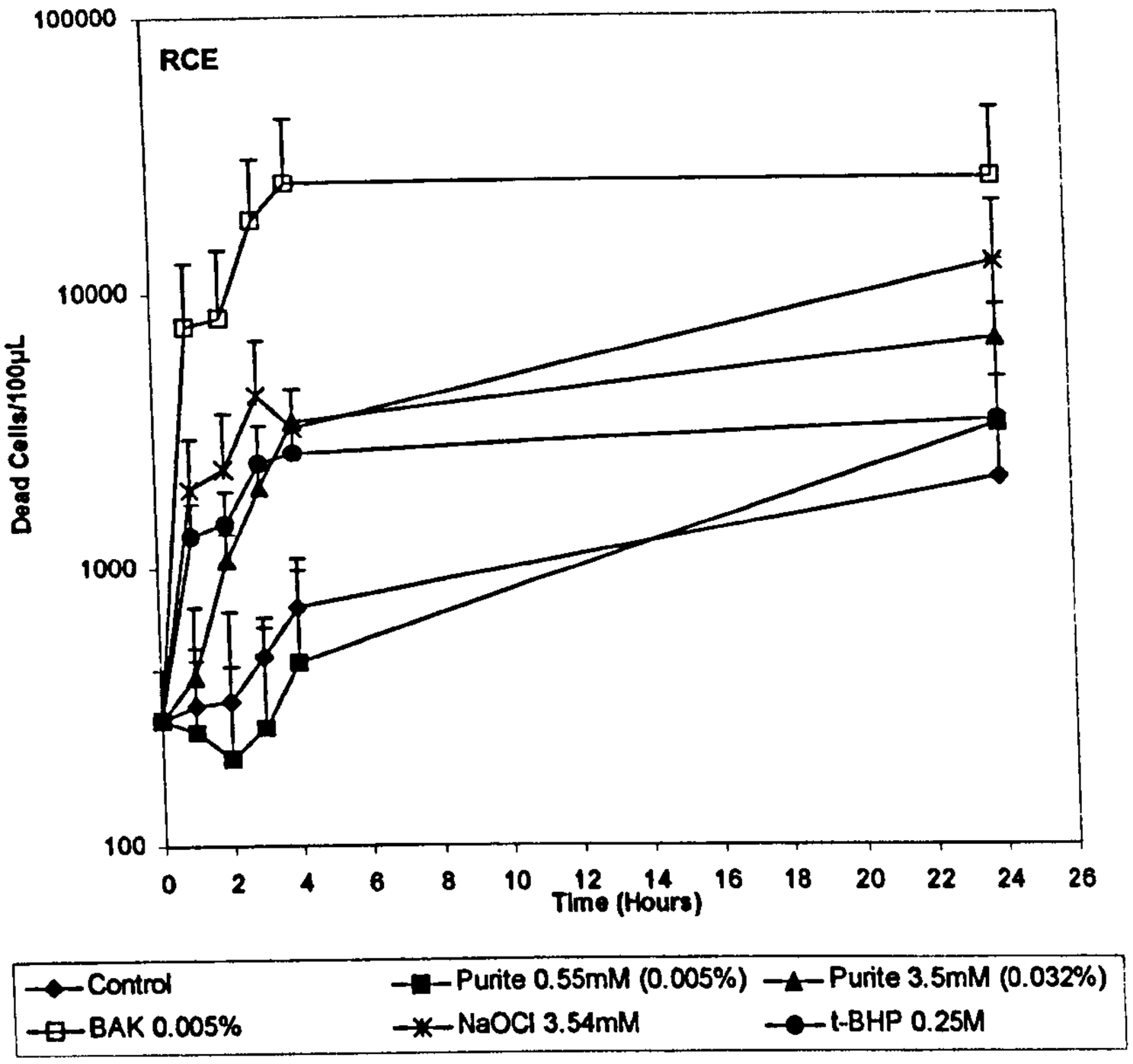


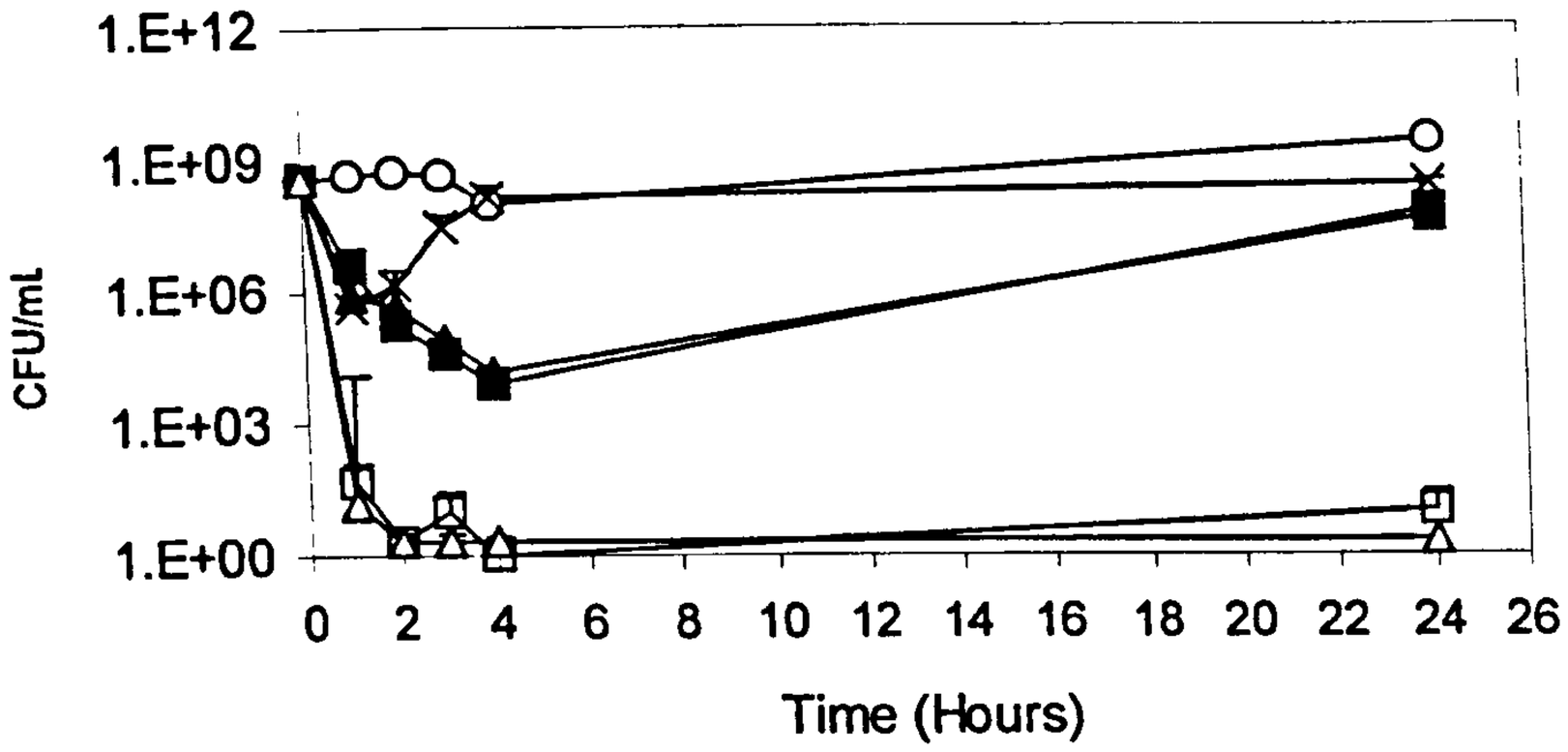
Figure 3.1. Effect of Oxidants and Preservatives on the Viability of Mammalian Cells Measured by Trypan Blue Exclusion. Graphs show plots of non-viable (floating) Rabbit Corneal Epithelial RCE (n=4) and Human Conjunctival WKD (n=4) cells during a time course of treatment (+SEM). Apparent low toxicities of BAK and tBHP are a result of complete cell lysis leaving few countable cells.

Microbial Cell Treatments.

Microbial cells were treated and viability/cell growth was assayed over the same time course as mammalian cells. Viability/cell growth was assayed by dilutional plating of cell suspensions to determine viable cells by the number of colony forming units (*C. albicans, S. aureus* and *P. aeruginosa)*, or by filtration, and measurement of dry mass (*Alternaria)*.

Figure 3.2 shows the effects of preservative treatments on survival of the four micro-organisms, represented as the number of viable cells (expressed on a log scale) remaining in the incubation at each time for all micro-organisms except for *Alternaria*, where dry weight was used to estimate cell growth. Each of the micro-organisms remained viable in the control cultures over 24 hours. Of the 3 micro-organisms assessed by the same method, *C. albicans* growth seemed to be the least affected, since more of the treatments used allowed significant levels of viability at 24 hours. 0.2% Purite™ did not completely kill *C. albicans* until 24 hours post-treatment, whereas *S. aureus* and *P. aeruginosa* were killed by 4 hours post-treatment. Using the same criteria, *S. aureus* was found to be the most sensitive micro-organism to the treatments used. Unfortunately, it was not possible to compare the viability of *Alternaria* spp. directly with the other micro-organisms, owing to the different methodology required to quantify the filamentous fungi. Measurement of weight can provide information on the amount of fungal growth achieved, but cannot indicate whether loss of viability has occurred. However, it is apparent that *Alternaria* was able to grow reasonably well in the presence of both 0.005% Purite™ and 3.54 mM NaOCl, while Purite™ at 3.54 mM, BAK at 0.005% and tBHP + Fe^{2+} all prevented significant growth over 24 hours. Thus it could be concluded that *Alternaria* was more resistant to 0.005% Purite™ than either of the bacteria, and possibly had similar resistance to *Candida* (approximately 10-fold increase in cell numbers / weight over 24 hours).

a) *Staphylococcus*



b) *Pseudomonas aeruginosa*

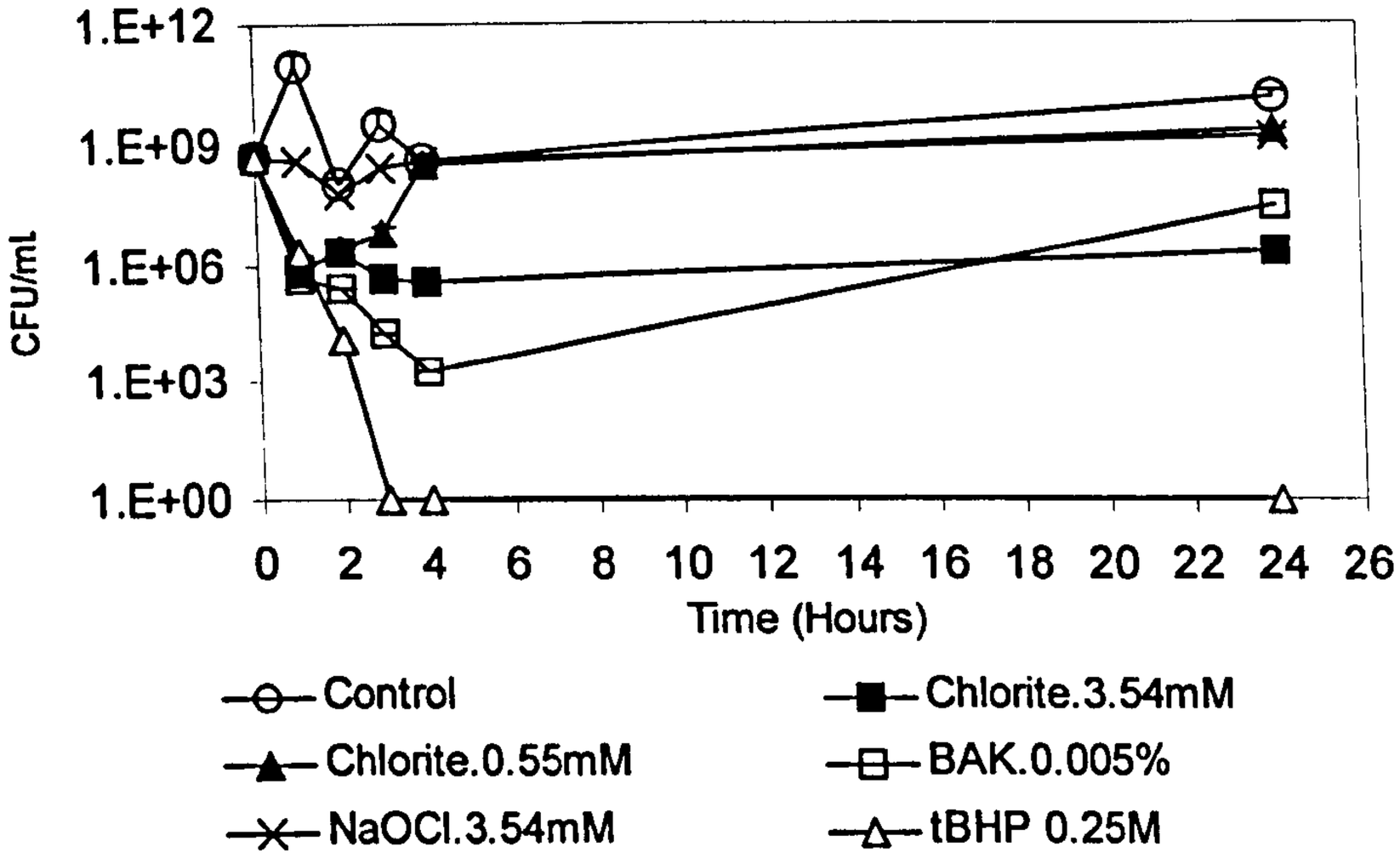
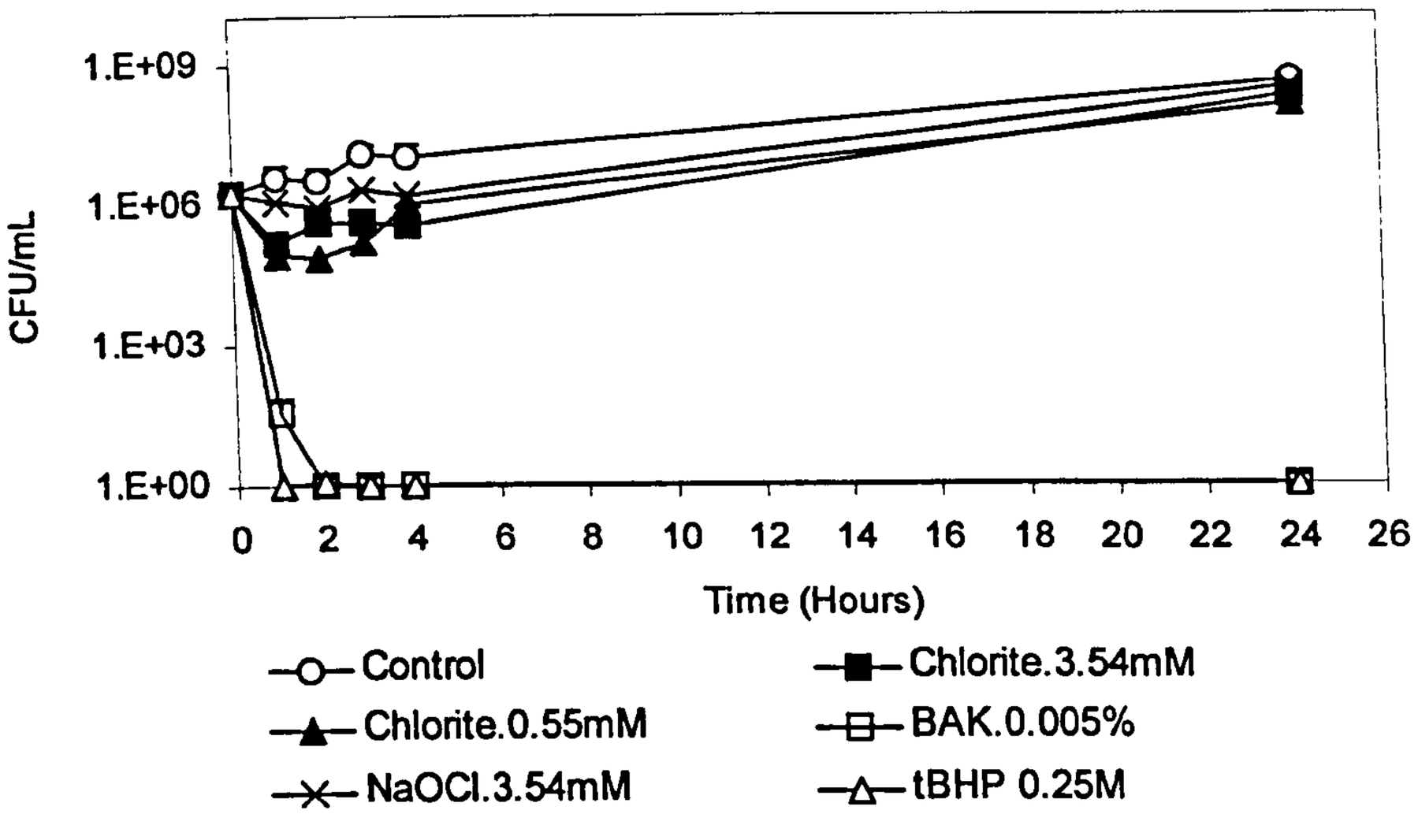


Figure 3.2. Effect of treatments on microorganism viability or growth (Cont'd over...)



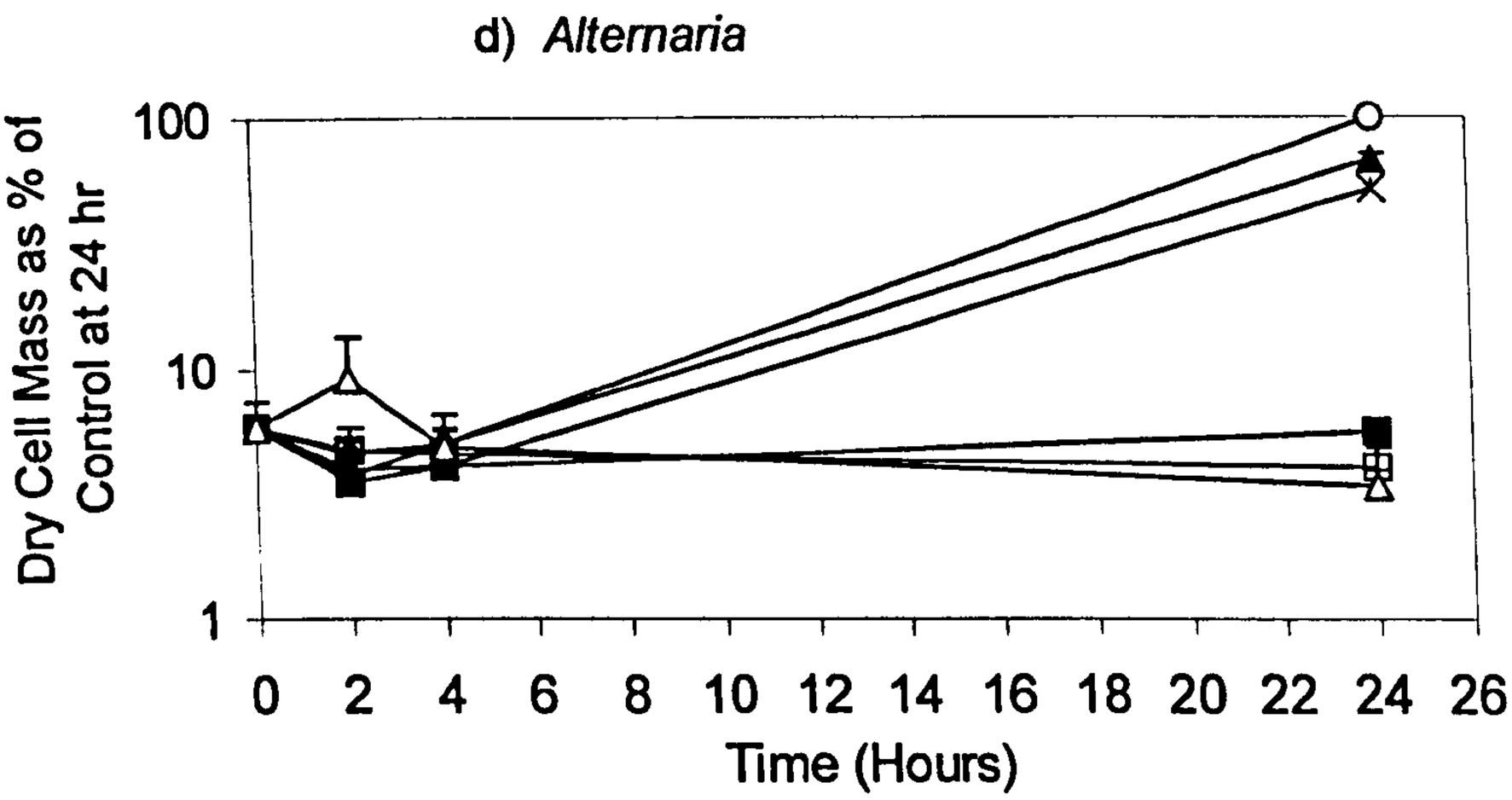


Figure 3.2. Effect of treatments on microorganism viability or growth. The graphs for *S. aureus*, *P. aeruginosa* and *C. albicans* show the number of colony forming units (CFU) remaining in the culture at each time point, equating to the number of viable cells. For *Alternaria* spp. the data is expressed in dry weight of cells as a percentage of the control flask at 24 hours.

Cytosolic Antioxidant Levels and Effects of Treatments.

Figure 3.3 shows the intracellular glutathione concentrations in all the organisms investigated in this study. It can be seen that the highest glutathione levels occurred in *C. albicans*, with an average of 3.53 ± 0.9 μmol/g. The mammalian cells also had relatively high levels, with RCE cells containing slightly more glutathione than WKD cells (2.6 ± 0.3 versus 1.65 ± 0.3 μmol/g). *P. aeruginosa* also had intermediate levels of glutathione (1.73 ± 0.15 μmol/g). *Alternaria* spp. had comparatively low levels of glutathione 0.33 ± 0.052 μmol/g, although still clearly in the detectable range. Whereas *S. aureus* had no detectable glutathione after 18h of culture.

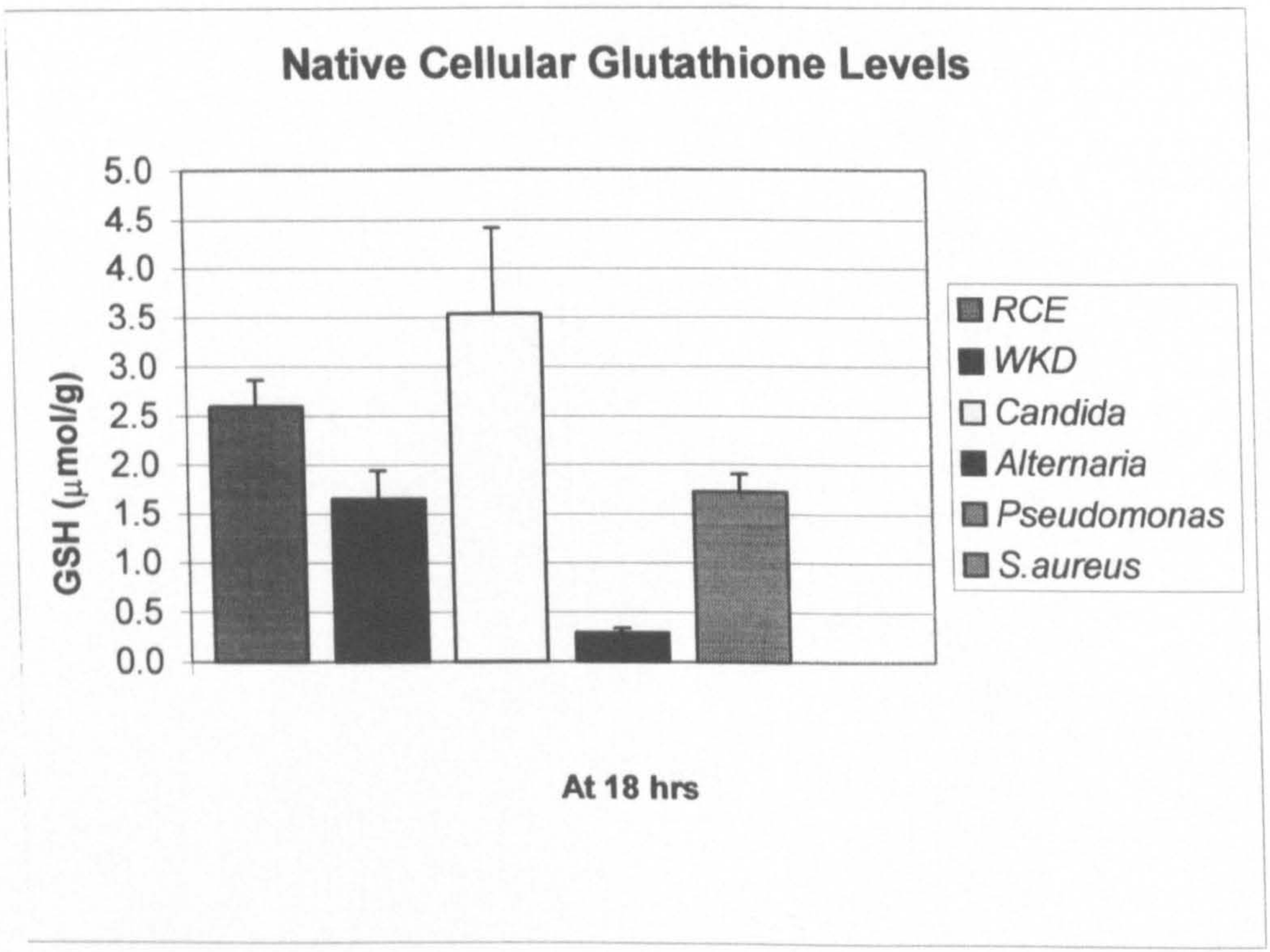


Figure 3.3. Native Cytosolic Glutathione Levels. (+SEM)

Note: Glutathione was not detectable in *S. aureus* in this assay.

Ascorbic acid occurred only at very low levels in *C. albicans* (approx. 0.3 µmol/mL cell water), and could not be detected at all in the mammalian cells or the bacteria. In fact, in *Candida* it is likely that the compound detected was the analogue erythroascorbate, which is known to occur in some fungi. Presumably the low levels of ascorbate in the WKD cells result from the inability of human cells to synthesize the compound from its precursor glucose. Attempts were made to increase the intracellular concentrations by supplementation, but these were unsuccessful; it is known that glucose can interfere with ascorbate uptake and that high intracellular concentrations are difficult to achieve in certain cell lines.

All treatments with the preservatives resulted in significant depletion of the total intracellular glutathione pool in RCE and WKD cells (Figure 3.4). The WKD cells were more sensitive to the treatments than RCE cells, with glutathione concentrations dropping almost to zero. However, the pattern of the response was the same in both cell lines: t-BHP and NaOCl causes the largest depletion, while Purite™ and BAK had less effect. It is presumed that BAK caused loss of intracellular glutathione by damaging the cell membrane, allowing release of glutathione into the medium. This is supported by the observation that BAK resulted in the highest glutathione levels measured in the medium following treatment. Treatments causing oxidation of glutathione to the toxic disulfide form (GSSG) can also lead to the active export of glutathione from cells and these increases were observed. However, treatments of WKD and RCE with Purite™ did not appear to increase the extracellular glutathione significantly when the cellular pool was found to be depleted. This may be due to oxidised glutathione breakdown as part of the stressed cellular homeostasis response.

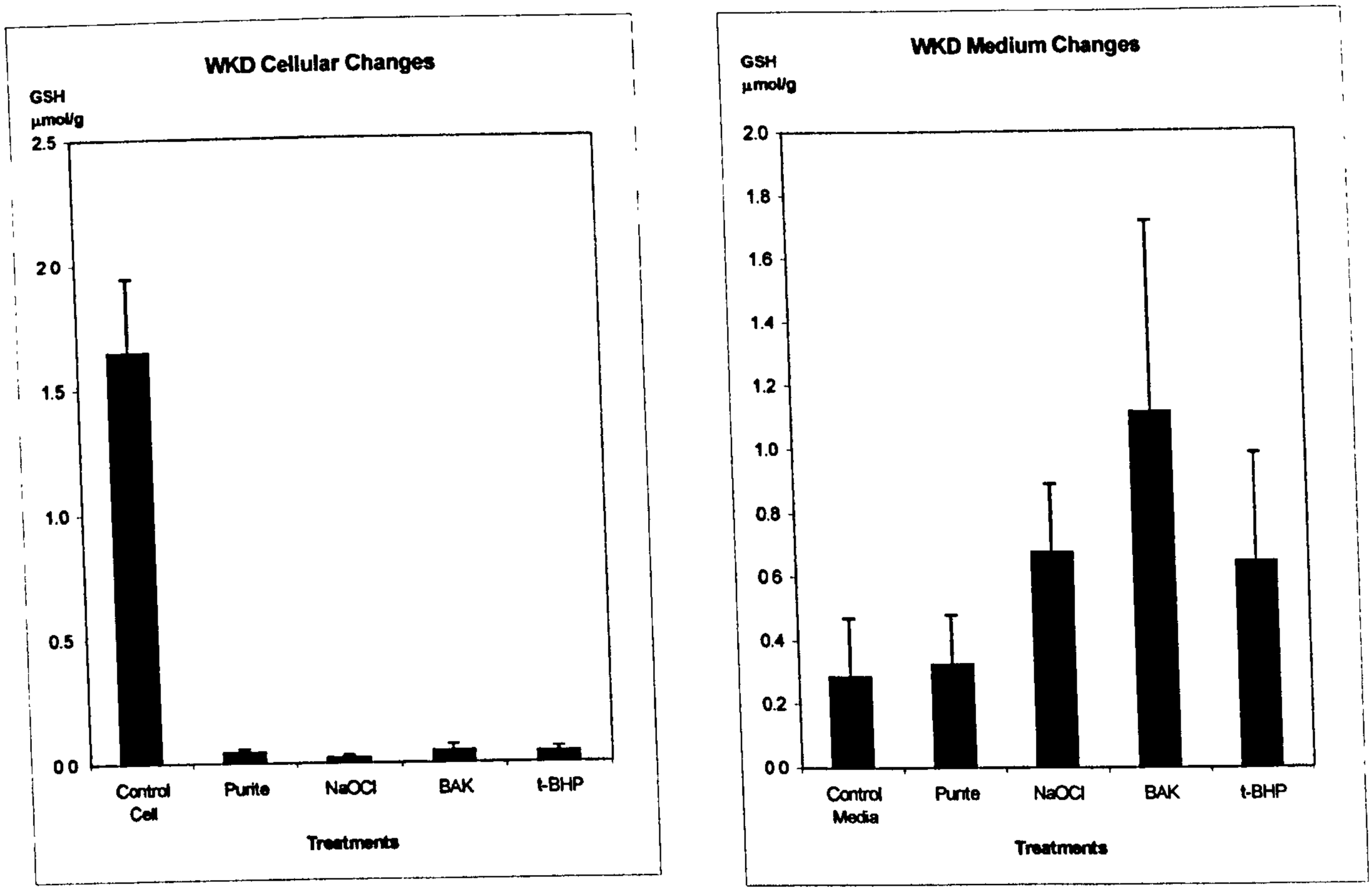
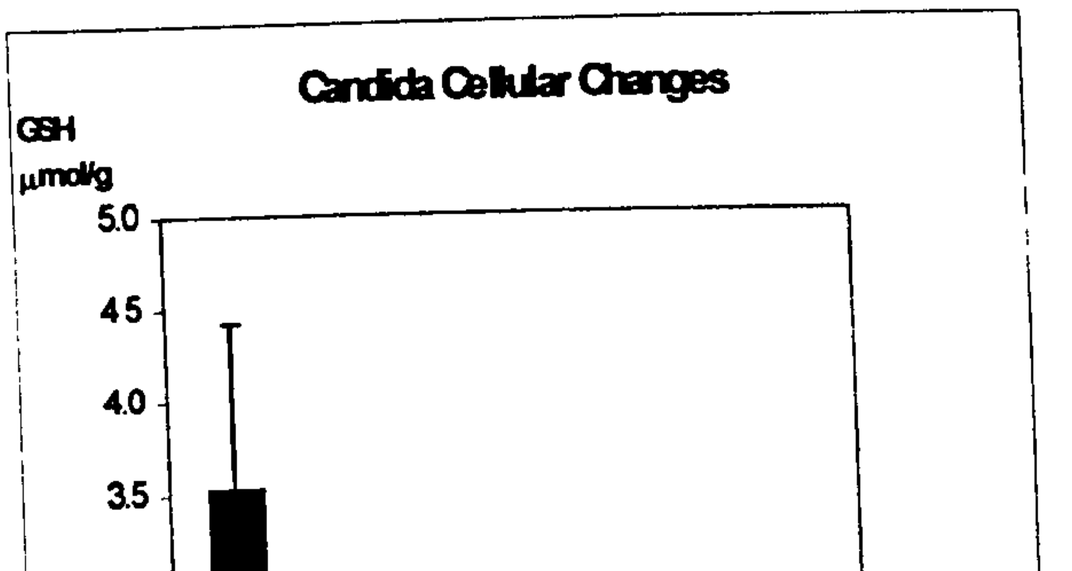


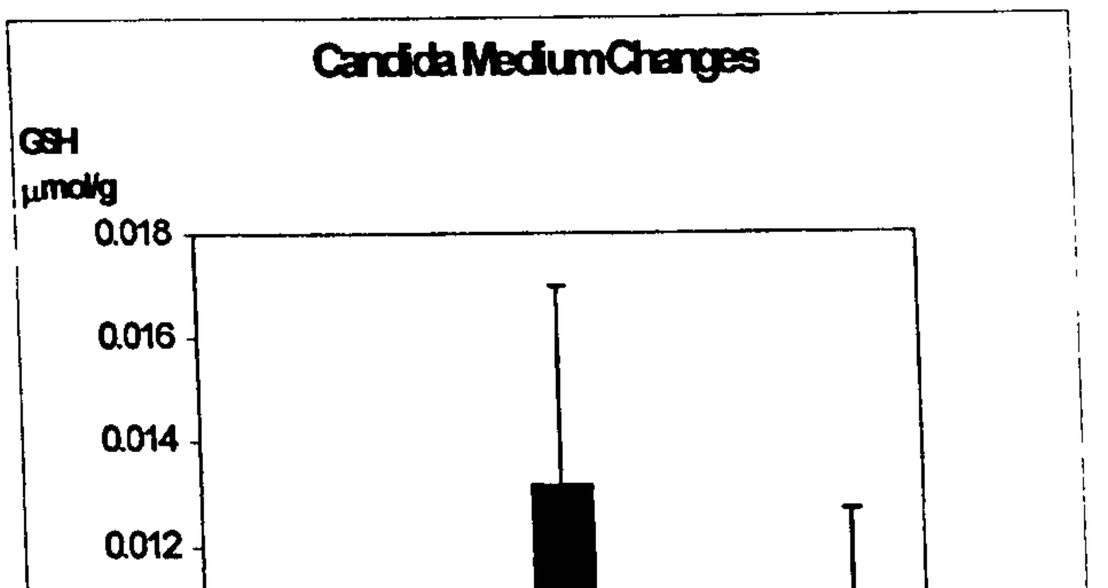
Figure 3.4. Changes in Total Glutathione on Treatment of Mammalian Cells with Preservatives. Measurements are for total glutathione (oxidised + reduced). Controls are untreated cells for comparison. Left hand panels show changes in intracellular glutathione for WKD (top) and RCE (bottom) cells (+SEM). Right hand panels show changes in glutathione in the extracellular matrix after treatment for the two cell types.

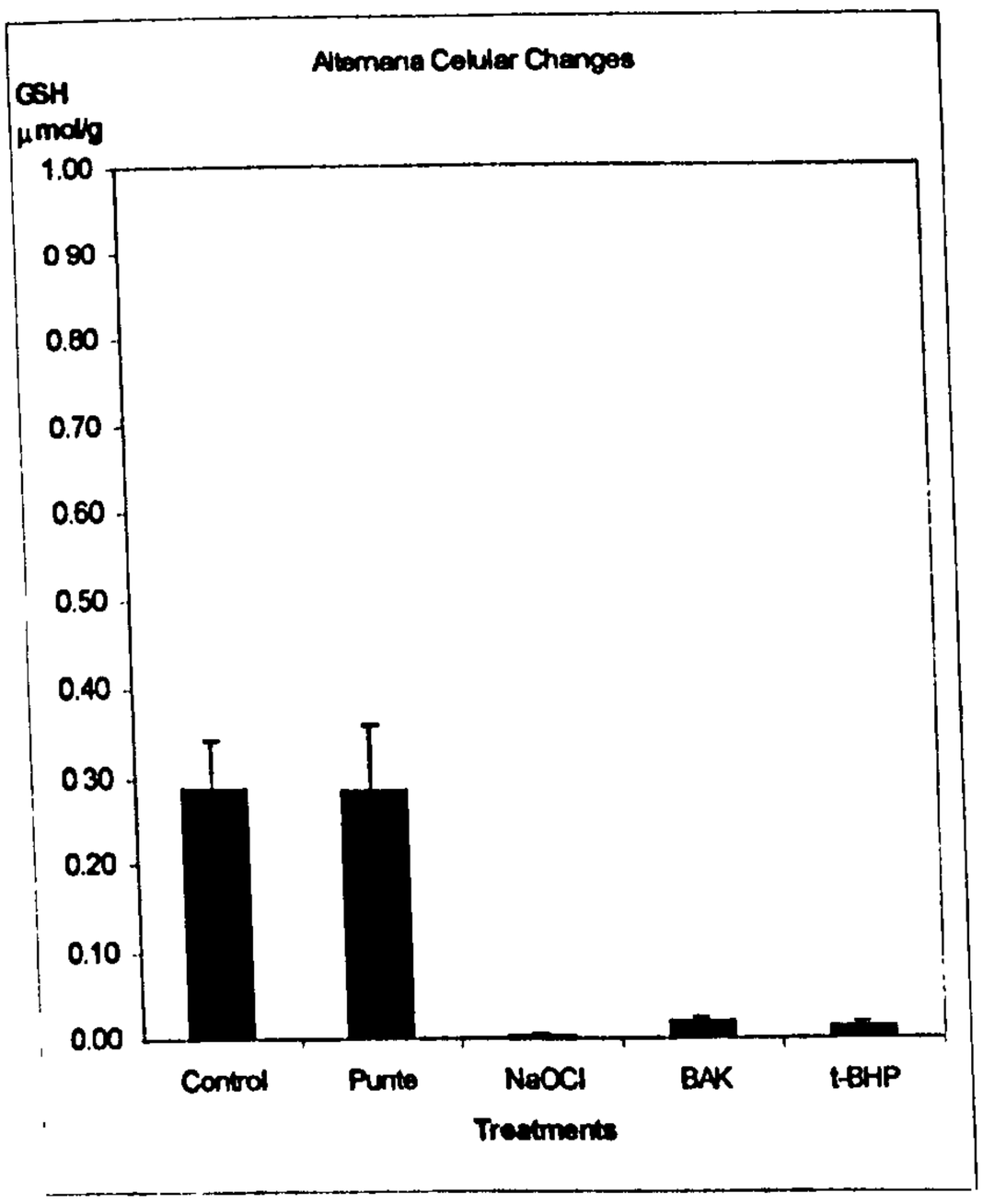
With *C. albicans* a similar pattern was observed in that NaOCl and t-BHP also caused the largest depletion of intracellular glutathione (Figure 3.5). In this case, however, treatments which decreased intracellular glutathione caused a corresponding increase in extracellular glutathione following these treatments. BAK also caused an increase in the glutathione level in the medium, although the intracellular depletion was much less severe than that induced by the two oxidants. Purite™ was slightly less effective at depleting intracellular glutathione than BAK, but again did not appear to cause any increase in extracellular glutathione. The very low levels of glutathione observed in the medium with *C. alicans* and *Alternaria spp.* with treatments relate in part to the presence of oxidative treatments in the medium, we found these oxidants affected the glutathione calibration curve, presumably due to inhibition of the enzyme glutathione reductase.

The response of *Alternaria spp.* to NaOCl, t-BHP and BAK was similar in pattern to that observed with all the other cell types, but the response to Purite™ was substantially different. Figure 3.5 shows that Purite™ treatment did not induce any depletion of intracellular glutathione. In fact, in a number of experiments the intracellular glutathione levels measured were actually higher after treatment with Purite™. The glutathione level in the medium was also unaffected, but this was also true of treatment with NaOCl, which caused the largest decrease in intracellular glutathione levels.

The effect of BAK, permeabalising cell membranes, was found to be the only agent to consistantly release glutathione into the treatment media in all. As BAK is a detergent-based preservative rather than an oxidant, it would not be expected that resistance to it would correlate with antioxidant content directly. However, increased membrane permeability may lead to loss of cellular glutathione by diffusion rather than export of toxic levels of reduced glutathione. Thus, indirectly depleting cellular glutathione.







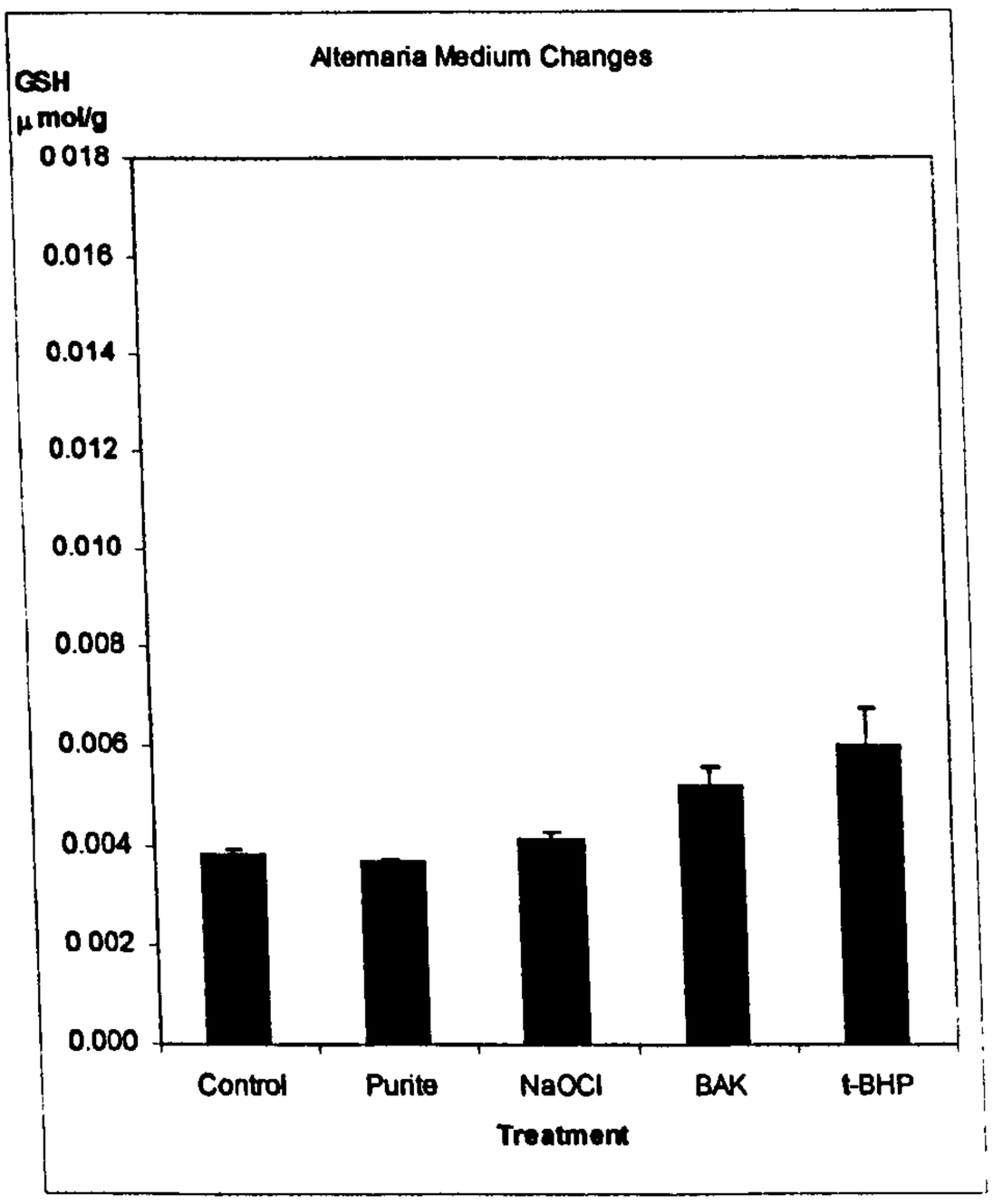


Figure 3.5. Bar Charts Showing Changes in Total Glutathione on Treatment of *C. albicans* and *Alternaria spp.* with Preservatives. Measurements are for total glutathione (oxidised + reduced). Controls are untreated cells for comparison. Left hand panels show changes in intracellular glutathione for *C. albicans* (top) and *Alternaria* spp. (bottom) cells (+SEM). Right hand panels show changes in glutathione in the extracellular matrix after treatment for the two organisms.

Discussion

The purpose of this investigation was to evaluate the cellular cytotoxicity of test organisms to a panel of different preservatives and control oxidants over a time course. The toxicity induced here, by the test preservatives and oxidants in the microorganisms and mammalian cells confirmed that the concentrations also used in simultaneous studies of Chapter 2 and 3 were in the appropriate range of concentrations. From this study it is now possible to correlate the results obtained in Chapters 2 and 3 to levels that result in cellular toxicity.

RCE cell lines have been previously shown as good models for ocular preservative investigations (Meyer & McCulley, 1991, Adamas *et al.*, 1992, Wang *et al.*, 2001, Burglassi *et al.*, 2001). The same is true for WKD (De Saint, 1999, Debbasch *et al.*, 2002). In both RCE and WKD cells, the most damaging treatment was found to be BAK, which caused de-adherence by 1 hour. As noted in the RCE and WKD cells, the apparent low toxicity of BAK was due to the fact that all the cells in the well had lysed and were uncountable by the Trypan blue method. Adamas *et al.* (1992) found similar results *in vitro* with BAK, with cell death at 15 minutes when combined with the chelating agent ethylenediaminetetra-acetic acid (EDTA). Debbasch *et al.* (2002) tested cells for only 15 minutes with 0.0001% and 0.01% BAK and allowed 24 hours recovery in normal growth media before measuring viability. They reported reduced intracellular glutathione and reactive oxygen species production. Debbasch *et al.* also observed a necrotic phenomenon at high concentrations of quaternary ammonium preservatives, whereas evidence for an apoptotic mechanism was found for lower concentrations. This toxicity observed *in vitro* can explain some of the ocular surface damage caused by long-term use of BAK preserved eye-drops. In RCE and WKD, NaOCl and t-BHP + Fe^{2+} were also found to cause rapid loss of viability over the short time course (1-4 hours).

Purite™ at 0.03% (3.5 mM) had a similar effect to NaOCl, but was less toxic after 24 hours. 0.03% (3.5 mM) Purite™ had quite high toxicity at 24 hours, but its effect was less severe over the first 4 hours. 0.005% Purite™ was no more toxic than the control treatment at the early time points. The lower Purite™ concentration had very little effect over the first few hours, but was slightly more toxic than the control after 24 hours.

Of the 3 microorganisms assessed by the same method, *C. albicans* appeared to be the most resistant organism, in that more of the treatments used allowed significant levels of survival at 24 hours. Using the same criteria, *S. aureus* was found to be most sensitive to the treatments used. Unfortunately, it was not possible to compare the resistance of *Alternaria spp.* directly with the other microorganisms, owing to the different methodology required. Measurement of weight can provide information on the amount of growth achieved, but cannot indicate whether loss of viability has occurred. However, it is apparent that *Alternaria* was able to grow reasonably well in the presence of both 0.005% Purite™ and 3.54 mM NaOCl, while Purite™ at 3.54 mM, BAK at 0.005% and tBHP + Fe^{2+} all prevented significant growth over 24 hours. The low concentration of BAK required to have its effect was previously explained in Chapter 1 where the low dilution coefficient η value of QAC's mean reaction kinetics are much less influenced by concentration. Thus, it could be concluded that *Alternaria* was more resistant to 0.005% Purite™ than either of the bacteria, and possibly had similar resistance to *Candida* (approximately 10-fold increase in cell numbers / weight over 24 hours). With all 4 microbes, 3.5mM NaOCl appeared to have relatively poor preservative ability. NaOCl had very little effect on the microbial cells. The effects observed here were very comparable to Tanners findings of disinfectants (1989). Tanner also assessed the effect of organic load, in the broth, upon the relative activity of hypochlorites. Tanner evaluated the presence of organic molecules, in test solutions, and discounted this as the major factor in the observed lack of efficacy. The presence of organic materials in a test solution have been shown to weaken the effects of sodium chlorite (Kemp and Schneider, 2000). The relative efficiency of QAC's and oxidizing agents to kill 99.9% of cells over a short time-course, less than 4 hours, observed here are comparable to the research by Tanner (1989). Tanner utilised pharmacopoael protocols and end-points for cell-kill in non-growth media. This suggests that although our protocol did not strictly follow these methods it is comparable to established methodology for evaluating anti-microbial efficiency.

C. albicans, which was found to have the highest resistance to oxidants generally, also had the highest glutathione levels. The mammalian cells appeared to have reasonable resistance to Purite™ compared to other preservatives, also had high

intracellular glutathione. However, *S.aureus*, which had no detectable glutathione, was particularly susceptible to the oxidants and preservatives. As the *Alternaria* data cannot provide information on loss of viability it is not possible to conclude that this organism was able to develop resistance following initial susceptibility, but it seems likely that it might. There is also some evidence that the micro-organisms may be able to adapt to remain viable in 0.005% Purite™: number of colony forming units decreased in *P. aeruginosa*, *S. aureus* and *C. albicans* over the first few hours, but after either 4 or 24 hours growth had recommenced. The effect of BAK, permeabilising cell membranes, was found to be the only agent to consistently release glutathione into the treatment media. However, increased membrane permeability may lead to loss of cellular glutathione by diffusion rather than export of toxic levels of oxidised glutathione. Thus, depleting cellular glutathione in a non-oxidative manner.

Over the shorter incubation times, Purite™ appeared to be a good antimicrobial agent, although at comparable concentrations its anti-microbial activity was slightly less than that of BAK. However, it was considerably less toxic to mammalian cells than BAK, even at higher concentrations, and especially over shorter incubations. This could reflect a slower mechanism of action allowing a short residual time on the ocular surface to remove Purite™ from the site of action before it has time to interact. BAK, on the other hand, is rapidly toxic and the residual time on the eye may be enough to cause chronic toxicity. Also, the low dilution co-efficient for BAK would mean that tear film dispersion would have little effect on its activity. This finding is supported by the clinical research study of Katz (2002). This study showed, over the long-term use, a lowered incidence of allergic conjunctivitis with increased safety and patient tolerability was achieved with a Purite™ preserved formulation over that of the same drug with BAK preservation. Purite™ also had much more appropriate differential toxicity than NaOCl, which was a poor antimicrobial agent but had quite high mammalian cell toxicity. The conjunctival cells (WKD) appeared to be generally more resistant to preservatives than the corneal cells (RCE). This may reflect the fact their physiological/anatomical location as they tend to be more exposed to air, and thus would be expected to have a higher

reliance on antioxidant protection that were being stressed (Riley, 1990; Riley and Wilson. 1993).

It is important to highlight that during these cytotoxicity experiments cell treatments were not neutralised post-incubation. Care must be taken in evaluating the levels of killing as they are an over-estimation of the cytotoxic effect. This is not as vital in mammalian cell counts as these were performed immediately after the treatment incubation time of interest. However, most of the microbial samples were grown for a further 24 hours in media after the treatment time ended. Aliquots of treated cells would been diluted by over well 100 times, and nearer 1000 times, original cell/preservative concentration to generate countable cell numbers on agar plates. This would have diluted the treatments in most cytotoxicity samples to sub-lethal levels. BAK, with its low dilution co-efficient, may have remained active in some of the samples where low cell numbers meant little dilution was performed. Also, due to the methodology employed here, a 100µL aliquot of *C. albicans, S. aureus* and *P. aeruginosa* were spread on petri-dishes with organic material in the agar that would have neutralised some of the remaining preservative/treatments. This is especially true of the oxidants. As noted above, organic materials have been shown to weaken the effects of sodium chlorite (Kemp and Schneider, 2000). These two factors combined negate many of the problems involved in not neutralising the treatments before dilutional plating and incubation; but some over-estimation of killing may have been observed.

Although a clear correlation between intracellular glutathione concentration and resistance to the oxidants and preservatives studied was not obtained, there does appear to be some relationships. Glutathione-dependent protection against lipid peroxidation has been shown to be vitamin E dependent (Haenen, *et al.*, 1992). The oxidation of α-tocopherol in test organism membranes may be, in part, a cause for the loss of glutathione concentration observed. Oxidation of glutathione to the disulfide form (GSSG) can also lead to the active export of glutathione (Romero & Canada, 1991), as the disulfide is toxic to cells (Wilhelm *et al.*, 2001). Chesney *et al.* (1996) observed that 1mol of GSSG will react further with 3.5-4 mol of hypochlorous acid and proposed this as a scavanger / non-enzymatic role.

Resistance may be indicated by the lack of *Alternaria* glutathione depletion upon treatment with Purite™. It is known that *Alternaria* is very resistant to Purite™ (e.g. contamination of stock bottles reported previously), and also to antimicrobial solutions used in the laboratory for the decontamination of glassware. The known oxidants depleted total glutathione in a predictable dose response manner that relates to detoxification. Thus, it appears likely that cellular protection from Purite™ treatments related, at least in part, to its ability to maintain intracellular glutathione levels upon challenge with this preservative, rather than the native level of glutathione. It is also observed that when the levels of intra-cellular and extra-cellular glutathione levels were combined in oxidatively treated cells the two levels did not equal the control. This may be due to a lack of sensitivity of the assay as the cellular glutathione was released into large volumes of media (10mL in mammalian cell treatments and 250mL in microbial treatments). However, as BAK showed a close correlation of compartmental glutathione levels this is unlikely. The theory that the oxidant treatments may have interacted with the DTNB assay were evaluated by performing calibration curves in the specific treatments and found to only have minimal effects. Thus, the lack of appearance of depleted cellular glutathione activity in the media must mean that Purite™ and other oxidants deplete the protective glutathione pool altogether.

Other protective mechanisms reported in microbial cells include altered membrane phospholipid profiles, to partition the agents from cell entry, or an adaptive response similar to those observed by Ferguson (1999) in *Escherischia coli.* Acidification of the cytosol was shown to protect DNA from electrophilic attack, growth rates slowed, and specific protein-thiol transcription factors were up regulated in *Escherischia coli* to induce resistance. Cabiscol *et al.* (2000) described the regulation of protective enzymes such as catalase and superoxide dismutase, small proteins like glutaredoxin and thioredoxin, and molecules such as glutathione. Cabiscol *et al.* (2000) indicate that bacterial genetic responses, to oxidative stress, are controlled by the antioxidant OxyR and SoxRS transcription factors. There is some evidence that micro-organisms can adapt their membrane phospholipid composition to attain resistance to sub-lethal doses of anti-microbial agents (Pechey *et al.*, 1974, Ikeda *et al.*, 1984; Imai *et al.*, 1975; Mechin *et al.*, 1999). The findings in Chapter 3

cannot address this, as this theory relates to sub-lethal doses and those tested in our studies were toxic. It would only be possible to elucidate such a theory by performing a full treatment dose response curve in each cell type followed by cellular phospholipid extraction and analysis.

There is evidence, from our findings, that several of the micro-organisms may be able to develop resistance and show growth following Purite™ treatment with low, sub-lethal doses, over 24 hours. Further work would be required to confirm this and investigate the molecular mechanisms responsible. Other researchers have shown that some microbial species resistant to sodium chlorite have specific chlorate reductase enzymes capable of detoxifying chlorates and chlorites by means of using them as substrates for respiration (Coates, *et al.*, 1999).

The response of the mammalian cells also supports the hypothesis that susceptibility to Purite™ relates to loss of cellular glutathione protection. WKD cells appeared to be more susceptible to the preservatives than RCE cells. In addition to having lower initial glutathione levels, their glutathione was more severely depleted on treatment with preservatives.

Chapter 4:

Native lipid profiles of mammalian cells and microorganisms and changes associated with preservative/ oxidant exposure.

Introduction

The aim of these studies was to characterise the membrane phospholipid profile of all the cell types investigated and to determine if the composition correlated to the relative resistance described in Chapter 3. From the *in vitro* results of Chapter 2, a full elucidation of the specific cellular phospholipid profile, in each organism, may offer an indication of membrane phospholipid susceptibility to oxidation upon subsequent exposure to preservative treatment *in vivo*. The basic surface compositional and structural differences between the bacterial, fungal and mammalian cells was described earlier (Chapter 2). It is also necessary to understand the actual susceptibility of the test organisms to oxidative change by oxidation mechanisms described in Chapter 1. Thus, this study also aimed to determine the effects of selected preservatives and positive control oxidants had upon the native phospholipid membranes of these organisms. Previous researchers have shown that this can be evaluated by performing lipid analysis of treated micro-organisms (Krasowska *et al.*, 2000) and mammalian cells (Augustina *et al.*, 1998; Spickett *et al.*, 2001) for oxidative modifications. These groups successfully determined lipid modifications from experiments with whole cells by assaying secondary oxidation reaction products, malonaldehydes, in the TBARS assay (Augustina *et al.*, 1998; Krasowska *et al.*, 2000) or direct detection of modified lipids by ES-MS analysis (Spickett *et al.*, 2001). Care must be taken interpreting TBARS results in whole cell treatments as thiobarbituric acid will react with oxidatively modified amino acids in samples to give positive results (Halliwell & Gutteridge, 1984).

Further to the observations of Chapter 3, researchers have reported experimental evidence that micro-organisms can adapt their phenotypic composition via metabolic regulation to attain resistance to lethal doses of anti-microbial agents (Jones *et al* 1989). Furthermore, Mechin *et al.* (1999) found that *Pseudomonas*

aeruginosa was able to modify the fatty acyl chain composition of membrane phospholipids upon culture in a QAC biocide using a stepwise increase in biocide concentration in the culture medium. Using this method the test organism was able to grow in a concentration five times greater than the control MIC. This correlated to the observed increased lauric acid and hydroxylated acyl chains, and decreased palmitic acid, generating increased membrane fluidity as an adaptive resistance strategy. Shortening in chain length (Rice and Olivier, 1992) and decreased unsaturation (Diefenbach *et al.*, 1992) of fatty acids have also been correlated to microbial stress responses.

Conventional analytical methods for characterisation of membrane phospholipids at the level of their individual molecular species, is typically a multi-step procedure (Patton *et al.*, 1984). Lipids are extracted by liquid-liquid extraction and chromatographically separated into phospholipid classes, often derivatised, and analysed by thin-layer chromatography, high-performance liquid chromatography or mass spectrometry methods. Mass spectrometric ionisation methodology has developed so that polar lipids, in principle, can be analysed using soft ionisation techniques such as field desorption, chemical ionisation, fast atom bombardment, or electrospray (ES-MS). Among the soft ionisation methods developed for analysis of polar bio-molecules, ES-MS represents a major breakthrough for biological MS because the technique does not require derivatisation reactions. It is highly sensitive with moderate experimental complexity, and provides reproducible results (Haroldsen and Murphy, 1987).

For investigation of the mechanisms of action of Purite™, a variety of cell types were chosen to represent the eye and micro-organisms commonly known to present a problem either through contamination of the solutions or ocular infection. These were the mammalian cell lines, rabbit corneal epithelial cell and human conjunctival epithelial cell, a Gram-positive bacterium, *Staphylococcus aureus,* a Gram-negative bacterium , *Pseudomonas aeruginosa,* a yeast, *Candida albicans*, and a filamentous fungus *Alternaria spp.*

This study aims to provide information on the different mechanisms by which the treatments may exert their toxic effects upon specific cell membranes.

Materials and Methods

Cell culture conditions for native lipid profiles.

Mammalian cell lines used were obtained from the European Collection of Cell Cultures (CAMR, Porton Down, UK). These were rabbit corneal epithelial cell, RCE (ECACC No. 95081046) and human conjunctival epithelial cell WKD (ECACC No. 88021103). RCE cells were cultured in DMEM:Ham's F12 medium supplemented with 2mM glutamine, 5µg/mL insulin, 10ng/mL EGF and 15% foetal calf serum (Gibco BRL, UK) until confluent in a 5% CO_2 incubator at 37°C. WKD cells were cultured in Medium 199 (Hanks) supplemented with 2mM glutamine and 10% foetal calf serum (Gibco BRL, UK). Cells were grown in 75cm^2 flasks until confluent in a 5% CO_2 incubator at 37°C.

Bacterial and *Candida* stocks were made on Protect storage beads (TSC Ltd, Lancashire, UK). *Pseudomonas aeruginosa, Staphylococcus aureus* were aerobically incubated in tryptone soya broth (Oxoid, Hampshire, UK) at 37°C for 18 hours in shake flasks after inoculation by a Protect storage bead (TSC Ltd, Lancashire, UK). *Candida albicans* (clinical islolate from Glasgow Royal Infirmary, UK) and *Alternaria spp.* (pharmacological isolate for Allergan Corp, California USA) were grown aerobically for 18 hours in YEPD broth; containing 1% yeast extract (Difco, Michigan USA), 2% bacto peptone (Difco, Michigan USA) and 2% glucose (BDH, Dorset, UK)(w/v). *Candida albicans* and *Alternaria spp.* were inoculated from stock organisms stored on Protect beads or from agar plates grown at 37°C and 26°C respectively.

Cells were harvested by centrifugation and washed twice with sterile phosphate buffered saline (PBS) pH 7.2. The cell pellet was then treated to extract total lipids by the stepwise addition of 1:1:1 chloroform/methanol/0.88% potassium chloride (v/v), (Sigma, UK) with sonication between additions for 15 minutes at 35°C, as a modification of the Bligh and Dyer (1959) method as described by Spickett *et al.* (2001). All solvents and chemicals were of analytical grade or equivalent. The lipid extracts were dried under nitrogen, stored at –70°C, and reconstituted in the appropriate solvent for analysis by ESMS or LC-MS.

Cell Treatments.

Mammalian cells were grown in 75cm^2 flasks to 90-100% confluence. Media was removed and the cells washed with PBS. Preservatives or oxidants were prepared in PBS. Cells were treated with various oxidants and preservatives at the following concentrations: sodium chlorite (Purite™) at 0.005%, 0.03% [=3.5 mM], 0.2%, 0.4%; benzalkonium chloride (BAK) at 0.005%, 0.2%; NaOCl at 3.5 mM pH 7.2; H_2O_2 + Fe^2+ at 0.1 M + 1 mM; and *tert*-butylhydroperoxide (tBHP) + Fe^2+ at 0.25 M + 1 mM; and incubated with the cells at 37°C for 4 hours. For control treatments phosphate-buffered saline was added instead. All reagents were of the quality appropriate to their use; either tissue culture, bacteriological or analytical grade.

Bacteria and fungi were grown in conical 1L flasks in TSB and YEPD respectively in a manner described above. Cultures were centrifuged and washed twice in sterile PBS. Cultures were re-suspended at either 10^7/mL (*Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans)* or 0.01g/mL (*Alternaria spp)* in treatments using an adaption of the McFarland (1907) Standard turbidity technique. Treatments were incubated in smaller, 250mL, flasks for 4 hours. Treatments were at the same concentrations as mammalian cells.

Cells were harvested and lipids extracted following the same protocol detailed above. The lipid extracts were dried under nitrogen, stored at –70°C, and reconstituted in the appropriate solvent for analysis by ESMS or LC-MS.

Electrospray Mass-spectrometry Analysis.

Direct injection and LC-MS were performed on a VG Platform mass spectrometer (Micromass, Cheshire UK) using a Shimadzu LC-10 (Kyoto, Japan) isocratic pump profile. Solvent flow rates of 20μL/min (9.1 methanol/water) and 100μL/min (71:5:7. methanol/hexane/0.1M ammonium acetate (v/v)) were used for direct injection and LC-MS respectively. Lipid extracts from eukaryotic cells were analysed by positive-ion MS and LC-MS. The source temperature was set to 75°C with a nebulising gas flow of 20 L/h and a drying gas flow of 400 L/h. All other settings were adjusted to give maximum ion intensity from stock lipids. Prokaryotic cell extracts were analysed by both positive and negative-ion MS and LC-MS.

All data was acquired and manipulated using the MassLynx software. Direct injection data was collected between 400-1000m/z with a sweep time of 5 seconds. Spectra were combined from 15 consecutive scans. Summed spectra were mean smoothed twice with a window of 0.3 Da. In LC runs of 40 minutes, peak top data were collected between 700 and 1000 m/z with a sweep time of 2 seconds.

Results

Native Lipid Profiles.

The chromatograms and combined spectra of lipids extracted from eukaryotic cells are shown in Figure 4.1. Interpretation of the chromatographs indicated the presence of specific phospholipid species. These were identified, by their mass/charge ratio calculated from the molecular mass plus the counter-ion (Na+23 H+1).

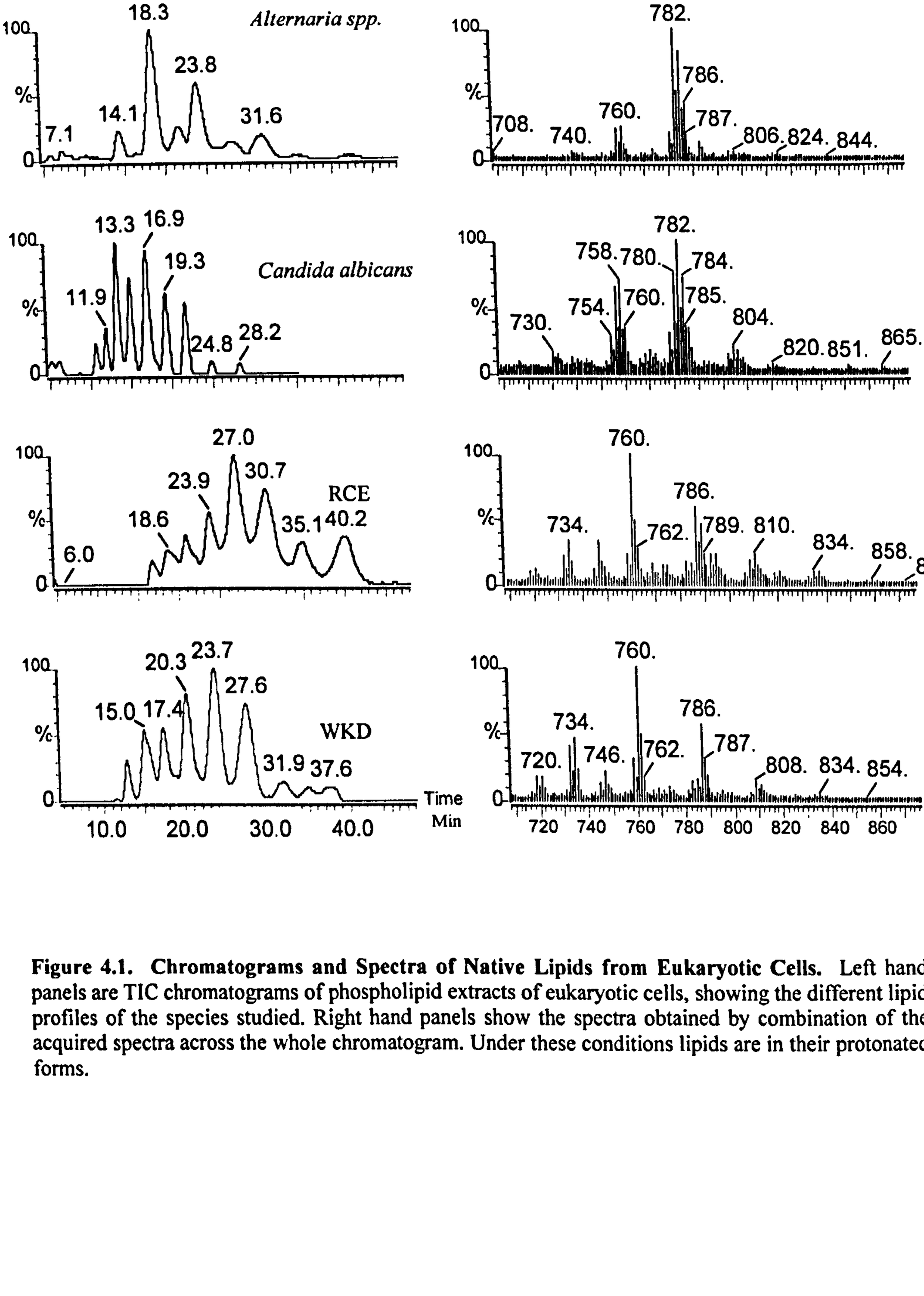


Figure 4.1. Chromatograms and Spectra of Native Lipids from Eukaryotic Cells. Left hand panels are TIC chromatograms of phospholipid extracts of eukaryotic cells, showing the different lipid profiles of the species studied. Right hand panels show the spectra obtained by combination of the acquired spectra across the whole chromatogram. Under these conditions lipids are in their protonated forms.

A summary of the major phospholipid species, identified from lipid extracts of the eukaryotic organisms, are shown in Table 4.1. It must be noted that when detecting unknown lipids in these experiments it was not possible to determine the acyl-chain length ratio so the total length and the number of double bonds have been determined. These data were identified from analysis of combined spectrum m/z values shown in the right panel of Figure 4.1. The majority of phospholipids identified in all these cells were PCs. These can readily be detected in positive ion mode, but some of the phospholipids had mass-to-charge ratios corresponding to PE's and PS's. The shortest phospholipid observed in these samples had 30 carbon acyl-chain length and was always saturated.

Acyl chains total length: no. double bonds.	Polar Head Group			
	WKD	**RCE**	**Candida**	**Alternaria**
30:0	PC (706)	PC (706)	PS (708)	PS (708)
32:0	PC (734)	PC (734)	PC + PS (734+736)	
32:1	PC (732)	PS + PG (732+721)	PC (732)	
32:2	PC (730)		PC (730)	
34:0	PC + PE (762+720)	PC (762)		
34:1	PC (760)	PC (760)	PC (760)	
34:2	PC (758)	PC (758)	PC (758)	PC (758)
36:0		PC (790)		PS (792)
36:1	PC + PE (788+746)	PC (788)	PC (788)	PC (788)
36:2	PC (786)		PC + PE (786+744)	PC (786)
36:3			PC (784)	PC + PE + PG + PS (784+742+773+786)
36:4			PC (782)	PC (782)
38:4		PC (810)	PC (810)	PC + PE (810+768)

Table 4.1. Major lipids observed in extracts of cells studied fungal and mammalian cell lipid composition.

Mass assignments, in brackets below, were based on protonated molecular weight of species in positive ion mode (All extracts n=3).

The RCE cell line contains mainly PCs with total fatty acyl chains of up to 38 carbons and four double bonds. Figure 4.1 shows masses with elution times within the chromatogram specific for the arachidonyl (PC 20:4, 16:0 or 18:0) species containing phospholipids in RCE controls. Other acyl-chains were found to be mono-di-, and poly- unsaturated. Some mono-unsaturated PS and PG were also found. From Figure 4.1, *Candida* was observed to have a chromatogram containing species with earlier elution times than the other organisms. On comparing the combined spectra of *Candida* to the other organisms it contained very similar lipid species. The difference in elution times of the *Candida* run, compared to the other chromatograms, may be due to day to day variation of column kinetics; or it is possible that the data collection was not started immediately after the HPLC injection. Thus, retention times appeared artificially earlier in the data collection than their actual column retention times. Interpretations of R_1 and R_2 chain length ratio were made by comparing differential elution times of the specific lipid species. Research using fragmentation techniques (Brugger *et al.*, 1997; Hoischen, *et al.*, 1997) have shown that both fatty acyl chains may contain double bonds. It is not possible to determine the ratio of chain lengths, and their specific level of unsaturation with the single quadrupole detector used here. Figure 4.1 shows that lipid extracts from WKD cells contained shorter total carbon acyl-chains than RCE cells. WKD phospholipid acyl-chains were found to be mono-, di- and unsaturated. WKD also had mono- and un-saturated PE present.

Lipid extracts of *Candida albicans* contained mostly PCs with total acyl-chain lengths of 38 carbons or less and only one was unsaturated. These appeared to have masses, and elution times in the chromatogram, for the phospholipids containing arachidonyl species (PC 20:4, 16:0 or 18:0). Some phospholipids with masses corresponding to PS and PE were also detected. *Alternaria spp.* contained less variety of PCs. The chromatogram and the spectrum of lipid extract of the PCs that were present, also appeared to be longer (total acyl-chains 34-38 carbons long) and mono-, di-, and poly-unsaturated. Interestingly, *Alternaria* also contains a broader spectrum of detectable PS, PE, and PG.

Lipid extracts of the bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa* contained only limited quantities of PC-containing phospholipids. These

could be detected in positive ion mode. Spectra were also acquired in negative ion mode to detect the other major cellular phospholipids (Figure 4.2). It was possible to observe PEs and phosphatidyl-glycerols (PGs), as well as PS using negative ion conditions. Some PS and PE phospholipid species detected in negative mode carry the same m/z. The non-fragmentation mass spectrometry methodology used in this study meant it was not possible to differentiate them. Table 4.2 summarizes the major phospholipids detected in the bacterial cells and where two possible species share a m/z both have been included.

Acyl chains total length: no. double bonds.	Polar Head Group	
	S. aureus	***P. aeruginosa***
29:0	PG (679)	
30:0	PG (693)	
30:1	PE (661)	
30:4		PC (702)
31:1	PE (675)	PC (740)
32:0	PG (721)	PE (690)
32:1	PC (754)	PE (688)
33:0	PG (735)	
34:0	PG (749)	PC (784) + PG (749)
34:1	PC (782)	PC (782) + PE (716) + PG (747)
34:2		PC (780) + PE (714)
35:0	PG (763)	PE (732)
35:1	PS (675)	PG (761)
36:0	PG (777)	PC (812) + PS (690)
36:1		PC (810) + PS (688)
36:2	PC (808)	PC (808) + PG (773)
37:1		PG (789)
38:1		PS (816)
38:2		PS (814)
39:0		PS (832)

Table 4.2. Major lipids observed in extracts of cells studied: Bacterial cell lipid composition (n=3).

S. aureus contained both odd and even numbered fatty acyl chains in PGs, most of which were fully saturated. The total acyl-chain carbon lengths ranged from 30 to 36. *S. aureus* also contained PC and PE with only di- unsaturated acyl-chains. *P. aeruginosa* predominantly contained PC and PE with mono- and di- unsaturated acyl-chains. The major saturated lipid in *P. aeruginosa* was PC.

Unfortunately it was not possible to carry out LC-MS in negative ion mode since the solvent system used for the HPLC contained ammonium acetate and was incompatible due to arcing in the electrospray source. A number of alternative solvent systems were tested using individual phospholipid standards (PE, PG and cardiolipin), but none of the solvents resulted in suitable retention of these lipids. While direct injection ESMS is adequate for the determination of a native lipid profile, it is less informative with regard to identifying oxidised phospholipid mixtures (as seen in Chapter 2).

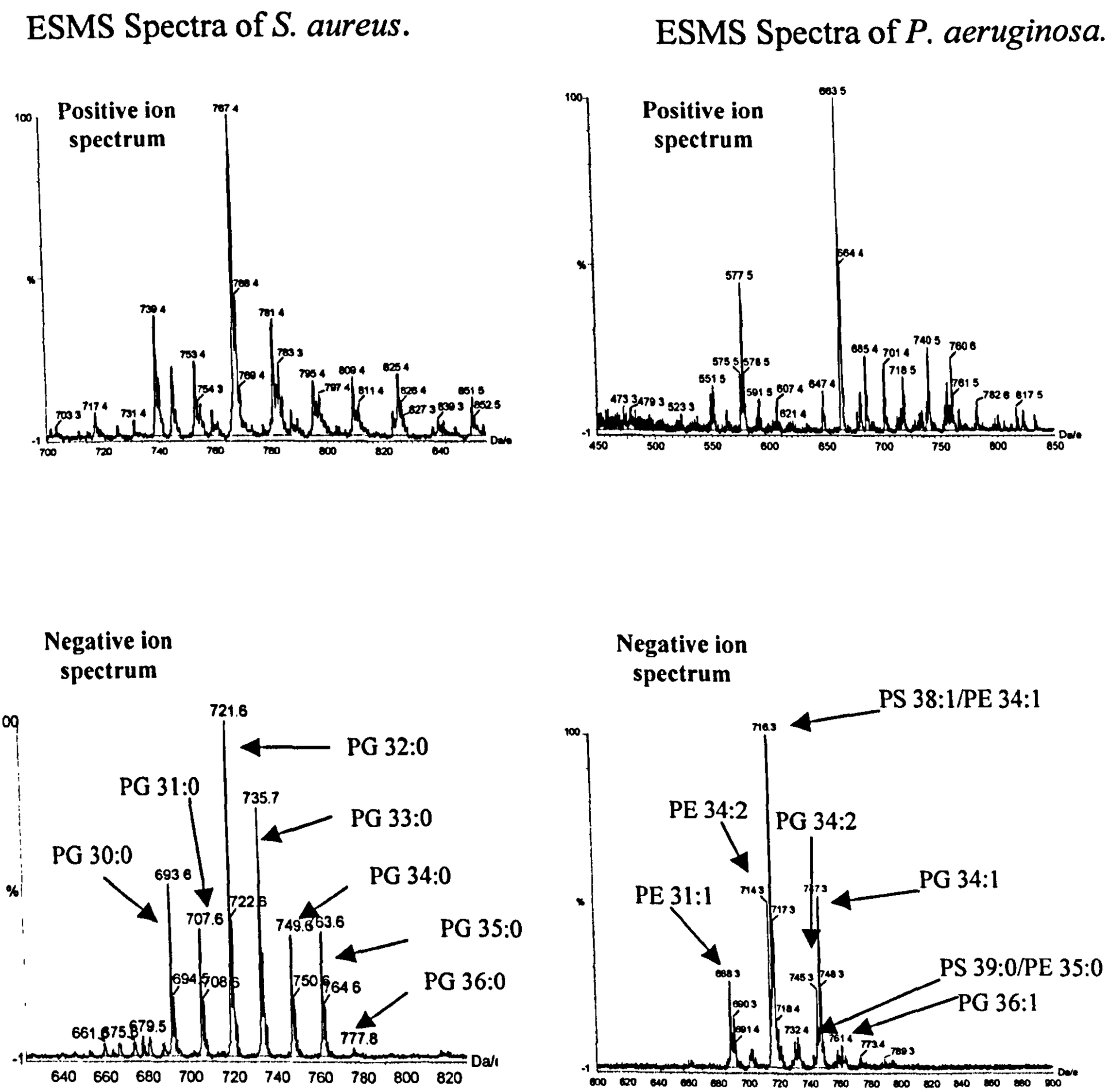


Figure 4.2. Direct Injection Spectra of Bacterial Lipids. Direct injection positive and negative ion ESMS of phospholipids extracted from *S. aureus(n=3)* and *P. aeruginosa(n=3)*. The upper panels show the positive ion mass spectra, with the lipids in their sodiated forms. The majority of the observed species are phosphatidyl cholines. Assignments are as seen before. The lower panels show the negative ion mass spectrum of the same extract. The main observed species are phosphatidyl ethanolamines (PE) and phosphatidyl glycerols (PG).

Lipid Profiles and Changes in Treated Cell.

Eukaryotic Cell Analysis.

An example of the chromatograms obtained, using MassLynx software, from the analysis of lipids extracted from treated *Candida* cells are shown in Figure 4.3

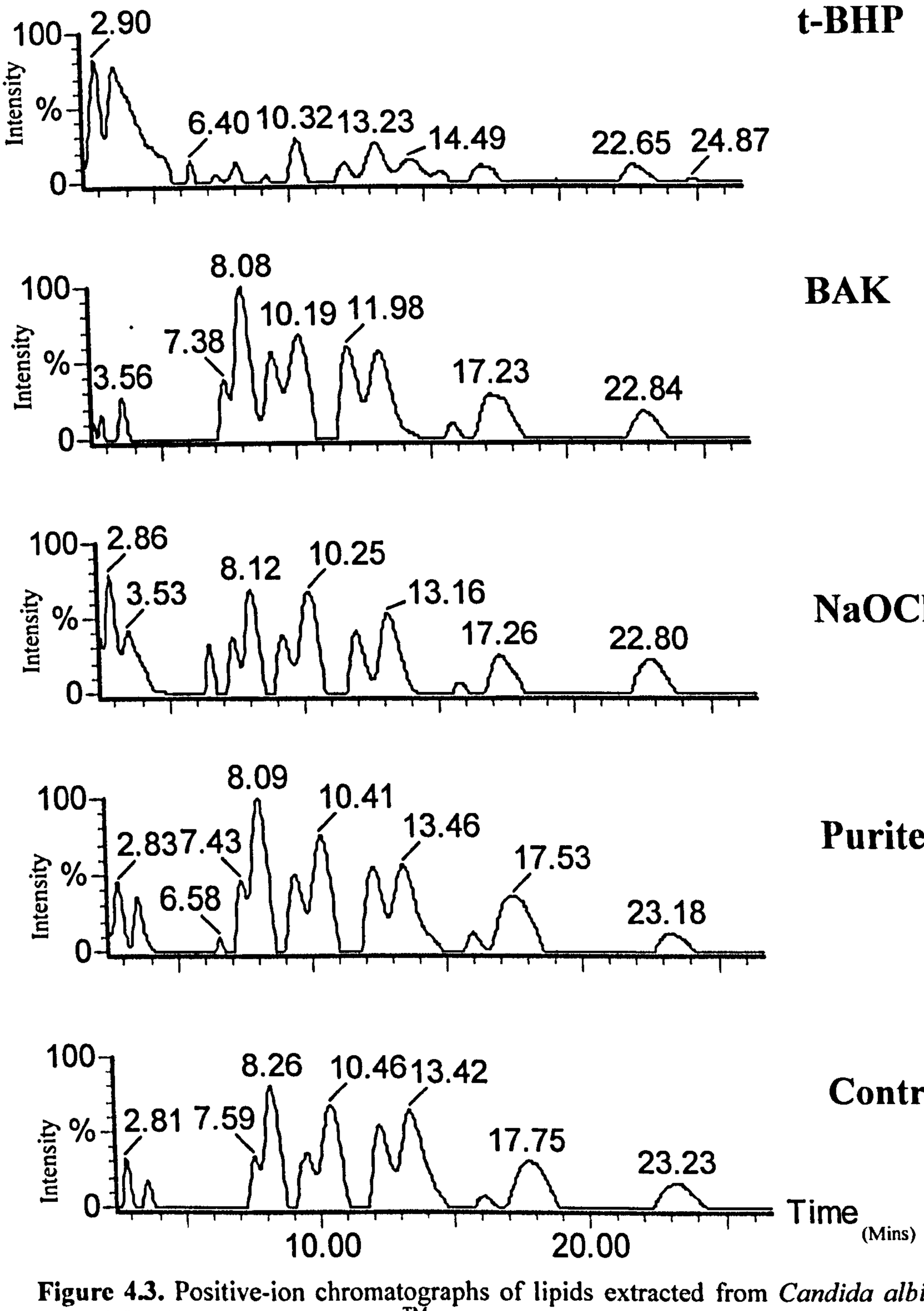


Figure 4.3. Positive-ion chromatographs of lipids extracted from *Candida albicans* treated for four hours with Purite™ at 0.4%; benzalkonium chloride (BAK) at 0.2%; NaOCl at 3.5 mM pH 7.2; and *tert*-butylhydroperoxide (tBHP) + Fe^{2+} at 0.25 M + 1 mM . Vertical axes scales in these chromatograms have been linked for comparison.

Figure 4.3 shows a typical pattern of lipid profile changes similar to that obtained with all of the cell types tested. It must be highlighted that, in any chromatogram, it is the area under the peak that indicates the relative amount of any given species, and not the peak height alone. It was possible to observe the loss of several native lipid species peaks, and the appearance of new peaks in the early chromatograms, in treated cell extracts when compared to the control. Hypochlorous acid was found to cause native peak depletion of species with retention times of approximately 8, 13.2 and 17.2 minutes. Figure 4.3 shows the common observation of results for extracted cellular lipids of cells treated with *t*-BHP. *t*-BHP was found to deplete most native phospholipids compared to control. However, as observed in vesicle treatments (Chapter 2), there was not a full commensurate increase in detectable reaction products in the early chromatogram. Purite™ and BAK did not generate any new peaks in the early chromatogram and very mild depletion, if any, of the native lipid peaks.

Using MassLynx software, combined spectra of the native phospholipids (species with retention times of 6 to 26 minutes) and early chromatogram phospholipids (oxidatively modified and lyso-lipid species with retention times of 2 to 6 minutes), were made to identify treatment induced changes when compared to controls. Hypochlorous acid was found to cause some native peak depletion. This was most readily observed within Figure 4.3 in the un-saturated phospholipid with mass/charge ratio (m/z or Da/z) 786 and the peak retention time of 17.2 to 17.8 minutes. From knowledge of the m/z and the column retention times of control lipid vesicles, in previous experiments, this is likely to be phosphtidyl-choline with 36 carbon total acyl-chain length with double unsaturation (PC-36:2). This observation was confirmed using MassLynx software for spectrum analysis, but it is not shown clearly in a summary spectrum such as Figure 4.4 (Right-hand panel). Analysis of the early chromatogram region indicates the oxidatively modified products, mono- and di- chlorohyrinated lipid species of native phospholipids plus 52 and 104 Da/e units respectively. These observations were supported by identification of the characteristic chlorine isotope peaks associated with chlorohydrinated phospholipid species.

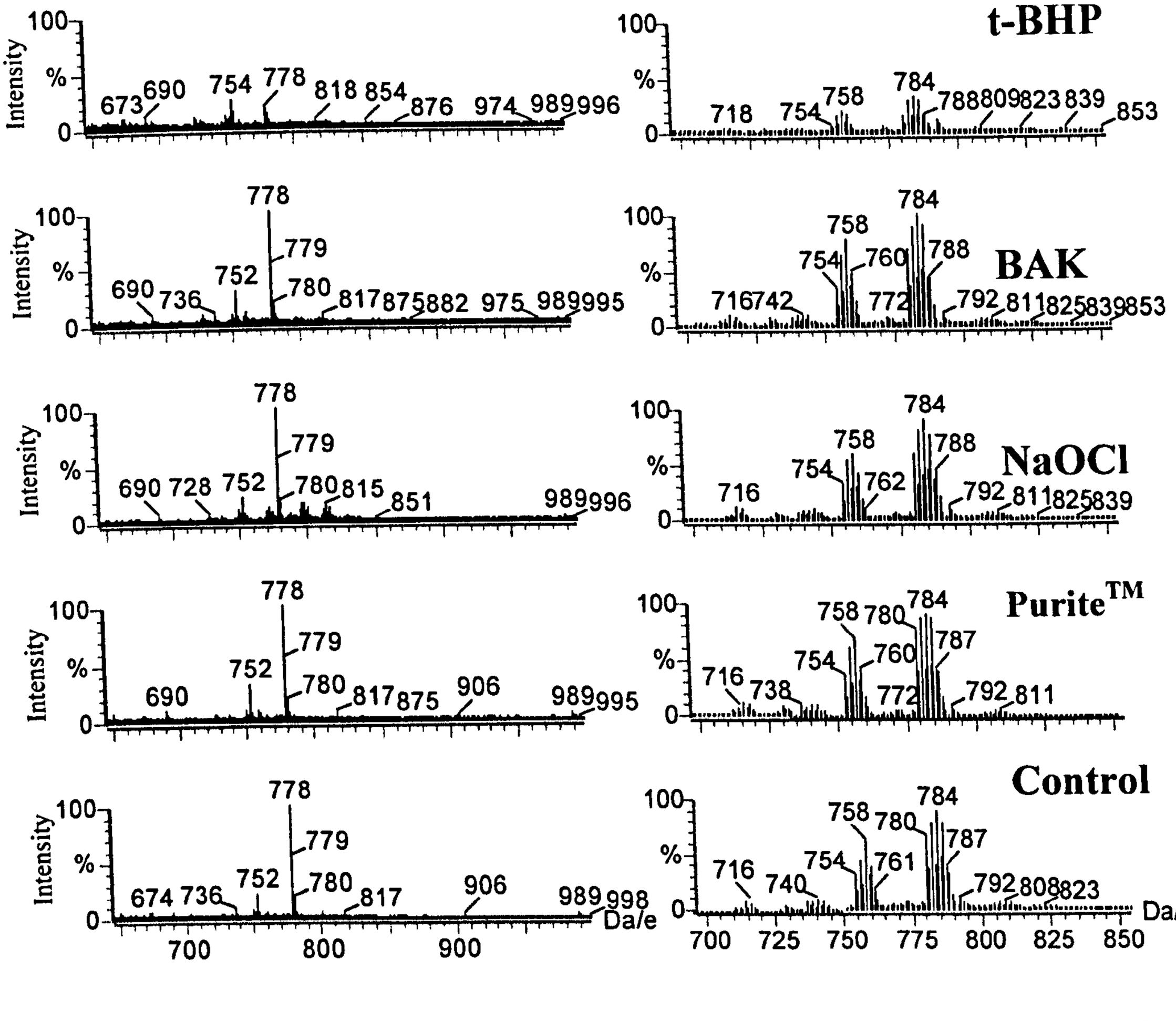


Figure 4.4. Combined spectra from phospholipid chromatograms (Fig 4.3) from *Candida albicans* treated with control oxidants and preservatives. Left-hand panel shows differences in lipids from the early portion of the chromatogram. Right-hand panel shows the profile of native lipids from the main body of the chromatogram.

The oxidised lipids (bis-hydroperoxides) and lipids with a single acyl-chain removed due to oxidative damage (lyso-lipids) were detected in the early chromatogram of t-BHP treated cell extracts as seen in Figure 4.4 (Left hand panel). Bis-hydroperoxides in lipids were identified in reconstructed spectra from the early chromatogram region at +32, 64 and 96 Da/e above their corresponding native lipid Da/e. Figure 4.4 further supports the observations in Figure 4.1 where, compared to control cell phospholipids, Purite™ and BAK, at all concentrations, had little effect in depleting native peaks. Treatments generating very few or no new peaks in the early chromatogram would suggest little oxidative modification of native lipid

species has arisen. Table 4.3 indicates the identified modifications in all eukaryotic cells types treated (n=4).

			Hydroperoxides of t-BHP		Chlorohydrins of NaOCl	
RCE	Native Phospholipid					
	M/z Native	**Lipid**	**+32**	**+64**	**+52**	**+104**
	760	(PC34:1)	792	824	812	
	786	(PC36:2)	818			
	810	(PC38:4)	842	874		
	836	(PC40:4)	868	900		
			Hydroperoxides of t-BHP		Chlorohydrins of NaOCl	
WKD	Native Phospholipid					
	M/z Native	**Lipid**	**+32**	**+64**	**+52**	**+104**
	732	(PC32:1)	764			
	758	(PC34:2)	790			
	760	(PC34:1)	792			
	786	(PC36:2)	818			
	792	(PS36:0)		856		
			Hydroperoxides of t-BHP		Chlorohydrins of NaOCl	
Candida	Native Phospholipid					
	M/z Native	**Lipid**	**+32**	**+64**	**+52**	**+104**
	756	(PC34:3)	788		808	
	758	(PC34:2)	790		810	862
	780	(PC36:5)	812		832	
	782	(PC36:4)	814		834	
	784	(PC36:3)	816			
	788	(PC36:1)	820		840	
	810	(PC38:4)			862	
			Hydroperoxides of t-BHP		Chlorohydrins of NaOCl	
Alternaria	Native Phospholipid					
	M/z Native	**Lipid**	**+32**	**+64**	**+52**	**+104**
	758	(PC34:2)			810	862
	780	(PC36:5)			832	884
	782	(PC36:4)	814		834	886
	784	(PC36:3)	816		836	888
	786	(PC36:2)	818		838	890
	792	(PS36:0)	824		844	
	810	(PC38:4)		874		
	822	(PC40:5)	854		874	

Table 4.3. Oxidation products detected in eukaryotic cell lines (n=4). Note: no oxidation products were detected in cells treated with Purite™ or BAK.

Prokayrotic Cell Analysis.

It can be seen in Figures 4.5 and 4.6 that the direct injection spectra of positive control oxidants, *t*-BHP and NaOCl, were found to generate hydroperoxides and chlorohydrins respectively in microbial cells. However, the appearance of oxidised phospholipids with these oxidants was not as apparent in the mammalian cells. In comparison with the clearly observed modifications of the phospholipid vesicles, the number of modified cellular native species were fewer than expected. This is probably due, in part, to cellular anti-oxidant protection and possibly due to loss of modified lipids due to homeostatic breakdown and removal. The concept that the cells would be able to resist t-BHP effects is unlikely as it is known that t-BHP cannot be neutalised by catalase (Riley and Wilson, 1993). In addition we have previously found that levels of the lipophilic chain breaking antioxidant (tocopherol) are low in all cell types tested. Observations suggesting modification had occurred, even in the mammalian cells, were that there was depletion of many native unsaturated and poly-unsaturated lipids despite the lack of appearance/detection of modified products in the chromatograms. This could be due to the cells removing these toxic molecules, poor extraction of these specific phospholipids in the chloroform/methanol separation, and possibly their poor detection in the single quadropole detector.

As reported earlier, it was not possible to develop the HPLC methodology to detect individual bacterial lipids successfully by negative mode LC-MS. Thus, the phospholipids extracted from treated bacteria were analysed by direct injection only. Only spectra were generated without the more powerful tool of chromatographs. Figure 4.5 and 6.6 show examples of direct injection spectra of lipids extracted from treated *S. aureus* and *P. aeruginosa* cells respectively.

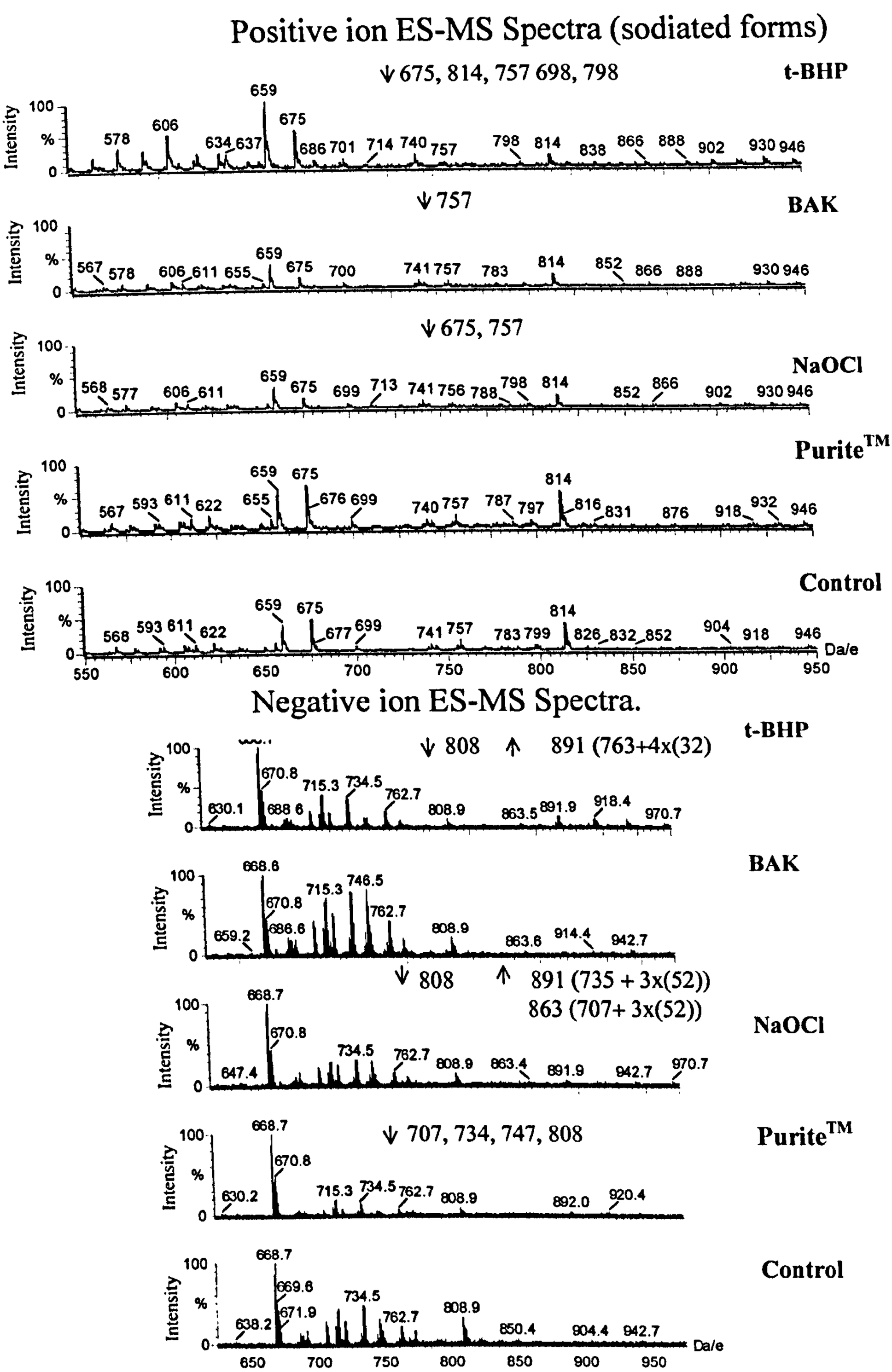
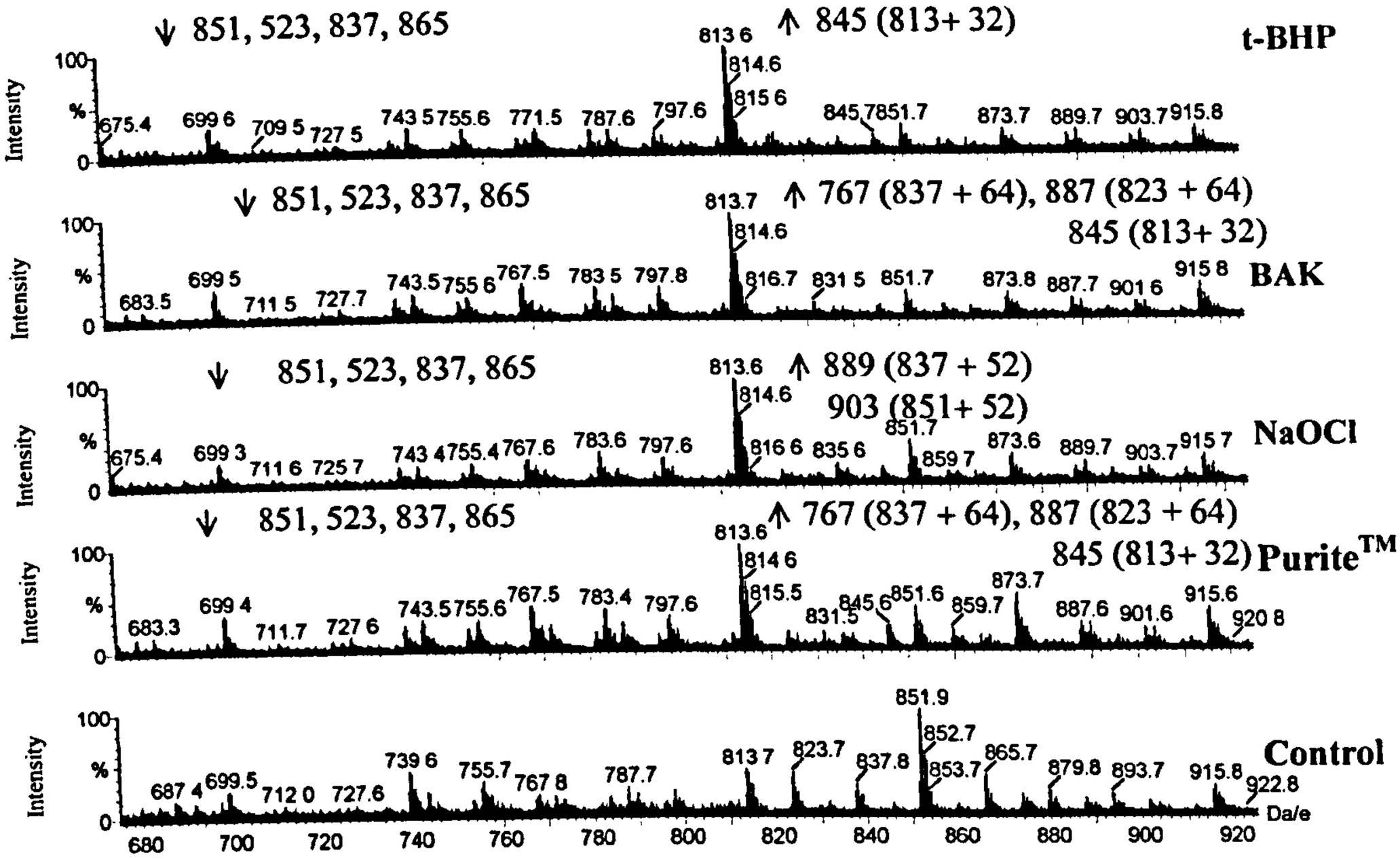


Figure 4.5 Direct-injection spectra of lipids from *Pseudomonas aeruginosa* treated for four hours with Purite™ at 0.4%; benzalkonium chloride (BAK) at 0.2%; NaOCl at 3.5 mM pH 7.2; and *tert*-butylhydroperoxide (tBHP) + Fe2+ at 0.25 M + 1 mM. Vertical axes scales in these chromatograms have been linked for comparison. Observed changes are noted above the spectra.



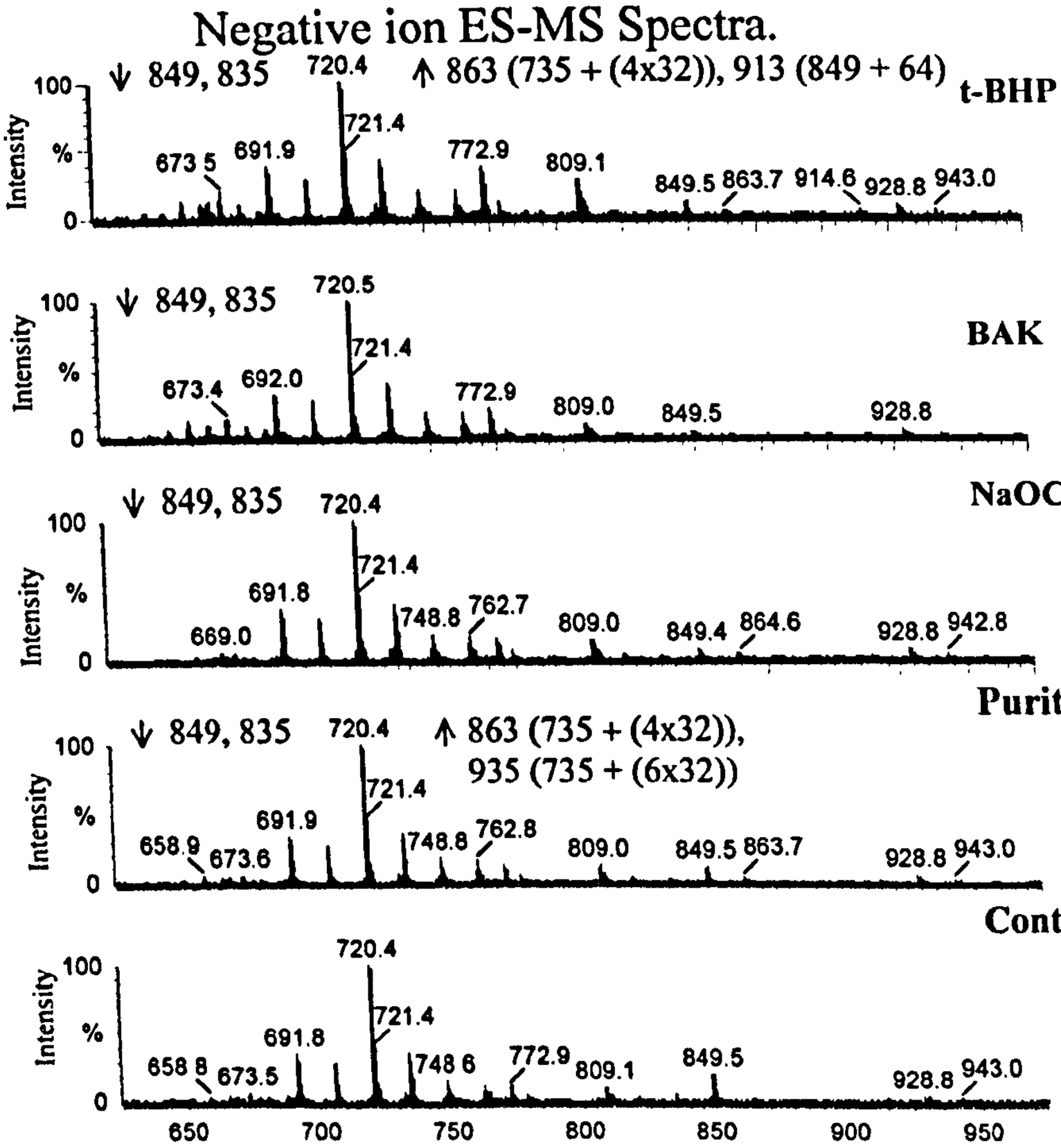


Figure 4.6 Direct-injection spectra of lipids from *Staph. aureus* treated for four hours with Purite™ at 0.4%; benzalkonium chloride (BAK) at 0.2%; NaOCl at 3.5 mM pH 7.2; and *tert*-butylhydroperoxide (tBHP) + Fe2+ at 0.25 M + 1 mM. Observed changes are noted above spectra.

It was less easy to observe and analyse results by direct injection with definitive clarity. As the complex phospholipids were mixtures analysed simultaneously there was a general loss of sensitivity in detecting the smaller constituents due to the signal to noise generated by the larger native species in the mixture. However, in Figures 4.5 and 4.6 it is still possible to observe the loss of some major native species (indicated by down arrows) and indicate the generation of lipid hydroperoxides, at multiples of +32Da, and chlorohydrins, at multiples of +52 Da (indicated by up arrows). The summarised results of oxidative modifications of bacterial lipids can be seen in Table 4.4. In contrast to eukaryotic mammalian cells and the other micro-organisms, Table 4.4 indicates that the *Staph. aureus* underwent oxidative modifications upon treatment with Purite™ and BAK. This may represent enhanced hydro-peroxidation of labile lipids upon cell membrane permeabilisation and cell death by the preservatives in a cell type. In addition we have shown *Staph. aureus* to lack glutathione and other major antioxidants that would have also been labile in the other cell types exposed to Purite™ and BAK.

Staph. aureus ***Control Treatment*** Native Phospholipid	Hydroperoxides with t-BHP		Chlorohydrins with NaOCl	
M/z Native	**+32**	**+64**	**+52**	**+104**
813	845			
837			889	
851			903	
849		913		
Staph. aureus ***Experimental Treatment*** Native Phospholipid	Hydroperoxides With Purite™		Hydroperoxides with BAK	
M/z Native	**+32**	**+64**	**+32**	**+64**
813	845		845	
837				
851				
767		831		831
823		887		887
849				
735				
Psuedo. aeruginosa ***Control Treatment*** Native Phospholipid	Hydroperoxides with t-BHP		Chlorohydrins with NaOCl	
M/z Native	**+32**	**+64**		
707				
735				
763				

Table 4.4 Summary table of modified lipid products from *Staph. aureus* (n=3) and *Psuedo. aeruginosa* (n=3) treated for four hours with Purite™ at 0.4%; benzalkonium chloride (BAK) at 0.2%; NaOCl at 3.5 mM pH 7.2; and *tert*-butylhydroperoxide (tBHP) + Fe2+ at 0.25 M + 1 mM.

Discussion

Electrospray Mass Spectrometry.

The aim of this investigation was to elucidate the specific phospholipid profiles of the mammalian cells, fungi, yeast, and bacterial species used throughout this study. This was achieved using soft ionization, ES-MS, of total cell lipid extracts with and without HPLC separation of complex lipid mixtures. The use of MS analysis, for membrane phospholipid profiling, has been undertaken by many researchers to achieve many goals. These include the identification of *Bacillus* species chemotaxonomic differentiation (Black *et al.*, 1997), adaptive responses involved in cytotoxic chemical resistance (Mechin, *et al.*, 1999), and the effects of oxidative damage (Spickett, *et al*, 2001). From the observations of these researchers, and those in Chapter 2, many of the interpretations have been made in this study.

Previous researchers have shown that phosphatidyl-cholines and ethanolamines (PC & PE's) are detectable by positive-ion MS (Spickett *et al.*, 1998; Brouwers, *et al.*, 1999). Phosphatidyl-ethanolamines, -serines, -glycerols, and -di-glycerols (PE, PS, PG and di-PG) species are better, or only, detectable in negative-ion mode with the solvent system used (Smith *et al.*, 1995; Kwon *et al.*, 1996; Black *et al.*, 1997; Brugger *et al.*, 1997). These observations agree with our findings and the chemical structure responsible for this is shown in Figure 4.7. The charged nature of the PC phospholipids limit their detection to positive-ion MS only.

Fragmentation analysis can give an insight into the exact phospholipid structure, but often the differences in profile between species are more fundamental than the differences in unsaturated:saturated R_1 and R_2 chain length ratio. One of the major advantages of fragmentation techniques is the positive elucidation of lipid polar head groups. It was not possible to give absolute identification of phospholipid species by ES-MS alone as PE and PS m/z overlap. However, the use of HPLC retention times can help identify different species of the same m/z. The adaptation of ES-MS to generate in-source fragmentation of the polar head-group may be of limited use, specifying PC's, PE's, and PS's by detection of the polar head group m/z fragment only. To fully identify the ratio of acyl-chain lengths and saturation hard ionization or tandem MS is required. Researchers (Brugger *et al.*, 1996; Smith *et al.*,

1995; Black, *et al.*, 1997) have fully analysed mammalian and bacterial membrane lipids by these techniques. Many of the results here have been further interpreted based on the additional strength of their analysis. This technology was not available at the time of analysis.

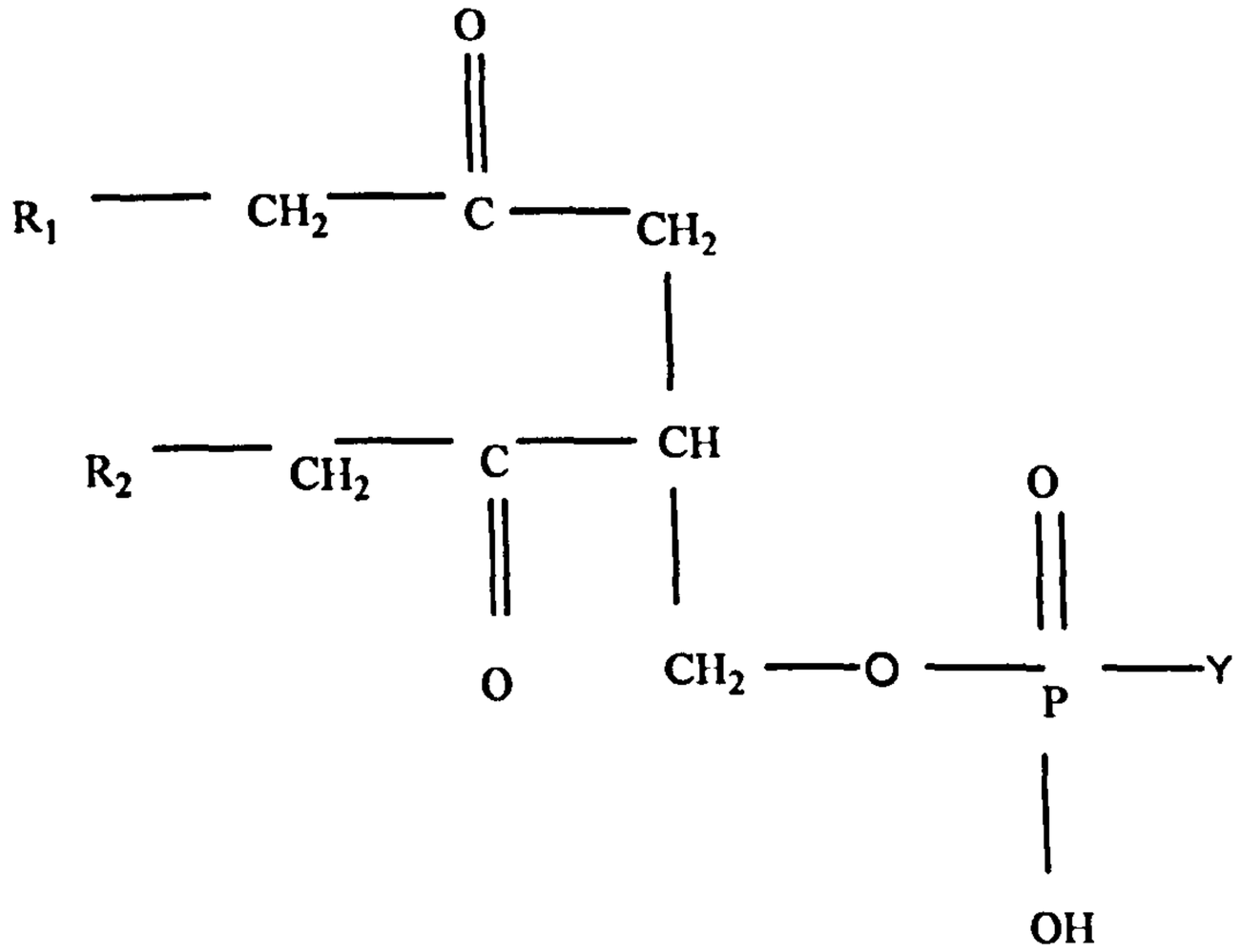


Figure 4.7. Chemical diagram of the structure of phospholipids.
Above: Ester bonds link the fatty acyl chains (R_1 and R_2) to the glycerol backbone,
Below: The some possible phospholipid head groups (Y).

Native Cellular Lipid Analysis.

The ocular cells used, RCE and WKD, were found to be rich in PC phospholipids. These observations broadly agree with the findings of Kwon *et al.* (1995) in pancreatic islet cells. Kwon investigated the role of sphingomyelin production from the abundant PC precursor in this cell line. Furthermore, there was good correlation to the m/z found in RCE and WKD and the parent species observed by Spickett *et al.* (2001) in leukocytes and Brugger *et al.* (1996) in chinese hampster ovary (CHO) cell lines. Brugger also used sample fragmentation to elucidate chain length ratio and unsaturation sites of detected phospholipid species. Brugger included internal standards of PC 28:0 in the ES-MS samples to generate quantitative analysis using a PC with chains shorter than CHO cells produce. Quantitative analysis was not performed for WKD or RCE, but it is interesting to note that the shortest chain observed by other studies were PC 30:0 and the masses observed by researchers fit well with the findings presented here. Spickett *et al.* (2001) used the same Luna C_8 HPLC column utilised in this study and defined the relative retention times of many of the lipid species observed in RCE and WKD extracts.

In the eukaryotic cells studied, *Candida* and *Alternaria,* the phospholipid membranes may not be significantly different to mammalian cells, in structure versus function status. Some researchers have analysed lipids from the *Candida* genus to define speciation (Abdi *et al.*, 1999). The analysis of Abdi overlooked PC content because it was reported to be high in all species tested and so concentrated upon the differences between fatty acid chains attached to the minor phosphatidyl-inositol, phosphatidic acid and PG phospholipids identified. However, Ghannoum *et al.* (1992) also identified PC as the major phospholipid constituent of *Candida albicans* and went further to describe the PC fatty acid composition as mainly C16 and C18. Kobayashi *et al.* (1987) used polar column gas-liquid chromatography to describe the levels of fatty acid saturation. They found the major saturated fatty acids of *Candida* to be C14:0, C16:0, and C18:0; with mono-unsaturated C16:1 and C18:1 and poly-unsaturated C18:2 and C18:3. This certainly agrees with the PC species of 32 to 36 total carbon lengths and 0,1,2,3 and 4 double bonds we observed in PC extracts of *Candida albicans*.

Little research has been reported on the analysis of *Alternaria,* or similar organisms. However Hoischen *et al.* (1997) has investigated phospholipid profiles of cell wall and wall-less forms of *Streptomyces hygroscopicus*. Hoischen only reported limited analysis of negative-ion results for PE, PG and lyso-PG. The filamentous fungi *Alternaria* was found to have phospholipids of a more varied nature. PC, PE, PG, and PS were present and there was a tendency for most phospholipids to be poly-unsaturated. Given the relative resistance of *Candida* and *Alternaria* to oxidative treatments (observed in Chapter 3), one surprising observation from this study was that there were more, potentially oxidisable, poly-unsaturated species found in *Candida* and *Alternaria* than WKD and RCE.

Mechin *et al.* (1999) found that *P. aeruginosa* ATCC 15442 consisted mainly of acyl-chains of C18:1 (40.8%), C16:0 (32.7%) and C16:1 (11.3%); with C10:0, C12:0 and C18:0 also detected. Odd numbered carbon acyl-chains were found in trace amounts only. These findings agree with the m/z data presented here and Mechin *et al.* (1999) states, 'The profile for the major fatty acids in *P. aeruginosa* ATCC 15442 was the same as that in other strains of this species'. *S. aureus* was found to contain significantly more saturated PG than any other organism tested. The identification of extracted membrane phospholipids from *S. aureus* had no direct comparable research in the literature for the species. However, the direct injection spectra are very similar to those found in *Bacillus spp.* (Smith *et al.,* 1995; Black *et al.,* 1997). Black *et al.* found that the C15:0 acyl-chain predominated in *Bacillus.*

One theory, already touched upon, generated from Chapter 3 observations was that organisms sensitive to oxidative preservatives may have an abundance of poly-unsaturated phospholipids in their native lipid profiles. However, the results from Chapter 3 (Time-course viability studies) indicated that organisms exhibiting resistance to Purite™ were in fact those that contained membrane phospholipids more susceptible to oxidative modifications and, therefore, probably possess other anti-oxidant mechanisms to generate resistance to oxidative damage within their membranes.

The hypothetical outcome from native lipid profile investigations would be that, specifically known resistant test organisms, i.e. the mammalian cells plus *Candida* and *Alternaria*, did not confer resistance to Purite™, or other oxidants, by having an abundance of saturated phospholipids in their membrane profile.

Preservative Effects upon Cellular Lipids.

In these investigations cells were treated with oxidants and preservatives, for four hours, then subsequently their lipids were extracted and analysed by electrospray mass-spectrometry. t-BHP and hypochlorous acid were used as positive control oxidants for detection of cell hydro-peroxides and chlorohydrin lipid products. Both oxidants were found to produce the type of lipid modifications observed previously. Like previous researchers (Spickett *et al.*, 2001; Carr *et al.*, 1996) the results obtained here have shown t-BHP was able to form lipid hydro-peroxides and HOCl lipid chlorohydrins in cellular treatments that were detectable by MS. However, the levels of lipid modification products detected by direct injection and HLPC MS did not reflect the loss of native lipid species in treatments. HOCl and t-BHP generated significant loss of unsaturated lipid signals in all cell types. Other researchers have performed cell treatments of micro-organisms with oxidants but have not analysed lipid modification by MS (Krasowska *et al.*, 2000). The use of TBARS assays and conjugated diene detection is not as informative as the methodology employed here. The lack of modified species detected could be due to the innate instability of these products in the cells. Thus, the loss of native lipid species was a key indicator of modification of membrane lipids by the treatments in this study. Purite™ and BAK were found to generate minor changes in native peak profile. In all cell types, the lipid modifications induced by Purite™ treatments were detected by MS as hydro-peroxides of native lipids. These were only slightly larger than the auto-oxidation observed in the untreated controls. This was unlikely to be due to the presence of transition metals, initiating the Fenton reaction, in the Purite™ formulation as the quality control data supplied by Bio-cide (Oklahoma, USA) would indicate that metal contents would have been lower than 1ppm in the final treatment. All oxidative modifications induced by BAK and Purite™ were restricted to the same native species as those passively oxidised in the

untreated control lipid extracts. In addition, BAK and Purite™ treatments modified native lipids to the same products as control cell lipid extracts and no treatment specific modifications to other native lipid species were observed. Thus, Purite™ treatments did not induce a greater number of multiple lipid oxidations of the same lipid compared to control. From these results it is possible to suggest that, in the cells tested here, Purite™ did not oxidatively modify cell lipids significantly. These observations support the findings of Chapter 2, where Purite™'s effects upon phospholipid vesicles were mild compared to HOCl and t-BHP.

The viability results of Chapter 3 allow the evaluation of phospholipid treatment observations at concentrations known to be cytotoxic. In Chapter 3, the organism found to be most sensitive to oxidative treatments was *Staph. aureus* and this was the only organism to have major extracted lipid modification upon treatments. *Staph. aureus* was also the only test organism to lack the cytosolic anti-oxidant glutathione. Ocular cells (RCE and WKD), *Candida albicans, Pseudomonas aeruginosa,* and *Alternaria spp.* were found to be more resistant to oxidative treatments in terms of both cytotoxicity and phospholipid oxidation. Upon treatment of the cells with toxic levels of oxidants, Purite™ was found to have only mild oxidative effects on cellular lipids compared to control treatments. Indeed, Purite™ was comparable to BAK in this respect. Thus, it is unlikely that the direct mechanism of Purite™'s cytotoxicity is through phospholipid membrane oxidation of cells.

Protective mechanisms to biocides that have been reported in microbial cells include altered membrane phospholipid profiles, to partition the agents from cell entry, or an adaptive response similar to those observed by Ferguson (1999) in *Escherischia coli.* There is some evidence that micro-organisms can adapt their membrane phospholipid composition to attain resistance to sub-lethal doses of anti-microbial agents (Pechey *et al.*, 1974; Ikeda *et al.*, 1984; Imai *et al.*, 1975; Mechin *et al.*, 1999). The findings in Chapter 3 cannot address this, as this theory relates to sub-lethal doses and those tested in our studies were toxic. It would only be possible to elucidate such a theory after performing a full treatment dose response curve in each cell type to elucidate the cytotoxicity dose response curve, followed by the same treatment dose response incubations with cellular phospholipid extraction and analysis.

Chapter 5:

The Effects of Ocular Preservatives on Total Glutathione Levels and Viability In Mammalian and Microbial Cells.

Introduction

Although the previous experimental chapters have generated many observations that have been discussed in their own context there are also limitations within the results that have to be considered when planning new studies. In Chapter 3, the use of different cytotoxicity methods between cell types tested for time course viability meant that care had to be taken when comparing the different test organisms. Also, Chapter 3 only evaluated the concentrations of treatments required to see effects observed on bio-molecules *in vitro* in Chapter 2 to confirm that they were indeed cytotoxic doses. These concentrations were also used in cell treatments in Chapter 4 to further determine the types of phospholipid modifications that Purite™ was able to induce in cells. Although these concentrations could be related to the effects observed in a mechanistic manner they perhaps did not reflect the concentrations used in ocular preservatives and lens disinfecting solutions.

Research has suggested that a low level of BAK exposure in WKD causes cell apoptosis and only necrosis at a higher concentration (De Saint, *et al.*, 1999). This is an interesting, concentration dependent, differential killing mechanism. Thus, it may be valuable to study and focus on the effects of a wider range of doses of oxidative and non-oxidative preservatives, on any given cell, to fully understand the range of possible sub-lethal to lethal cellular responses and mechanisms of action involved.

In Chapter 4, it was observed that the oxidative preservative Purite™ could generate very few changes to destabilise cell membrane lipids and this was too much lesser degree than positive control oxidants. Chapter 3 indicated that cellular cytotoxicity/resistance to Purite™ and other oxidative treatments correlated to maintaining glutathione homeostasis within the cell. Furthermore, in this study, glutathione appeared to be the most abundant anti-oxidant in the panel of mammalian

cells as well as some of the micro-organisms. These specific species have been shown in Chapter 3 to have resistance to Purite™. Thus, it is important to understand more about a wider range of treatment dose response effects on glutathione depletion and the relationship to cellular cytotoxicity.

For this dose response study, after discussions with Allergan, the incubation period was adjusted to 6 hours as this is the minimum time of contact lens disinfection. Thus, the higher doses tested would evaluate the possible application of contact lens disinfection whilst continuing to determine the effects of lower concentrations as ocular preservatives. After discussions on the reviews of Hugo (1967), who correlated treatment uptake isotherms with the molecular mechanism of action of specific biocides, the benefits of starting to monitor Purite™ uptake from the incubation media were evaluated. It was decided to assay Purite™ levels in the post-incubation media as this may indicate how much Purite™ the cells have consumed to generate the cytotoxic and oxidative effects. Thus, the toxicity of Purite™ to mammalian cells and micro-organisms could be compared to cellular glutathione depletion.

The aim of this study was to determine the resistance of the cell types, used previously, to the panel of preservatives, including the compound Purite™, and control oxidants over a wide range of concentrations. Cellular adenosine tri-phosphate (ATP) levels are closely regulated, since ATP is necessary for survival in all living organisms. This molecule, therefore, provides an accurate measurement of the number of living cells within a population. Monitoring the levels of cytotoxicity, by a cytosolic ATP method could overcome some of the limitations of Chapter 3. Thus, measuring the levels of cellular oxidative stress induced by treatments would generate results that could be used subsequently to compare the differences between lethal and non-lethal doses, as measured by a single ATP technique, for the cell types tested. The most successful technique for ATP measurement is the luciferin-luciferase bioluminescent assay, as it is highly sensitive and accurate over six orders of magnitude (Crouch *et al.*, 1993 & 2000). This is 1000 fold more sensitive than cell proliferation/cytotoxicity fluorescence techniques. The observed changes of cellular glutathione could be compared to cellular cytotoxicity, as measured by ATP

luciferin/luciferase bioluminescence, for the same preservative dose response parameters.

However, certain antimicrobial agents may exert effects on microbial bioenergetics. At sub-lethal doses, specific biocides have been shown to uncouple oxidative phosphorylation and the respiratory chain to generate a non-linear correlation of viability to cytosolic ATP but linearity returns at concentrations the MIC (Denyer, 1998).

The bioluminescent reaction of cytosolic ATP with Luciferin-Luciferase have been developed by many companies into test kits assaying cytotoxicity and cell proliferation by luminescence detection (Cunningham, 2001). The principle of the reaction is as follows:

$$ATP + Luciferin + O_2 \xrightarrow[Luciferase]{} Oxyluciferin + AMP + PPi + CO_2 + Light$$

The reaction involved is catalysed by firefly luciferase, yielding a light intensity linearly related to ATP concentration.

To focus these studies further reselection of treatments and test organisms were decided. The new panel of treatments for dose response studies, Purite™, BAK and H_2O_2, were selected for their relevance to ocular pharmaceutical preservation. The preservative sodium perborate was of increasing importance as a passive preservative (Abelson and Washburn, 2002) and has been described as producing 55ppm H_2O_2 as its mechanism of action (personal communication with Allergan). The test organisms were RCE, WKD, *Candida albicans, Pseudomonas aeruginosa* and *Alternaria,* to reflect organisms showing resistance to Purite™ in Chapter 3, and *Staphylococcus aureus* as an organism lacking detectable glutathione.

The aim was to determine whether cellular glutathione is involved, and if this is a potential mechanism of Purite™ action and resistance, as suggested in Chapter 3. This would then give an insight to relate the differences in preservative toxicity profiles to antioxidant status between the cell types tested, in a mechanistic approach.

Materials and Methods

Dose Response Evaluation of Preservatives and Oxidants Affecting Cytosolic Glutathione.

Mammalian Cell Treatments for Cytosolic Glutathione Changes.

Mammalian cells, RCE and WKD, were grown to 90-100% confluence in 75cm^2 tissue culture flasks as described in Chapter 3. Mammalian cells were washed with sterile PBS. 10mL of fresh, serum free culture media containing the preservative or oxidant test solutions, were added to the cells in tissue culture flasks, and these were incubated for 6 hours at 37°C. All cellular treatments were incubated in dark conditions. The preservatives tested were 0-6000ppm H_2O_2, BAK and Purite™.

The adherent cells were detached using Acutase (icT, California, USA) and washed twice with PBS in a pre-weighed universal tube. Wet cell weight was recorded for each pellet. Cell cytosolic contents were extracted by the addition of 200µL 5% trichloroacetic acid (TCA)(w/v) to precipitate cellular protein, followed by freeze thawing and centrifugation to remove cell debris. All samples were centrifuged for one minute at 10,000 × g (in a microfuge). Supernatants were stored at 4°C and assayed within two hours.

Microbial Cell Treatments for Cytosolic Glutathione Changes.

Pseudomonas aeruginosa, Staphylococcus aureus and *C. albicans* were grown for 18 hours at 37°C in conical flasks. Bacteria were cultured in TSB and fungi in YEPD broth. *Alternaria* was grown at 26°C in conical flasks in YEPD. Cultures were washed twice in PBS and resuspended to 1x10^{8}cells/mL (or 0.01g/mL) in preservative treatments. Test organisms were incubated for 6 hours at their original culture temperature. After incubation cells were centrifuged and washed twice in PBS in a pre-weighed universal tube. The final wash solution was removed, and the cell pellet weight calculated. The pellet was resuspended in a 1.5mL of PBS and cell contents were extracted by cell membrane disruption (Constant Systems) at 20 kPa. 0.25mL 20% trichloroacetic acid (TCA)(w/v) was added to 0.75mL of pressed cell suspension (5% final TCA), to precipitate cellular protein, followed by

centrifugation to remove the cell debris. Supernatants were stored at 4°C and assayed within two hours.

Dose Response Evaluation of Preservatives and Oxidants Effecting Viability

Mammalian Cell Treatments for Cytosolic ATP Changes.

The WKD and RCE cells were seeded at $1x10^4$ cells per well in 100µL of media on 96 well µClear luminometer plates (Greiner, UK) and allowed to adhere overnight. Additional wells on the plate were seeded with 100µL of serial dilutions of the $1x10^5$ cells per mL stock, to generate a calibration curve. Culture media was removed and cells were washed with PBS. Treatments of 0-6000ppm (0-0.6%) H_2O_2, BAK and Purite™ in serum free media were placed in specific wells and incubated for 6 hours at 37°C in the dark.

At the end of the incubation, treatments were removed, and retained, and cells washed with PBS. Retained treatments were collected and final Purite™ levels in the treatment media was assayed as described below. Well contents were assayed for cellular ATP levels as a measure of cytotoxicity using a CellTitre-Glo™ (Promega, UK) assay kit and a LUMIstar Galaxy (BMG, UK) microplate luminometer. The protocol followed that of the Instructions for Use and Notes publications specifically written for the test kit (Riss *et al.*, 2002). Further validated on the LUMIstar Galaxy machine (Hoffman *et al.*, 2001). CellTitre-Glo™ Substrate and Buffer stock vials were stored at 4°C until required. For each 96-well plate a vial of substrate and buffer stock were removed and warmed to room temperature. The two vials were gently mixed to form the CellTitre-Glo™ Reagent. 100µLs of CellTitre-Glo™ Reagent was pipetted into each well of the 96-well plate and mixed on a shaker for 5 minutes. The entire plate was then read on a LUMIstar Galaxy (BMG, UK) luminometer. The machine was set to read in well mode taking 3 consecutive 1 second light recordings per well. The average relative light units per second (RLU/s) for each well/sample was calculated from the three readings and the average blank well RLU/s subtracted. Cell calibration curves on each plate were used to convert the RLU/s to result in the percentage of cells remaining in each well. Cell number reduction was calculated as a percentage of control.

Microbial Cell Treatments for Cytosolic ATP Changes.

Candida, Staphylococcus, Pseudomonas, and *Alternaria* were grown, treated, and incubated with preservatives in the same manner described for glutathione analysis (above). At the end of incubation, 100 mL of treated culture was placed in wells of a 96 well luminometer 0.2 μm filter plate (Millipore, UK). Additional wells on the plate were seeded with 100μL of serial dilutions of the control cells to generate a calibration curve. Treatments were removed and cells washed with PBS by vacuum manifold (Pall, UK) filtration. Post-treatment media was retained and final Purite™ levels assayed (as described below). 100μL of sterile water was placed in each well to re-suspend the cells.

The well contents were assayed for cellular ATP levels as a measure of cytotoxicity using a ViaLight MDA (Lumitech, UK) assay kit on a LUMIstar Galaxy (BMG, UK) microplate luminometer set to read in well mode at 25°C. Bactolyse was stored at 4°C and warmed to 25°C as required. ATP Monitoring Reagent (AMR) was reconstituted and 5mL aliquots stored frozen at –20°C. 100μL of Bactolyse was injected into the wells and the plate was incubated for 5 minutes with shaking. For one 96-well plate one 5mL AMR aliquot was thawed and warmed to 25°C when required. 20μLs of AMR were then injected into each well and the luminesence measured for 3 one second readings after a two second delay. RLU readings were then converted to cell percentages in the manner described above.

Total Glutathione Analysis.

Total glutathione was determined by the DTNB-recycling spectrophotometric assay first described in Chapter 3. All solutions were stored in the dark, on ice. The amount of glutathione in the samples was determined from the standard curve in which the GSH concentration was plotted against the rate of change in absorbance at 412 nm. Glutathione concentration was calculated in micromoles per g of cells extracted.

Purite™ Uptake Analysis.

For incubations with Purite™, the loss / consumption of Purite™ was determined by analysis of the Purite™ concentration in the supernatant after harvesting of the cells for glutathione analysis at the end of the treatments. Purite™ concentration was measured by adding 50µL of sample/blank to 50µL of 2% potassium iodide (w/v) and 1500µL 50mM HCl. The reaction of sodium chlorite (Purite™) with iodide, under acidic conditions, to yield iodine, was detected spectrophotometrically at 350 nm. The level of Purite™ in a sample was calculated from a standard curve of Purite™/$NaClO_2$ diluted to the range 1-100ppm (0.0001%-0.01%). Samples outside of the 1-100ppm range were diluted, 1 in 10 or 100, and final concentrations calculated taking the dilution into account.

Statistical Analysis.

Statistical analysis of the results was performed in Minitab using Mann-Whitney U tests. Each dose response was carried out (n=6) and the significance level set to $P>0.05$.

Results.

Dose response studies were carried out to investigate the use of intracellular ATP levels as a measure of viability, using a luminescence assay. This technique allowed the viability of both mammalian cells and micro-organisms to be measured in the same way, thus providing directly comparable information. A large number of different assay kits for ATP-viability measurements exist, some of which are designed specifically for use with bacteria or micro-organisms. We have carried out validation tests with several kits to check that the changes in ATP concentration determined correspond to the number of viable cells present (Figure 5.1). This was compared to the method previously used such as plating for colony forming units and example methods using the kits (Riss *et al.*, 2002). The ATP method was found to be accurate, precise and faster than the traditional methods used in Chapter 3. By performing cellular ATP standard curves on every 96-well plate, the method appears to be sensitive and reliable. We are confident that the percentage of cells compared to controls, shown in Figures 5.2 and 5.5, are comparable to cellular ATP for all the organisms under investigation. The results of preservative treatments on the intracellular glutathione concentration, with the same range of preservatives used in cytotoxicity studies, in RCE, WKD, *Alternaria spp, Pseudomonas aeruginoas* and *C. albicans* was determined and results are shown in Figures 5.2 and 5.5.

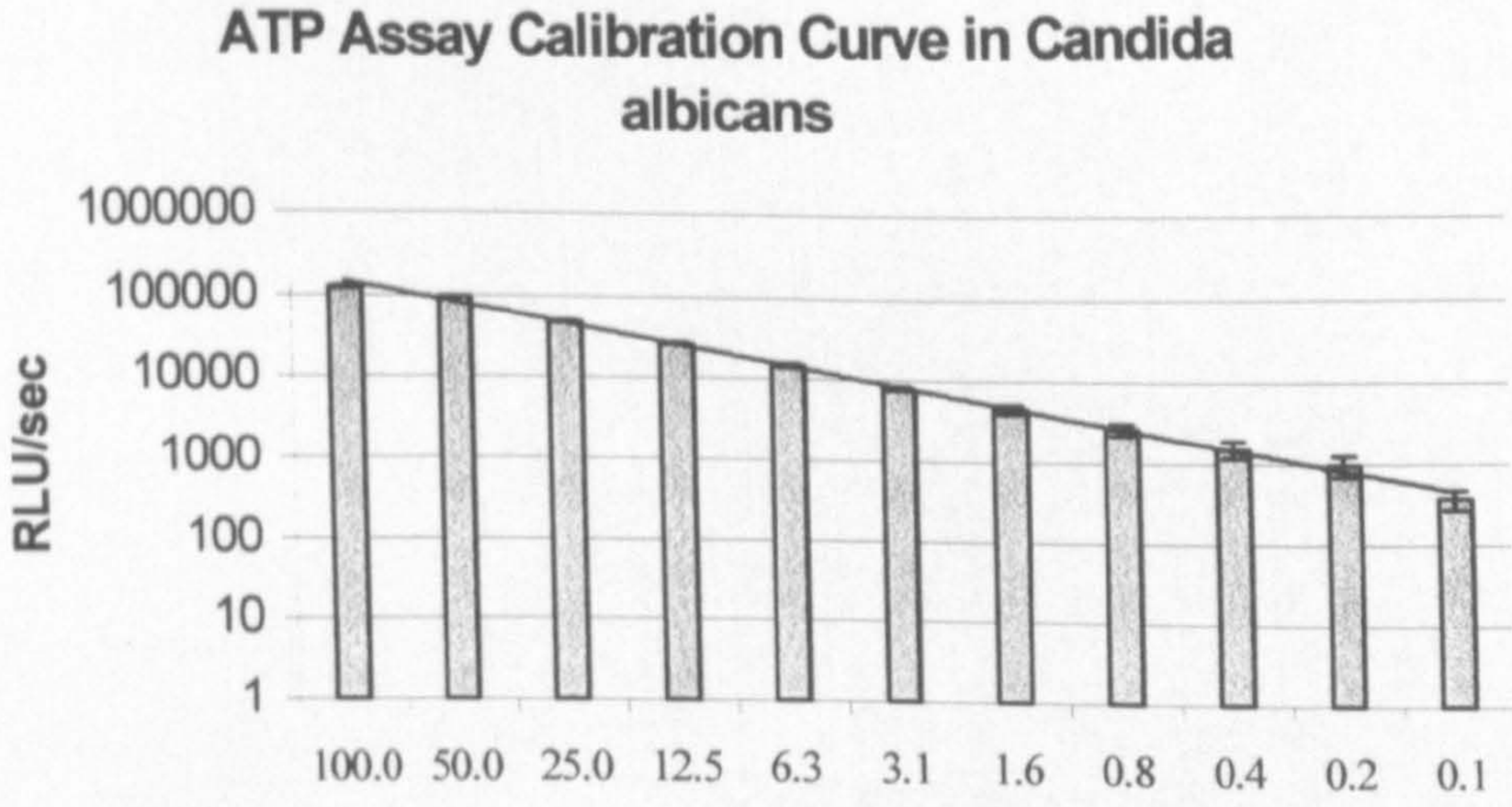


Figure 5.1 Example of an ATP Luciferin/Luciferase Luminescence calibration curve of *Candida albicans*. Luminescence, as measured by Relative Light Units per Second (RLU/sec), +/- standard error, is plotted against serial dilutions of fungal cells.

Mammalian Cell Investigations.

Results of Mammalian Cell Treatments for Cytotoxic and Glutathione Changes.

The response of RCE cell ATP levels to the preservatives was investigated, and the results are shown in Figure 5.2 (Top). This shows the dose response of RCE cells to Purite™ and BAK over the range 0-750 ppm and hydrogen peroxide over a range 0-6000ppm. It can be seen that BAK is highly toxic to the cells, with the lowest concentration used (5 ppm) already causing loss of 50 % of the intracellular glutathione (Figure 5.3). Neither Purite™ nor hydrogen peroxide affected the glutathione at their lowest treatment concentration (5 ppm), but at higher concentrations hydrogen peroxide had more severe effects than Purite™ on these cells, with total glutathione depletion occurring at 50 ppm for hydrogen peroxide, versus 500 ppm for Purite™. The response of RCE cells to Purite™ and hydrogen peroxide is significantly different ($P>0.05$). Figure 5.2 (Top) shows that the loss of viability in these cells corresponds closely with the loss of intracellular glutathione for treatment with BAK and hydrogen peroxide. With Purite™, the cell viability curve is shifted slightly to the left of the glutathione curve, showing that cell death did not begin until after depletion of glutathione was evident. Measurement of the Purite™ remaining in the incubation medium at the end of the treatment period (Figure 5.4, Top) shows that regardless of the initial concentration of Purite™ in the treatment, approximately the same percentage, 40-50%, is depleted by incubation with the cells.

Figure 5.2 (Bottom) shows WKD cellular ATP as a measure of cytotoxicity. These results show a similar pattern to RCE. Purite™ was noted to be the least toxic to both ocular cell lines compared to the other preservatives at lower concentrations. The effect of BAK treatment on the cell line WKD on cellular glutathione (Figure 5.3 Bottom) was very similar to that observed with RCE cells with complete intracellular glutathione depletion induced by treatment with 25 ppm. However, WKD cells appeared to be slightly less resistant than RCE to Purite™ and more resistant to hydrogen peroxide, with total glutathione loss with the Purite™ not even occurring at the highest concentrations used. At concentrations below 100 ppm

Purite™ was less damaging than hydrogen peroxide, whereas above this point the opposite is true.

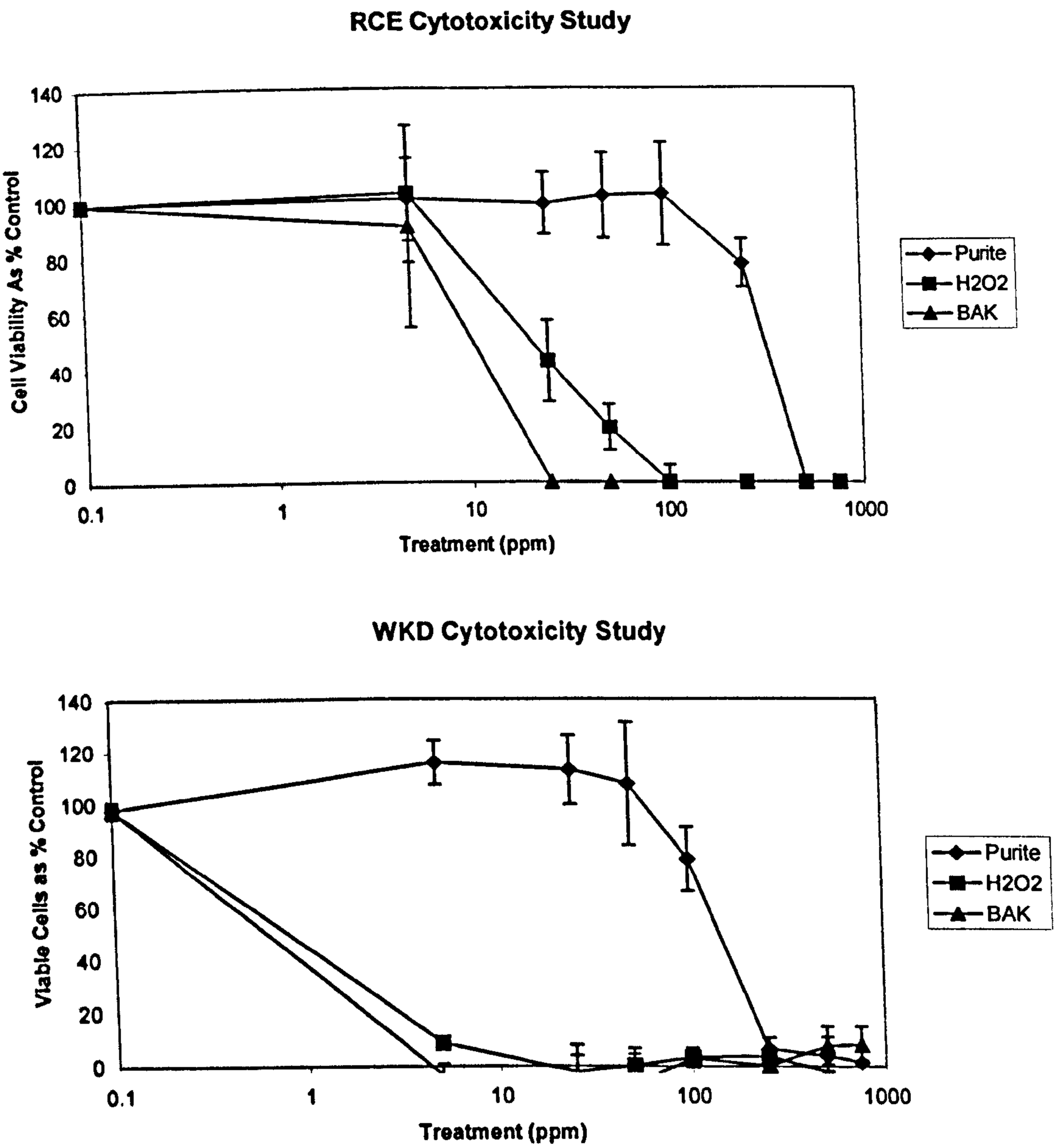
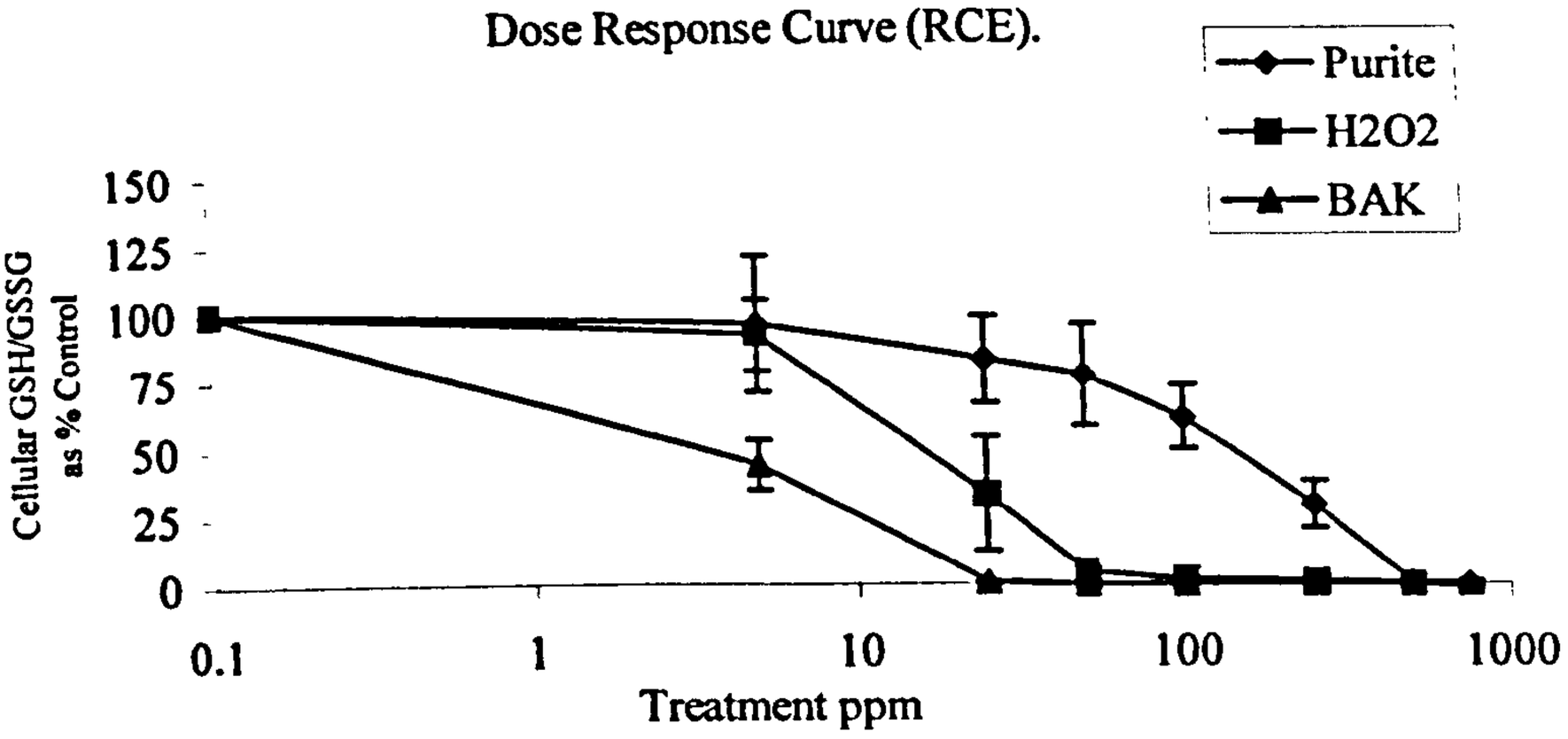


Figure 5.2. Changes in Cellular ATP as a Measure Of Cytotoxicity Upon Treatment of Mammalian Cells with Preservatives. Measurements were for ATP as measured by Luciferin/Luciferase Luminescence. Untreated cells were diluted to form standard curves. These were used in calculating the treatment results as a percentage of viable cells remaining compared to control. Graphs show changes in viability upon treatments for RCE (top) and WKD (bottom) cells (+/- SEM).



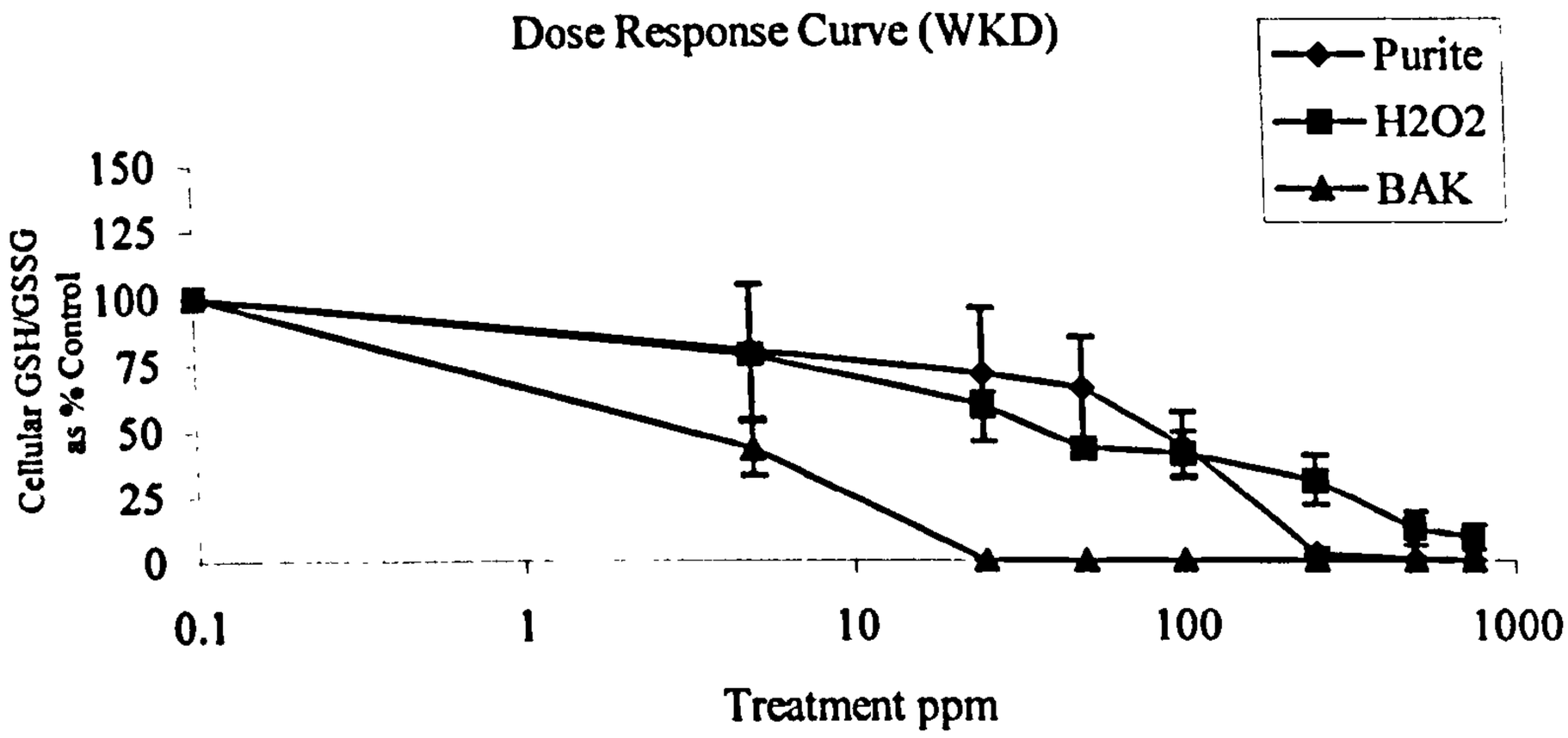
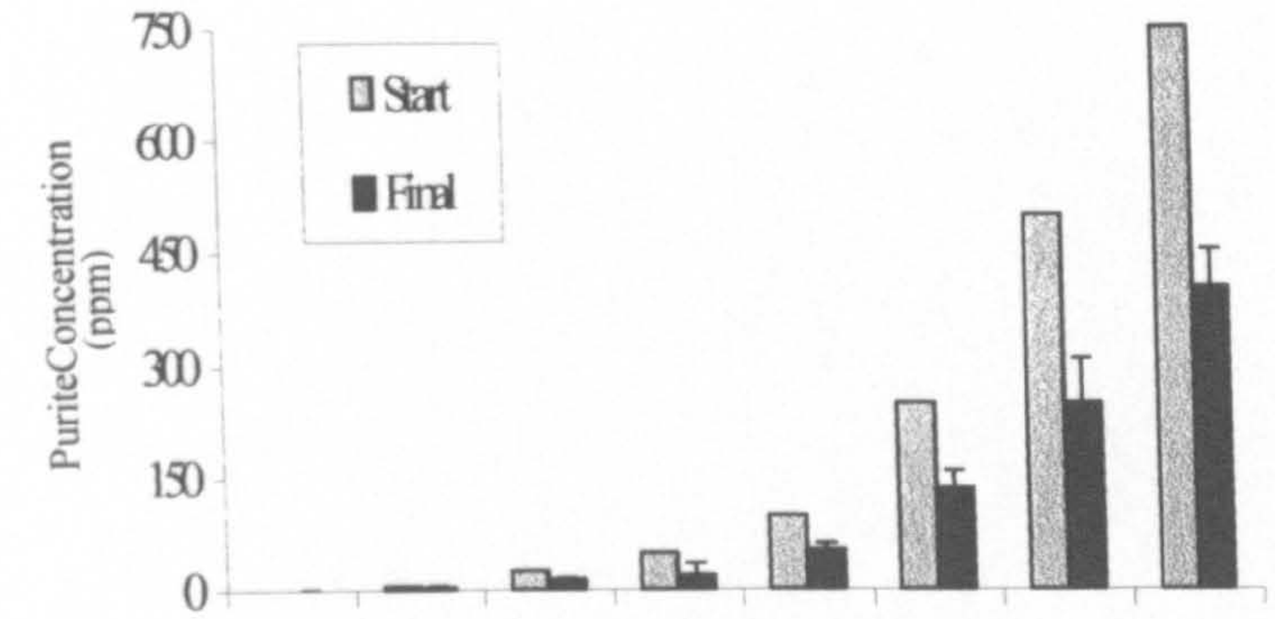
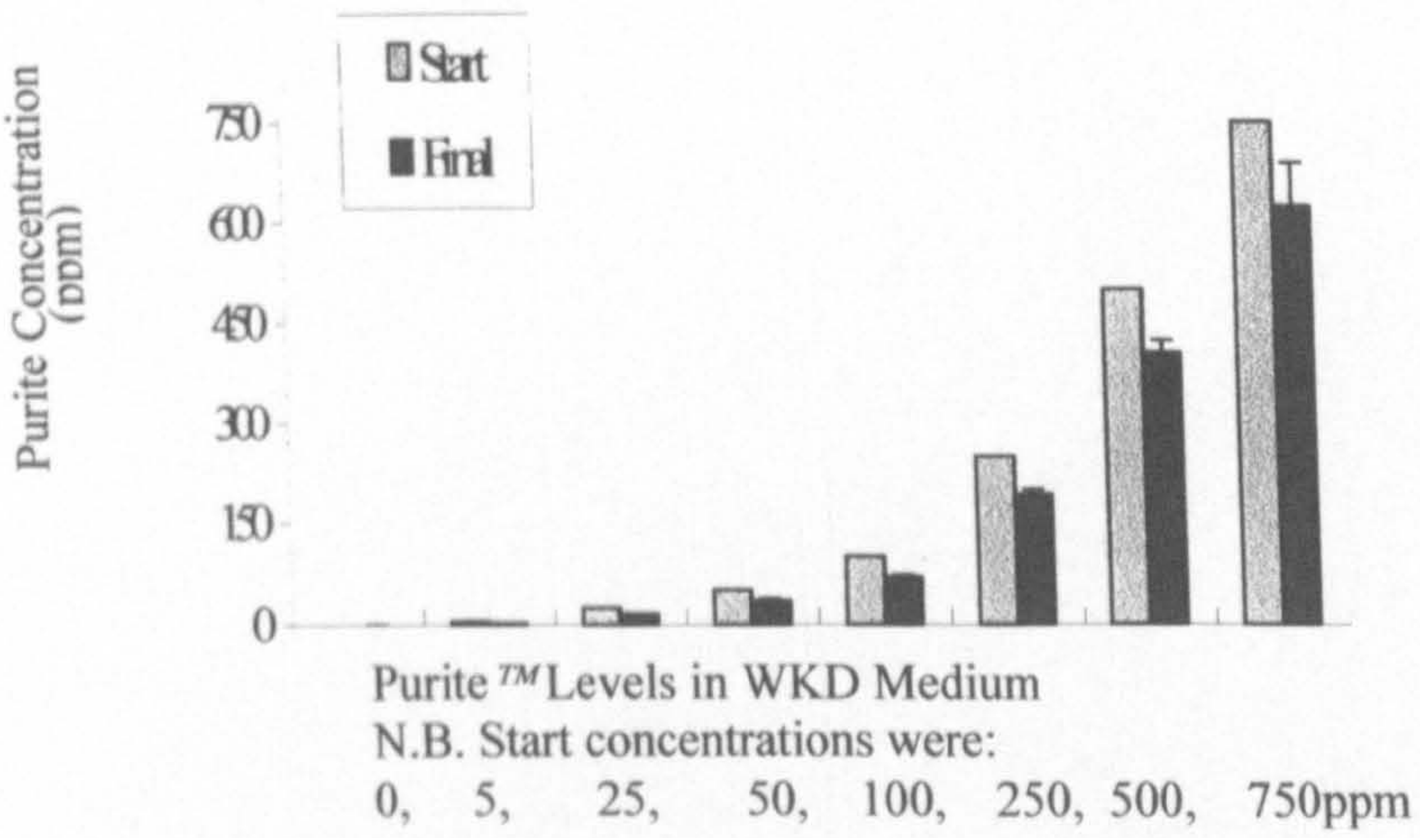


Figure 5.3. Changes in Cellular Glutathione as a Measure of Oxidative Stress Upon Treatment of Mammalian Cells with Preservatives. Controls (untreated cells) were used in calculating the treatment depletion results as a percentage of the total Glutathione (GSH+GSSG) compared to control. Graphs show changes in intracellular glutathione for RCE (top) and WKD (bottom) cells (+/- SEM).

A difference was also noted in the disappearance of Purite™ from the medium of WKD cells (Figure 5.4, Bottom). In contrast to RCE there was a substantially smaller proportion of Purite™, 10-20%, lost at any particular treatment concentration. This observation may be due to the difference in native total intracellular glutathione levels in the different cell types tested. Previous studies, reported in chapter 4, have shown that native intracellular glutathione concentrations in the mammalian cells were relatively high, with RCE cells containing slightly more glutathione than WKD cells (2.6 ± 0.3 versus 1.65 ± 0.3 μmol/g).



Purite ™ Levels in RCE Medium (ppm).
N.B. Start concentrations were:
0, 5, 25, 50, 100, 250, 500, 750ppm



Purite ™ Levels in WKD Medium
N.B. Start concentrations were:
0, 5, 25, 50, 100, 250, 500, 750ppm

Figure 5.4. Changes in Media Purite™ Levels Upon Treatment of Mammalian Cells for Six Hours. Controls are untreated cells (0ppm). Graphs show changes in Purite™ from Start to Final RCE (Top) and WKD (Bottom) cell treatments (+SEM).

Microbial Cell Investigations

Cell Treatments for Cytotoxic and Glutathione Changes.

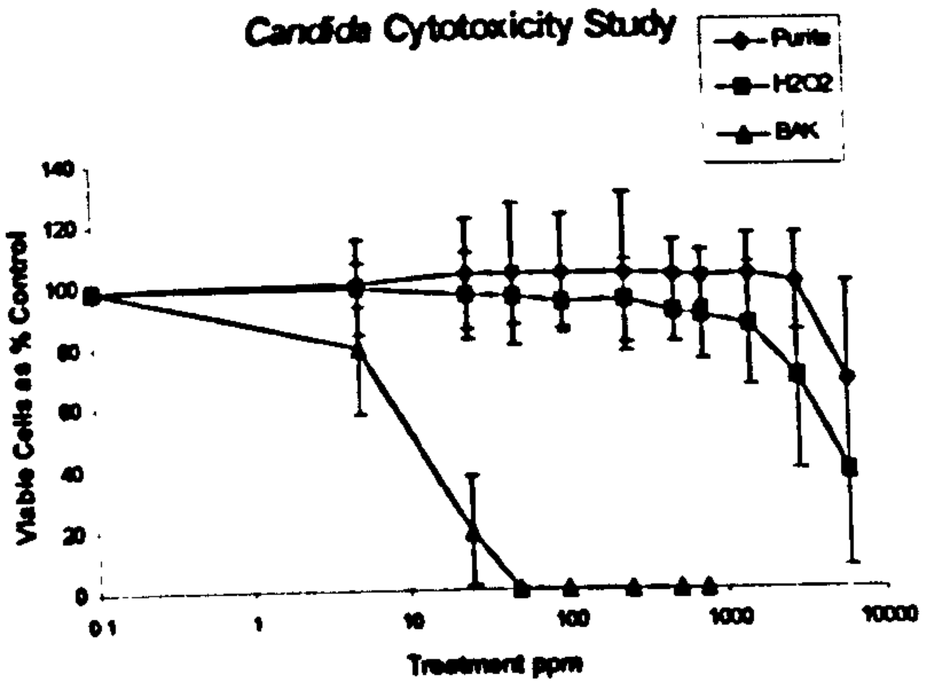
The *C. albicans* dose response of ATP, glutathione and Purite™ uptake upon treatment with test preservatives is shown in Figures 5.5-5.7 respectively. This organism showed considerably higher resistance to all the preservatives than mammalian cells, and it was necessary to extend the range of treatments used, up to 6000ppm, in order to define the response curve for hydrogen peroxide and Purite™. Figure 5.6 shows that treatments with hydrogen peroxide between 5 and 1500 ppm resulted in a large increase in glutathione levels above the control. A similar phenomenon was observed with Purite™. Here, increases in cytosolic glutathione levels were smaller in Purite™ treated cells than in peroxide. This stress type response occurred between 5 and 500 ppm. Glutathione levels and decreased below control levels at Purite™ concentrations above 1500 ppm. Purite™ depletion in the media corresponded to a small proportion of the initial Purite™ concentration in the treatment at low concentrations, and only increased to approximately 50% at the highest treatment level (6000 ppm). This depletion from the media may reflect a higher resistance of *Candida* to oxidative preservatives and correlates to the highest native glutathione level of all of the test organisms. Native intracellular glutathione concentrations were highest in *C. albicans*, with an average of 3.53 ± 0.9 μmol/g. *P. aeruginosa* had intermediate levels of glutathione 1.73 ± 0.15 μmol/g and *Alternaria* spp. had comparatively low levels of glutathione 0.33 ± 0.052 μmol/g.

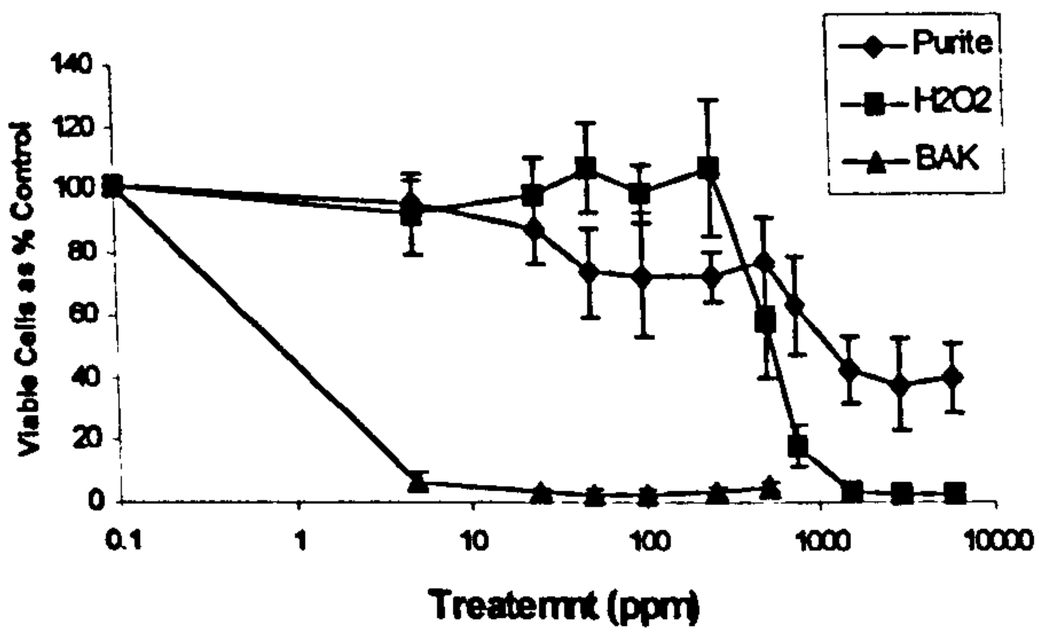
No up-regulation of glutathione was observed with BAK, which caused total depletion of cellular glutathione at 50 ppm. These glutathione responses were reflected by the ATP/cytotoxicity results of Figure 5.5. As in the mammalian cells the Purite™ cell viability curve is slightly to the right of the glutathione curve, showing that cell death did not begin until after depletion of glutathione was evident. This observation further supports the theory that glutathione is important in the resistance of cells to Purite™ treatment.

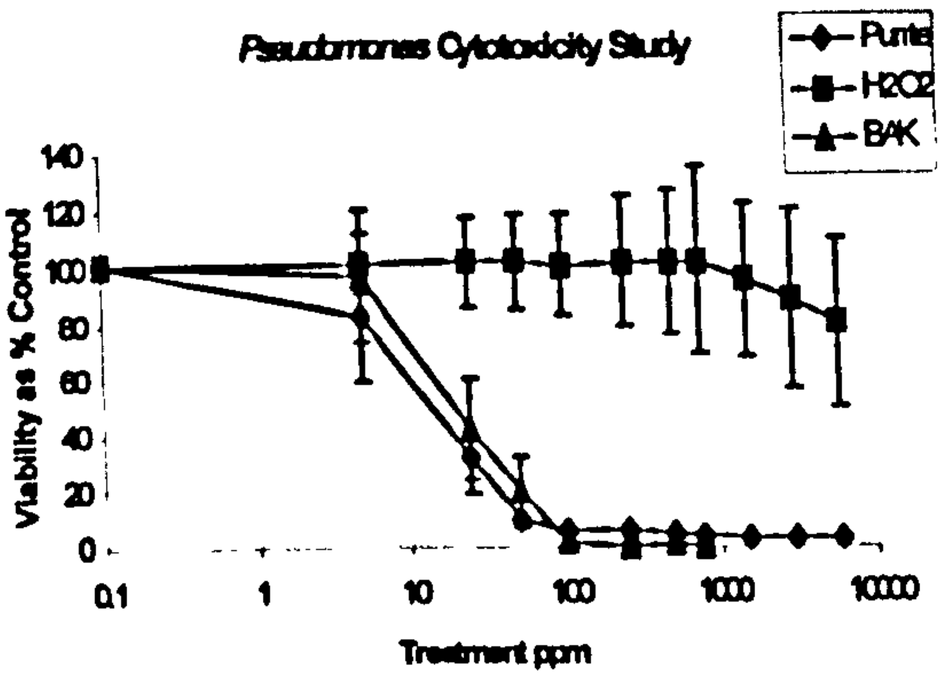
The response of *Alternaria* spp. glutathione levels to the preservatives is shown in Figure 5.6. It can be seen that, like *C. albicans*, *Alternaria* is much more resistant to the preservatives than the mammalian cells. However, its response is clearly different in several ways. It is more sensitive to BAK, with intracellular

glutathione levels already dropping at 5 ppm, and almost completely depleted at 25 ppm. It does not exhibit a stress response to hydrogen peroxide, but does show a significant increase in glutathione at low levels of Purite™. It is also more sensitive than *C. albicans* to hydrogen peroxide and Purite™, with depletion of glutathione becoming evident at 750 and 500 ppm respectively. Interestingly there was a complete lack of Purite™ depletion in the medium (Figure 5.7) with *Alternaria spp.* This was observed consistently in all experiments.

The other bacteria showed poor susceptibility to peroxide (Figure 5.5) even at 6000ppm. BAK and Purite™ toxicity was observed at 5ppm and over 99% kill ocurred by 100ppm. Figure 5.5 shows the loss of viability in these cells corresponds closely with the loss of intracellular glutathione for treatment with BAK and hydrogen peroxide. Again, with Purite™, the cell viability curve is slightly to the right of the glutathione curve, showing that cell death did not begin until after depletion of glutathione was evident.







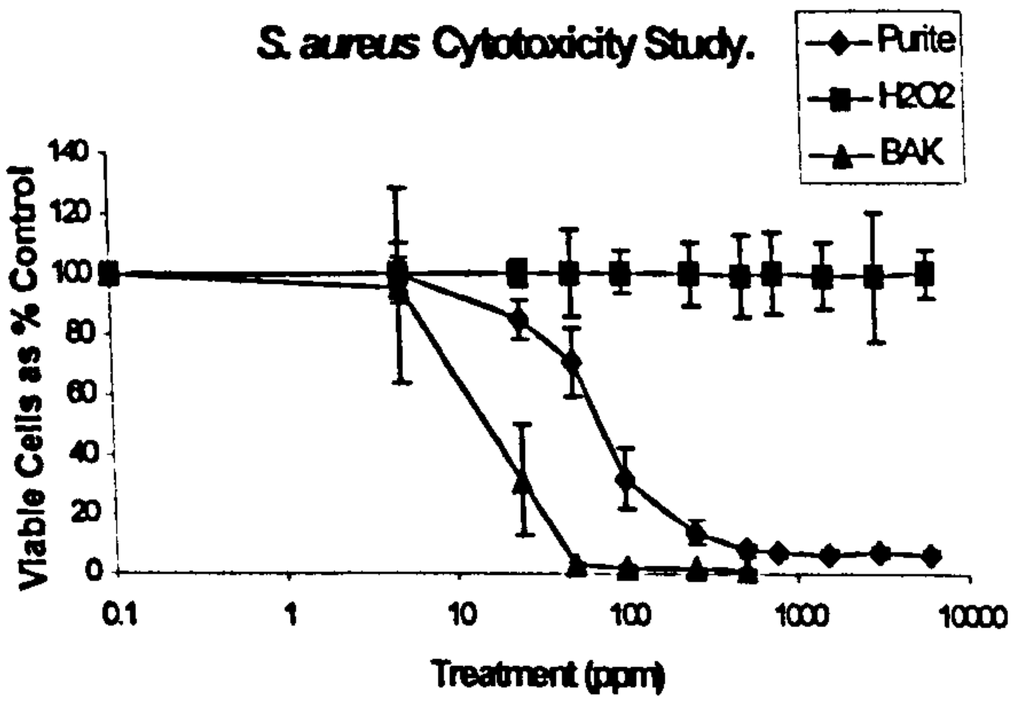


Figure 5.5. Changes in Cellular ATP as a Measure Of Cytotoxicity Upon Treatment of Bacteria, Yeast and Fungal Cells with Preservatives. Measurements were for ATP as measured by Luciferin/Luciferase Luminesence. Control, untreated cells, were diluted to form standard curves. These were used in calculating the treatment results as a percentage of the viable cells remaining compared to control. Graphs show changes in viability upon treatments for eukaryotic *Candida* and *Alternatia* (top) and prokaryotic *Pseudomonas and Staphylococcus* (bottom) cells (+/- SEM).

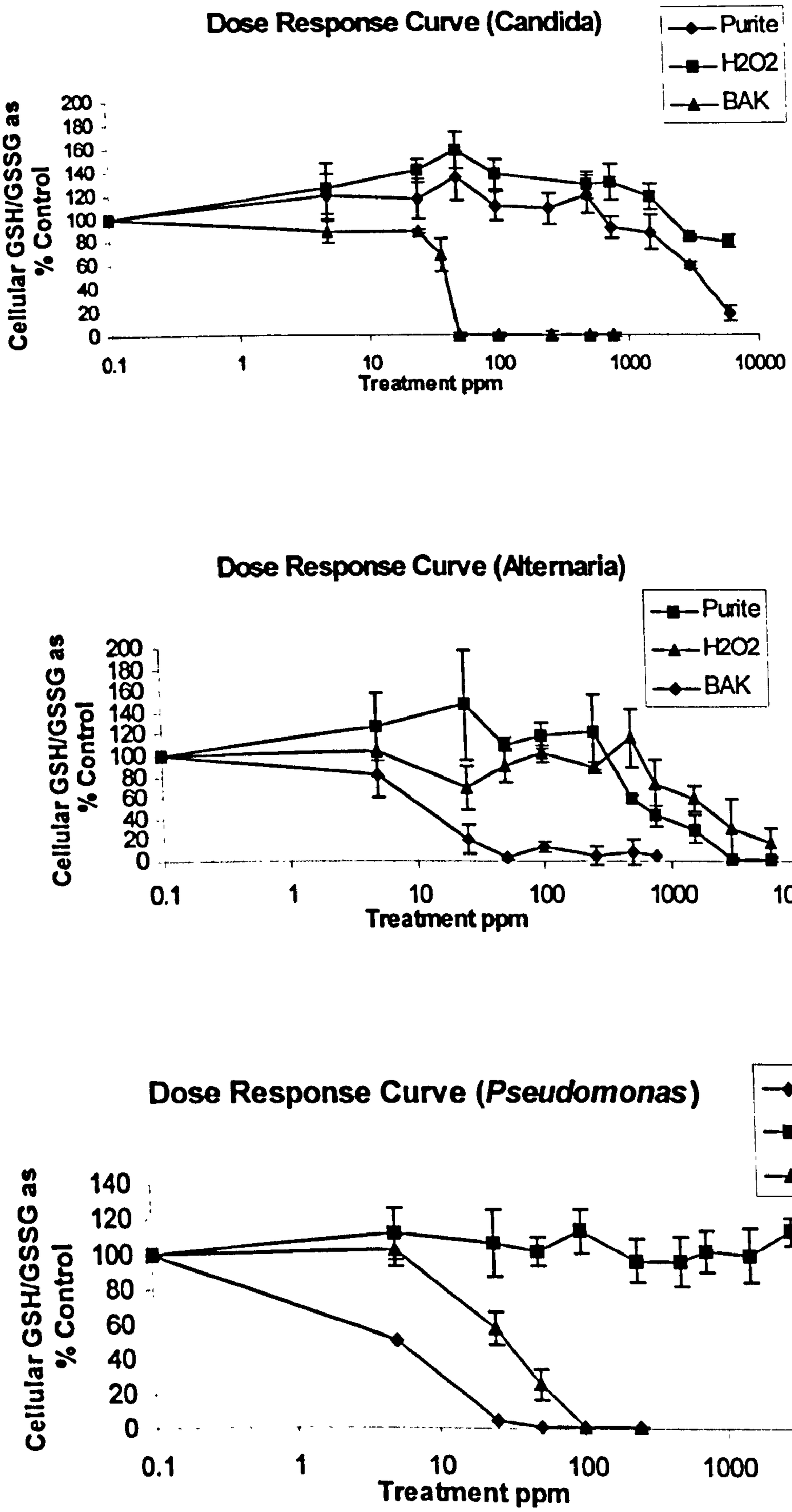
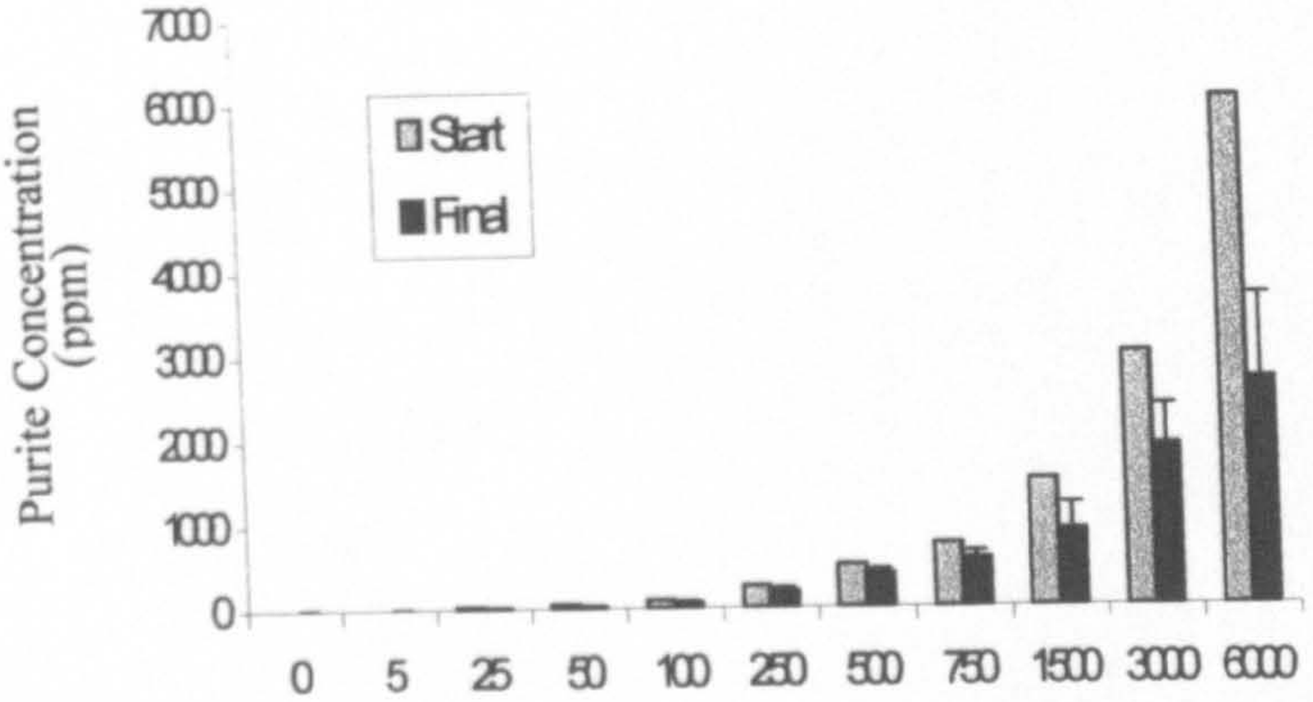
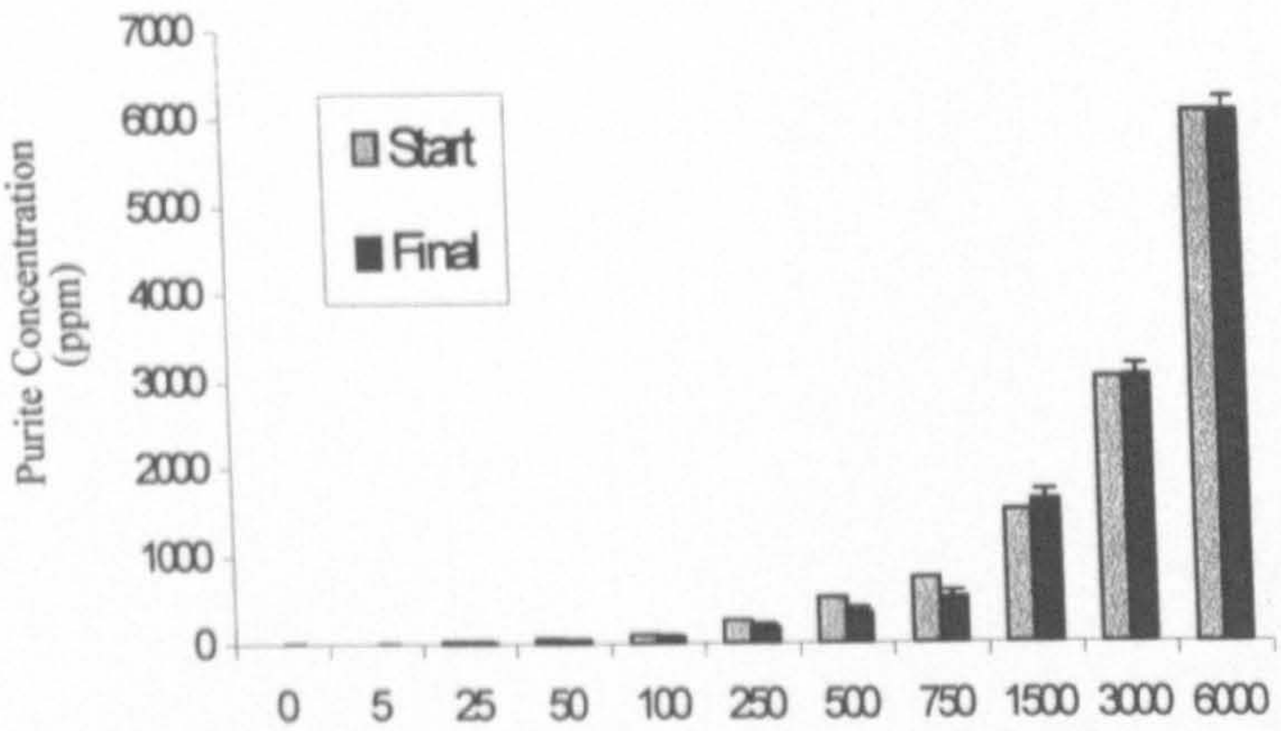


Figure 5.6. Changes in Cellular Glutathione as a Measure of Oxidative Stress Upon Treatment of Microbial Cells with Preservatives. Controls are untreated cells used in calculating the treatment results as a percentage of the Total Glutathione (GSH+GSSG) compared to control (+/- SEM).





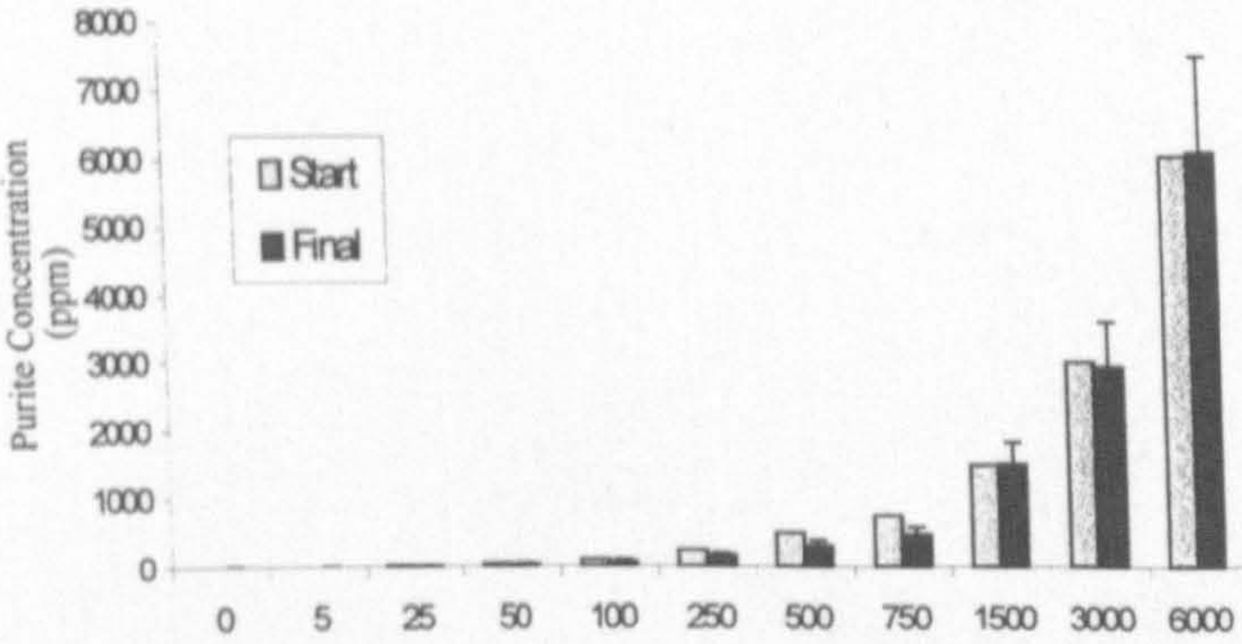


Figure 5.7. Changes in Media Purite™ Levels Upon Treatment of Microbial Cells for Six Hours. Controls are untreated cells (0ppm). Graphs show changes in Purite™ from Start to Final *Candida* (Top), *Alternaria* (Middle) and *Pseudomonas* (Bottom) cell treatments (+ SEM).

Statisitical Analysis of Dose Response Data.

Mann Whitney Test.

GSH Response: Significance at P ≥0.05

Cell Type	Purite ™ to BAK	Purite ™ to H_2O_2
RCE	25, 50ppm	50,100,250ppm
WKD	25,50ppm	250,750ppm
Candida	25,50,100,250,500,750ppm	
Alternaria	25,50,100ppm	
Ps.aeruginosa	5,25ppm	5,25,50,100ppm

ATP Response: Significance at P≥0.05

Cell Type	Purite ™ to BAK	Purite ™ to H_2O_2
RCE	25,50,100,250,750ppm	25,50,100,250ppm
WKD	5,25,50,100ppm	5,25,50,100ppm
Candida	25,100,250,500ppm	500,750ppm
Alternaria	5,25,50,100,250,500ppm	750,1500,3000,6000ppm
Staph.aureus	25,50,100,250,500ppm	25,50,100,250,500 750,1500,3000,6000ppm
Ps.aeruginosa		5,25,50,100,250,500, 750,1500,3000,6000ppm

Table 5.1 Showing significant treatments in GSH dose response curves (above) and ATP cytotoxicity dose respone curves (below).

Discussion

Experimental studies on a cell model *in vitro* are generally regarded as the most sensitive means of testing the effects of a compound (Tripathi *et al.*, 1992). We have found that the ATP luciferin-luciferase bioluminescence assay was a reliable and useful technique for the comparison of cytotoxicity between the different cell types. The microbial results of this chapter were validated by, and found to be comparable, to the dilutional plating methodology previously employed in previous chapters. They also agree with the recent work of Geerling *et al.* (2001) for assaying natural tear substitutes on primary human corneal cells. Furthermore, researchers have shown WKD and RCE to be good model systems for ocular research in pharmaceutical interaction and cytotoxicity. De Saint *et al.* (1999) tested the effect BAK had upon the expression of apoptotic markers in WKD. They incubated WKD with BAK in range of 1-1000ppm solutions for 10 minutes and studied the continuing effects on viability, p53, DNA content and Fas proteins over the next 72 hours. Use of RCE cultured cell lines has been reported by Wang *et al.* (2001) who investigated the effect of BAK preserved and unpreserved isopropyl unoprostone upon trans-epithelial electrical resistance.

Overall it is clear from the results that Purite™ is the least damaging preservative in terms of its depletion of glutathione and cytotoxicity in both ocular cell lines. Although at concentrations of 250 ppm and above it did prove slightly more aggressive than hydrogen peroxide to WKD glutathione depletion. From the results of statistical analysis, as seen in Table 5.1, compared to Purite™ the pattern of cytotoxic response was significantly different to H_2O_2 and BAK ($P>0.05$) in all cell types tested, except between Purite™ and BAK in *Pseudomonas*. Purite™ has not been researched in such a way previously. However, Simmons *et al.* (1991) did test an oxy-chloro disinfectant on explant corneal cells and found similar levels of low toxicity using exclusion assays. Of the treatments tested, BAK was observed to cause the most cytotoxic effects on ocular tissue. This agrees with the results reported in the clinical research of Katz (2002). In this study a comparison of long-term effects (12 months) of the anti-glaucoma drug, bromidine, were evaluated with either Purite™ or BAK as the preservative with patients. The bromidine-Purite™ preserved

formulation was found to have a favourable patient safety and tolerability profile (n=383) compared to bromidine-BAK (n=383) with a reduced incidence of allergic conjuctivitis and better satisfaction and comfort rating. The relative toxicity of BAK and H_2O_2 to ocular cell lines has been extensively researched. Geerling *et al.* (2001) found that natural substances, such as saliva and serum, formulated as tear substitutes were more toxic to cultured corneal cells than pharmaceutical formulations. Geerling *et al.* also reported that at 100ppm BAK preserved formulations were more toxic than unpreserved treatments when cellular cytotoxicity was measured by the highly sensitive ATP luciferin-luciferase luminescence method. Furthermore, De Saint *et al.* (1999) observed BAK induced cell death in WKD at 1ppm exposures for 10 minutes. This complements our findings of total cell death at 5ppm. Tripathi *et al.* (1992) evaluated the effects of 10ppm Thimerosal, 100ppm BAK, 50, 30 and 1ppm H_2O_2 upon human corneal primary cell lines. This study found all treatments to be toxic to *in vitro* cultures over 5 hours.

Purite™ was more effective at damaging glutathione metabolism in the micro-organisms than hydrogen peroxide. These results support the observations of the Chapter 3 NMR studies where the incubation of GSH with Purite™ and H_2O_2 showed Purite™ had a higher affinity for oxidising GSH. Resistance to H_2O_2 in the microbial cells could possibly be due to up-regulated levels of catalase in these cells as a stress response. Other researchers have investigated catalase expression and described the controlled up-regulation under H_2O_2 stress in *Candida albicans* (Nakagawa *et al.*, 1999; Frederick *et al.*, 2001), *Staph. aureus* (Horsburgh *et al.*, 2001) and *Pseudomonas aeruginosa* (Ochsner *et al.*, 2000). Higher catalase levels have been reported in the conjunctiva than in the cornea (Mayer, 1980) and this may be responsible for the observation that WKD cells had relatively higher resistance to glutathione depletion following treatment with hydrogen peroxide than RCE.

The response of *C. albicans* to the oxidative preservatives, by the observed up-regulation of glutathione levels, over 100% (Figure 5.6), in sub-lethal treated cells, strongly suggests that both Purite™ and hydrogen peroxide act by inducing oxidative stress and damaging the cell. Whether the type of oxidative stress Purite™ and H_2O_2 induced is via a similar mechanism is not clear. However, the differences observed in the glutathione response of *Alternaria* to Purite™ and hydrogen peroxide

suggests that it might not be. Other researchers have shown that sodium chlorite can produce singlet oxygen and the hydroxyl radical in the presence of lactic acid (Bagchi *et al.*, 1992). The results suggest that the increase in glutathione levels, presumably through up-regulation of the enzymes of glutathione metabolism, may contribute a mechanism of resistance to the oxidising preservatives. The results of the viability dose response curves support this point, and provide more evidence that maintaining intracellular glutathione determines the viability of the cells under Purite ™ stress.

Many different pathways of microbial oxidative stress responses have been reported in both the microbes studied here and other model bacterial systems. The changes in glutathione production during yeast to mycelium conversion in *Candida* under stress have been reported by Manavathu *et al.* (1996). They observed that although glutathione levels were modulated down, glutathione reductase, peroxidase and S-transferase remained normal. Moreover, academic research into the genetic expression of transcriptional activators of anti-oxidant pathways of microbes has been well researched.

Carbiscol *et al.* (2000) has described the response of *Escherichia coli* to peroxide and superoxide stress. Prokaryotic cells contain complex proteins which are able to repair some covalent modifications caused by oxidants that alter primary protein structure. The transcriptional activators OxyR and SoxRS are found in an inactive form in most *E.coli* growth phases under autoregulation. Upon oxidative stress, from peroxides and superoxides, OxyR and SoxR/RS become activated respectively. In *E.coli* the OxyR gene controls, among others, the genes encoding for hydroperoxidase (HP-I) (catalase) glutaredoxin, glutathione reductase and a protective DNA-binding protein (Dps). The SoxRS regulon contains at least ten genes, including those encoding for Mn-SOD, glucose-6-phosphate dehydrodrogenase and ferredoxin. One of the most frequent modifications is the reduction of oxidised disulphide bonds. The genetic response enables micro-organisms to resist the damaging effects of toxic agents when they are first pre-exposed to low doses (Davies, 1986).

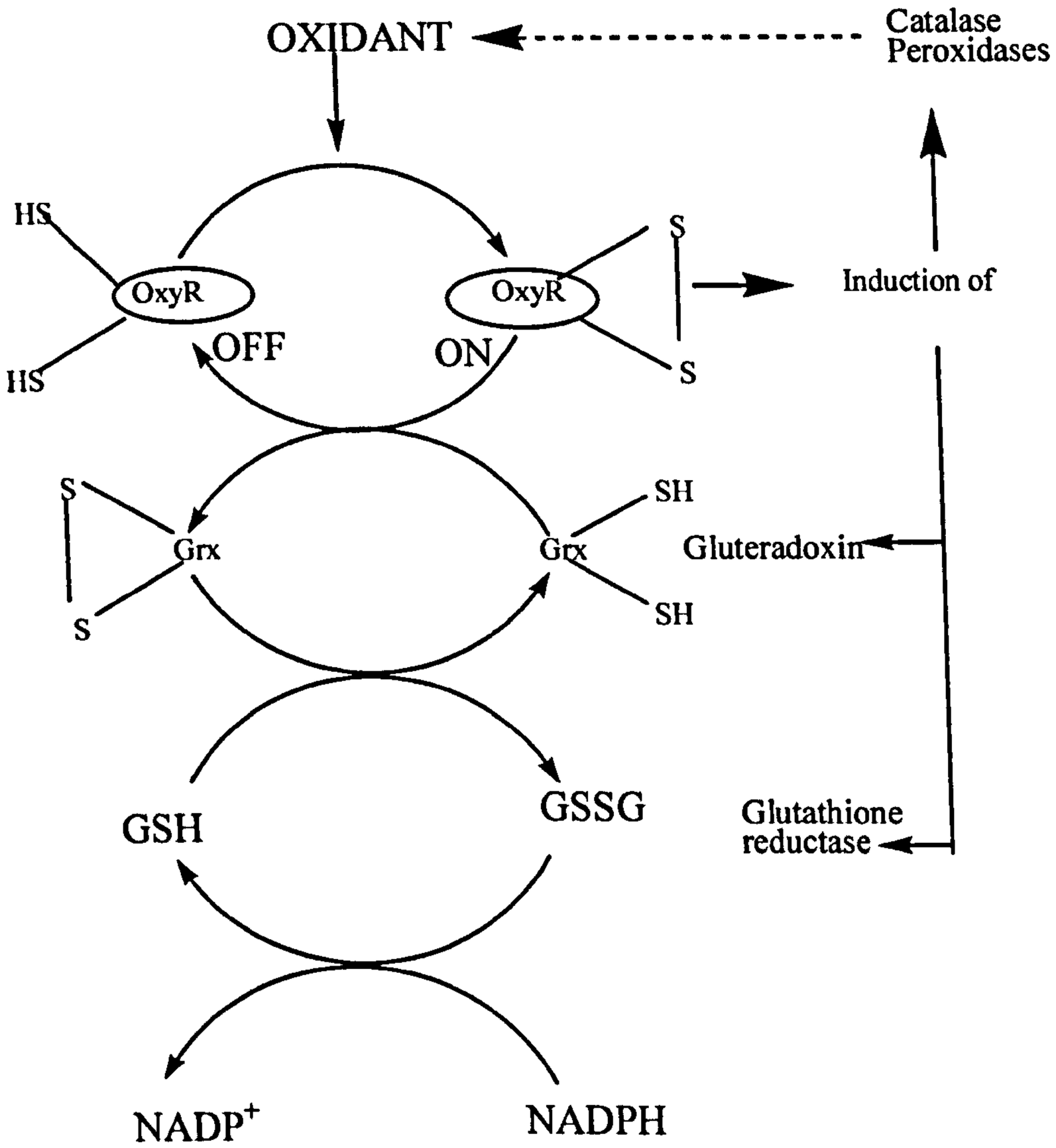


Figure 5.8. Pathways of OxyR activation by oxidative stress induced disulphide bond formation.

Such resistance pathways have been described in *Pseudomonas aeruginosa* (Ochsner *et al.*, 2000), *Staph. aureus* (Horsburgh *et al.*, 2001) and *Candida albicans* (Nakagawa *et al.*, 1999). The pathway is controlled by the formation and reduction of disulphide bonds as a rapid response to oxidative conditions shown in Figure 5.8. However, there has been no reported research on the control of glutathione biosynthesis in microbes under oxidative stress that would have generated the increased cytosolic glutathione in *Candida* and *Alternaria* treated with Purite™ and H_2O_2 observed in this study.

Purite™ may activate transcriptional systems to produce resistance in a cell. Examples of microbes possessing potential resistance pathways that can be up-regulated upon exposure to Purite™ include the nitrate-reducing bacteria. Researchers have isolated over 19 nitrate-reducing organisms that contain a (per)chlorate-reducing pathway. This includes a chlorite dismutase which is described in some bacteria to detoxify chlorite to chloride and oxygen (Coates *et al.*, 1999, Kengen *et al.*, 1999). Although many of these species are facultative anaerobes they were not able to use the nitrate-reducing pathway for energy production, they may still use oxygen as a terminal electron acceptor. Thus, alike to the microbial strains used, here all chlorite dismutase positive organisms were capable of aerobic respiration. However, it is not known if the microbes used in this study had a chlorate-reducing pathway. Work by Katz and Narkis (2001), on removal of by-products of chlorine dioxide disinfection found that chlorate and chlorite could be chemically removed non-enzymatically from water using ferrous salts. The reaction they proposed meant that the molar ratio in the reaction was $1ClO_2^-$ to $3.31Fe^{2+}$ for complete chlorite reduction in the reaction shown below :

$$4\,Fe^{2+} + ClO_2^- + 10\,H_2O \rightarrow 4\,Fe(OH)_3 + Cl^- + 8\,H^+$$

From the results of Purite™ uptake it appears that this reaction does not occur *in vivo.* The results of the Purite™ depletion measurements in treatment media showed that Purite™ was not depleted by *Alternaria*, in contrast to significant depletion by *Candida*, the most resistant micro-organism, and the mammalian cells RCE and WKD. Researchers have suggested that microbial resistance may also be, in part, a result of a slower metabolic rate of a specific organism (Hugo, 1967). If this was partially responsible for the relative resistance of *Alternaria* it may help explain the lack of Purite™ uptake and viability at high Purite™ concentrations. It was observable that in most organisms Purite™ consumption results showed a similar pattern. Prior to performing this study, it could have been proposed that at increasing Purite™ concentrations (above toxic levels) a lower percentage of the total amount would have been consumed. However, the same percentage of Purite™ continued to be removed from the media throughout, and the percentage of removal was specific to each organism. One possible reason for this is that each organism uses a

ubiquitous pathway or substance to convert Purite™ to another product up to an equilibrium point proportional to the native amount of that cytosolic reagent. Further work into the chemistry of Purite™ reactions in the presence of cells would be required to determine how the Purite™ is depleted and whether other chlorine compounds are produced. However, it appears that the native glutathione concentration correlates well to the relative amounts of Purite™ depletion by the different cell types tested.

The different patterns of dose-dependant resistance to glutathione depletion and cytotoxicity between the cell types tested here suggest that there may be more than one single mechanism of cellular resistance to Purite™. The dose response curves obtained provide a strong platform to investigate the role of specific biochemical pathways in Purite™ resistance. The effects of metabolic inhibitors, especially of the antioxidant pathways, could further elucidate the specific mechanisms of Purite™ interaction in these cells. These studies have been performed and can be found in Chapter 6.

Elucidation of the specific biochemical pathways that generate an organisms resistance to Purite™ will enable researchers to attempt to improve the effectiveness of Purite™ as a preservative, and to increase our understanding of the low toxicity in mammalian cells. This may eventually allow its application as a disinfectant in addition to enhancing the preservative capabilities of Purite™ formulations when used in combination with other synergistic compounds.

Chapter 6:

The effect of glutathione metabolism inhibitors on cellular cytotoxicity response to Purite™.

Introduction.

From the observations and the results of investigations in the previous chapters evidence showed that the antioxidant pathways offer species specific resistance to the cellular cytotoxicity induced by Purite™. Chapter 4 showed the presence of cytosolic glutathione in test organisms exhibiting increased resistance to Purite™. Furthermore, from the results of Chapter 5, cytotoxic effects were preceded by the loss of cytosolic glutathione from the test organisms, with increasing Purite™ concentration. This chapter aims to evaluate the relative role that various specific antioxidant pathways play in Purite™s resistance/detoxification in each test organism.

Depletion of Glutathione by Inhibitors.

One of the objectives of this study was to inhibit components of glutathione metabolism using specific inhibitors thus allowing the assessment of their importance in the organisms' resistance to Purite™. Many researchers have described the biochemical pathways of glutathione metabolism. Pathway specific inhibitors have been identified and their effects evaluated. An outline of the reaction pathways, and the sites where inhibitors of interest act, are shown in Figure 6.1.

The inhibitor buthionine sulfoximine (BSO) is well characterised as an inhibitor of glutathione synthesis. It reacts irreversibly with glutamate cysteine lyase, the first enzyme on the pathway of glutathione biosynthesis, which is thought to be the rate-limiting step of synthesis (see Figure 6.1, Haddad, 2000). This inhibitor has been used extensively in research on mammalian cells and also on some micro-organisms (Tokutake *et al.*, 1998; Baker *et al.*, 1996). Glutathione reductase is responsible for regenerating the reduced form of glutathione (GSH) in the

glutathione redox cycle. Carmustine (1.3-bis(2-chloroethyl)-1-nitrososurea/BCNU) inhibits the enzyme glutathione reductase (Starke *et al.*, 1985), resulting in accumulation of oxidised glutathione (GSSG) which has limited anti-oxidant capabilities, as shown in Figure 6.1.

In addition to studying the importance of glutathione in Purite™ resistance, the role of catalase, a ubiquitous enzyme antioxidant that detoxifies hydrogen peroxide, is another possible pathway of cellular Purite™ detoxification. Catalase can be inhibited by aminotriazole, as shown in Figure 6.1.

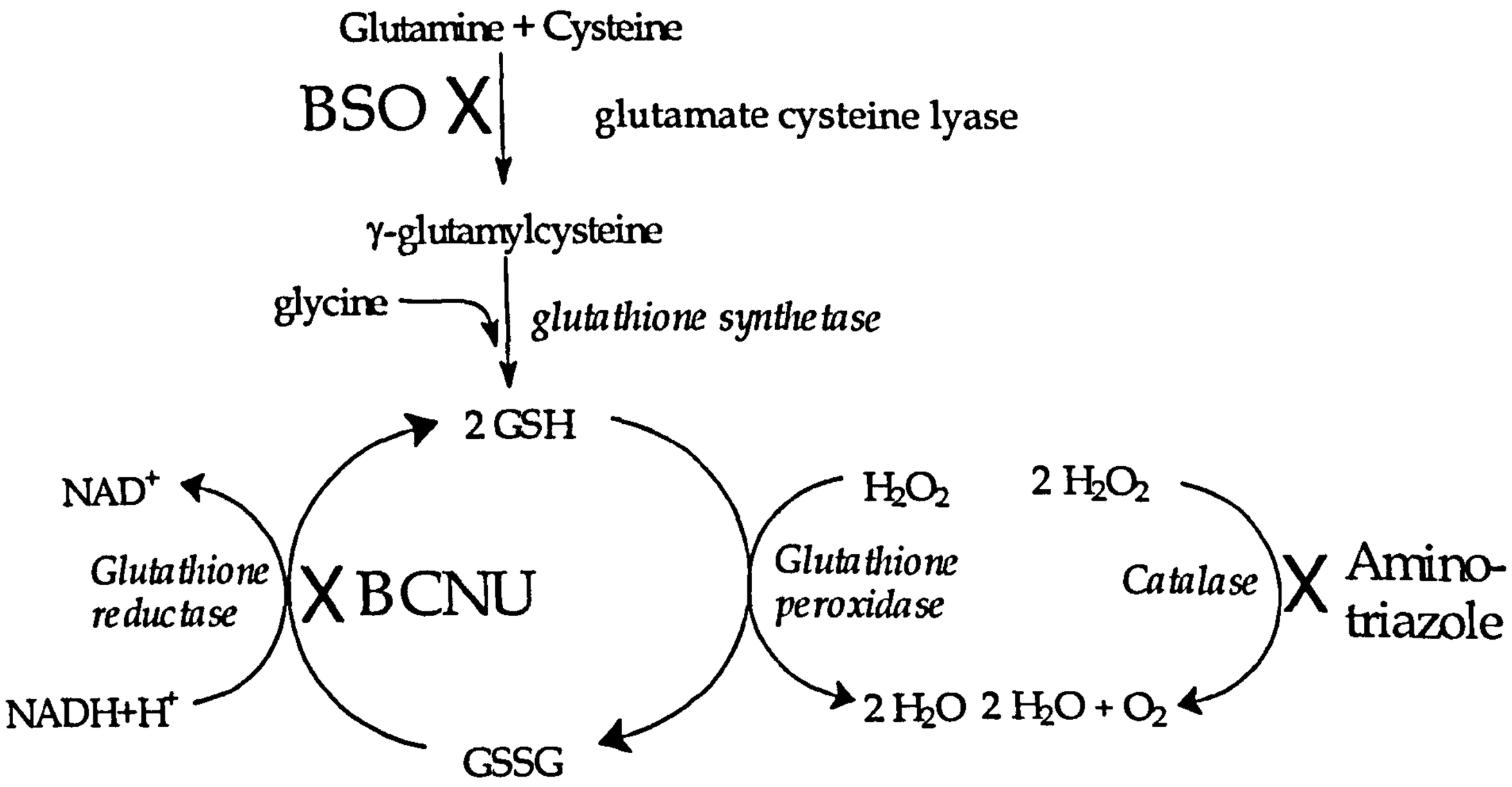


Figure 6.1. Biochemical pathways of antioxidant metabolism. The glutathione inhibitors buthionine sulfoximine (BSO) and carmustine (BCNU), and catalase inhibitor aminotriazole are shown.

Experimental Rationale.

From the knowledge that glutathione and catalase are essential for normal aerobic metabolism, it would not have been logical to deplete these bio-molecules completely. However, many researchers have shown that approximately 50% depletion of these molecules can occur in a cell with 100% viability (Lee *et al.*, 2001; Starke *et al.*, 1985). Thus, studying the dose response/cytotoxic effects of Purite™ in the presence of cells with 50% depletion of native cytosolic glutathione and catalase would be desirable. The Purite™ viability dose response curves obtained in Chapter 5 provide a powerful tool for comparing inhibitor effects in the test organisms. The test organisms were selected to represent each type known to contain high concentrations of native glutathione. The cell types selected for investigation were RCE, *Candida albicans, Alternaria spp,* and *Pseudomonas aeruginosa*. The levels of cellular cytotocity induced by Purite™ in inhibited cells could, thus, be compared to control ATP/viability dose response curves for specific effects of each inhibited pathway.

Materials and Methods

Inhibitor Evaluation

Depletion of cellular glutathione by BSO and BCNU.

Sterile 50mM stocks of BSO and BCNU (Sigma, UK) were made in sterile PBS, pH 7.4, and ethanol (Fisher, UK). For cell treatments, ten times final concentration stocks were made from 50mM-0.2mM of each treatment fresh on the day of experimentation.

RCE cells were grown to 90-100% confluence in 75cm^2 tissue culture flasks and then washed with sterile PBS. 10mL of fresh, serum free, culture media, containing the inhibitor test solution, was added to the cells in the tissue culture flasks. These were incubated for 6 hours at 37°C. Adherent (viable) cells were detached using Acutase (icT, California, USA) then washed twice with PBS in a pre-weighed universal tube. Wet cell weight was recorded for each pellet. Cell cytosolic contents were extracted by the addition of 200µL 5% trichloroacetic acid (TCA) to precipitate cellular protein, followed by freeze thawing and centrifugation to remove the cell debris. All samples were centrifuged for one minute at 10,000 × g (in a microfuge). Supernatants were stored at 4 °C and assayed within two hours.

Pseudomonas aeruginosa and *C. albicans* were grown for 18 hours at 37°C in 2.5L conical flasks. Bacteria were cultured in 250mL of TSB and fungi in 250mL of YEPD broth. *Alternaria* was grown at 26°C in 2.5L conical flasks in 250mL of YEPD. Cultures were washed twice in PBS and resuspended to 1x10^{8}cells/mL (or 0.01g/mL) in 10mL of inhibitor treatments in sterile 50mL centrifuge tubes. Test organisms were incubated for 6 hours at their original culture temperature in the dark. After incubation cells were centrifuged and washed twice in PBS in pre-weighed universal tubes. The final wash solution was removed, and the cell pellet weight calculated. The cell contents were extracted by cell membrane disruption as described in Chapter 4. Supernatants were stored at 4°C and assayed within two hours.

Essentially, RCE, *Candida albicans, Alternaria spp,* and *Pseudomonas aeruginosa* cells were prepared in a manner described above then treated with inhibitors, both singularly and in combination, for six hours under the same conditions as Chapter 5 (glutathione dose response protocols).

Glutathione Assay.

Post incubation cells were harvested and assayed for glutathione by the methodology first described in Chapter 4. Glutathione concentration was calculated in micro-moles per g of cells extracted.

Dose response curves of glutathione against inhibitor dose were plotted and experiments stopped when the concentration of inhibitor required to reduce native levels of glutathione by 50% had been determined.

Depletion of cellular catalase by aminotriazole.

The principle of this bio-assay was to elucidate the aminotriazole concentration, in each specific organism, required for a previously sub-lethal hydrogen peroxide concentration to cause 50% toxicity. Thus, the determination of 50% catalase depletion would be achieved.

Fresh sterile 1M stock of aminotriazole (Fluka, UK) was made in PBS pH 7.4 for each day's experimentation. For cell treatments, two times final-concentration of working-stocks were made from 1M-10mM for each treatment.

RCE, *Candida albicans, Alternaria spp,* and *Pseudomonas aeruginosa* cells were washed and prepared for inhibitor treatments in the presence of hydrogen peroxide at the organism-specific concentration that was shown to be just sub-lethal in the cytotoxicity studies of Chapter 5. The final concentrations of hydrogen peroxide used in these catalase inhibitor studies were 5ppm for RCE, 100ppm in *Candida albicans,* 250ppm in *Alternaria spp,* and 1000ppm in *Pseudomonas aeruginosa.*

RCE cells were seeded at $1x10^4$ cells per well in 100µL of media on 96 well µClear luminometer plates (Greiner, UK) and allowed to adhere overnight. Additional wells on the plate were seeded with serial dilutions, of the $1x10^5$ cells per

mL stock, of 100μL to generate a calibration curve. Culture media was removed and cells were washed with PBS. 100μL of aminotriazole (1-750mM) in serum free media and 5ppm hydrogen peroxide were placed in specific wells and incubated for 6 hours at 37°C in the dark. Treatments were removed and cells washed with PBS by vacuum manifold (Pall, UK) filtration. 100μL of sterile PBS was placed in each well to re-suspend the cells

Candida, Psuedomonas, and *Alternaria* were grown, treated, and incubated with the catalase inhibitor in the same manner described for glutathione analysis (above). 10mL of the micro-organisms at 0.01g/mL, in 50mL centrifuge tubes were then treated with the inhibitor in the presence of hydrogen peroxide. At the end of the incubation, 100μL of treated culture was placed in wells of a 96 well luminometer 0.2 μm filter plate (Millipore, UK). Additional wells on the plate were seeded with serial dilutions of 100μLs, of the control cells, to generate a calibration curve. Treatments were removed and cells washed with PBS by vacuum manifold (Pall, UK) filtration. 100μL of sterile water was placed in each well to re-suspend the cells.

Catalase Inhibition Bio-assay.

The concentration of aminotriazole required to induce 50% cytotoxicity in the specific test organisms previously exposed to sub-lethal hydrogen peroxide concentrations was assayed by ATP Luciferin/luciferase luminescence.

At the end of the RCE cell incubations, treatments were removed and cells washed with PBS. Well contents were assayed for cellular ATP levels as a measure of cytotoxicity using a CellTitre-Glo^{TM} (Promega, UK) assay kit and a LUMIstar Galaxy (BMG, UK) microplate luminometer as described in Chapter 5.

The washed 96-plate well contents were assayed for microbial cell ATP levels as a measure of cytotoxicity using a ViaLight MDA (Lumitech, UK) assay kit on a LUMIstar Galaxy (BMG, UK) microplate luminometer as described in Chapter 5.

Dose response curves of cytoxicity against inhibitor dose were plotted and the concentration of inhibitor required to reduce viability to 50% of control was determined.

Cytotoxicity dose response of Purite™ in the presence of inhibitors.

Purite™ Dose Response In The Presence Of Glutathione Inhibitors.

The specific concentration of inhibitor required to deplete native glutathione to 50% in each of the test organisms was then used in the Purite™ dose response studies. The final concentrations of inhibitors in these Purite™ treatments were 10μM BSO + 5μM BCNU in RCE, 2mM BSO + 25 μM BCNU in *Candida albicans,* 2mM BSO + 400 μM BCNU in *Alternaria spp,* and 2mM BSO + 200 μM BCNU in *Pseudomonas aeruginosa.*

Purite™ dose response curves of cytotoxicity in the presence of glutathione inhibitors, depleting 50% of native levels, were performed. This followed the same methodology as chapter 5. Post-incubation treatment medium was retained for Purite™ consumption analysis.

Purite™ Dose Response In The Presence Of Catalase Inhibitors

The specific concentrations of the inhibitor required to deplete native catalase to 50% in each of the test organisms was then used in the Purite™ dose response studies. The final concentrations of aminotriazole used in these Purite™ treatments was 400mM in RCE, 100mM in *Candida albicans,* 150mM in *Alternaria spp,* and 450mM in *Pseudomonas aeruginosa.*

Purite™ dose response curves of cytotoxicity in the presence of catalase inhibitor, depleting 50% of native levels, were performed. This followed the same methodology as Chapter 5. Post-incubation treatment medium was retained for Purite™ consumption analysis.

Purite™ Uptake Analysis.

For incubations with Purite™, the loss or consumption of Purite™ was determined by analysis of the Purite™ concentration in the supernatant after harvesting of the cells for glutathione analysis at the end of the treatments. Purite™ concentration was measured by adding 50µL of sample/blank to 50µL of 2% potassium iodide and 1500µL 50mM HCl as described in Chapter 5.

Statistical Analysis

Statistical analysis of results was performed in Minitab using Mann-Whitney U tests. Each dose response was repeated three times and significance level set to $P>0.05$.

Results

Inhibitor Evaluation

Depletion of cellular glutathione by BSO and BCNU.

BSO and BCNU were tested singularly and then in combination. When BSO alone was tested on all organisms, some species showed resistance to glutathione depletion. *Candida* was incubated with BSO concentrations of up to 20 mM with little depletion of the intracellular glutathione level, as observed in Figure 6.2. Similar resistance of microorganisms to BSO induced glutathione inhibition was found with *Alternaria*, *Pseudomonas* (Figure 6.2) and even with the mammalian cell line (data not shown). It was not clear whether the inhibitor was not reaching the cytosol, and was, therefore, ineffective, or if in the absence of applied oxidative stress the cells were able to maintain their glutathione levels via the redox cycle.

Analogous experiments were carried out using the inhibitor of the glutathione redox cycle, carmustine (BCNU). This inhibits the enzyme glutathione reductase, which is responsible for regenerating the reduced form of glutathione (GSH), as shown in Figure 6.1. It was found that this compound in concentrations up to 200μM did cause some reduction in glutathione levels in *Pseudomonas* and *Alternaria*, but these cells still appeared to be very resistant (Figure 6.3). However it was possible to observe glutathione depletion in *Candida* with 100μM BCNU.

Due to the lack of depletion observed in most of the test organisms to singular glutathione inhibitors, combinations of the BSO and BCNU were incubated with cells to determine if inhibiting both pathways overcomes the resistance observed with BSO and BCNU singularly.

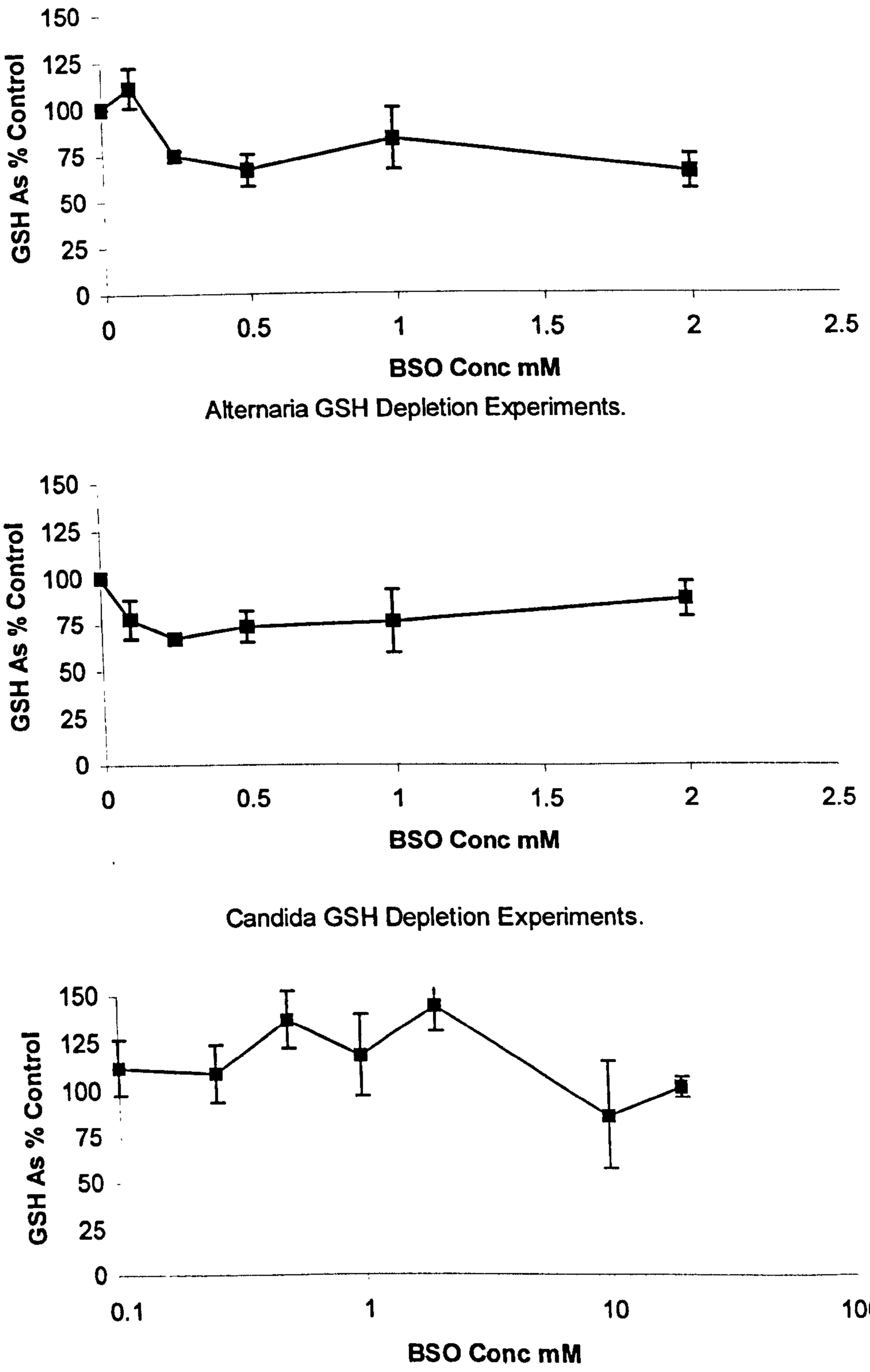
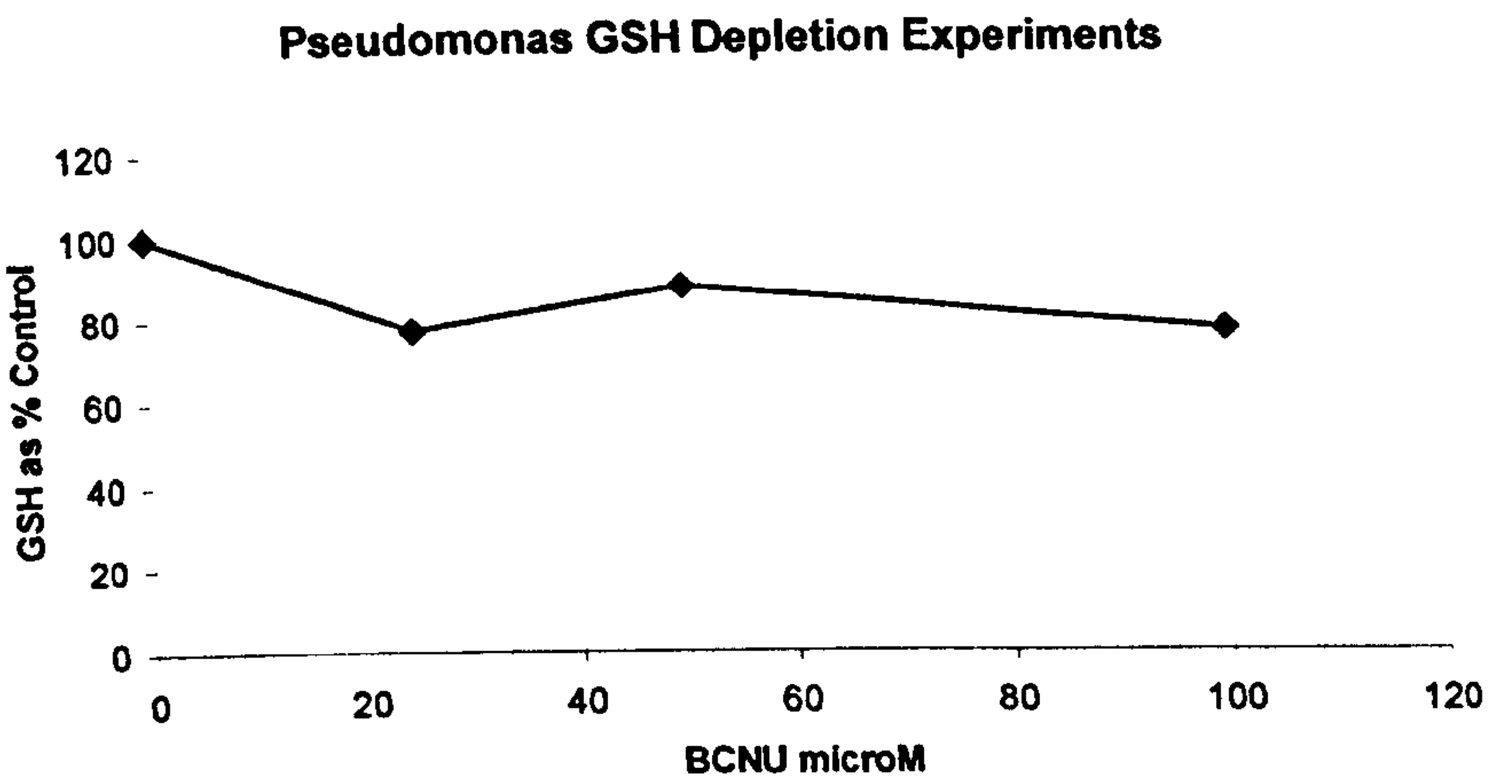
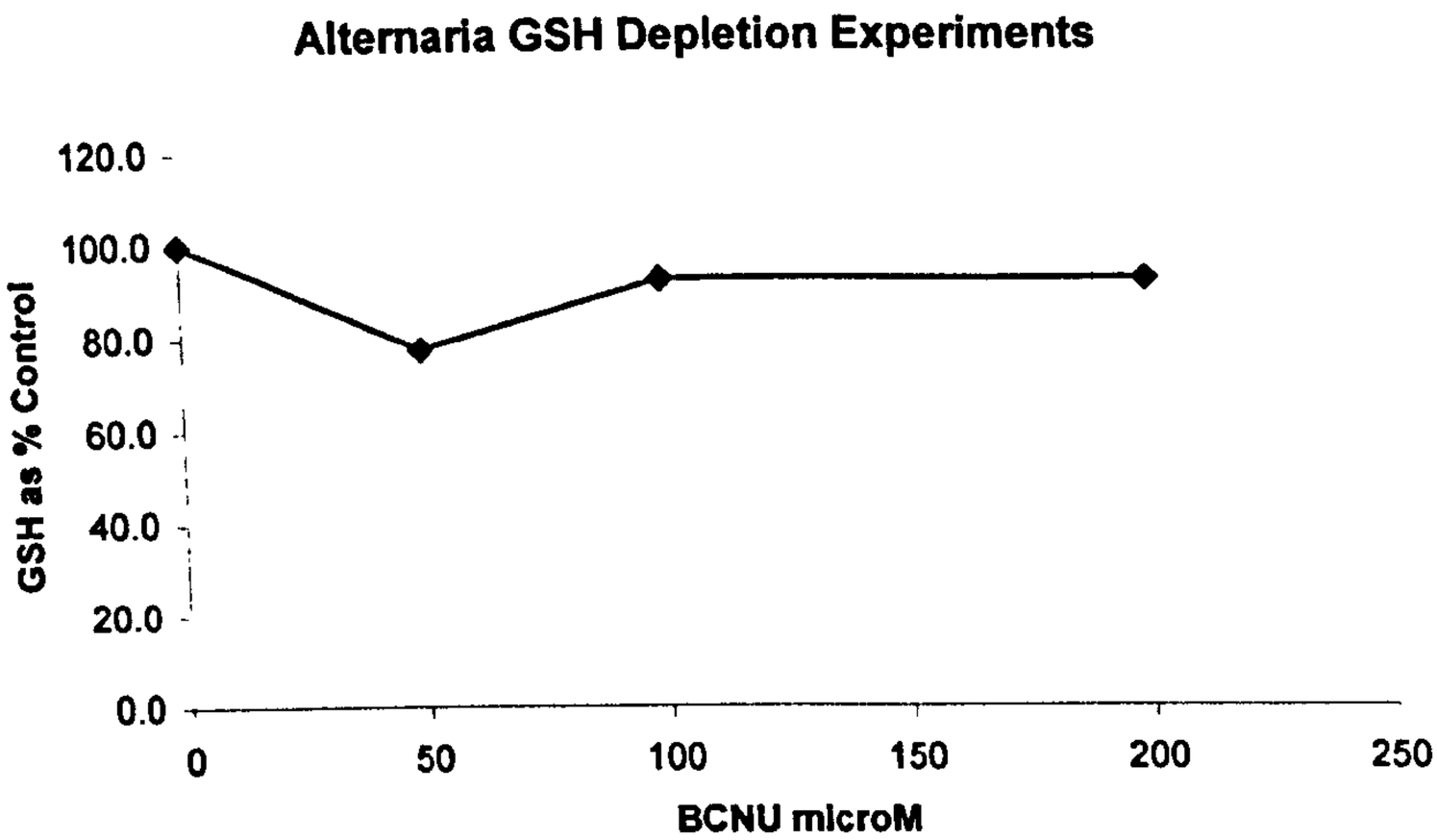


Figure 6.2. The effect of BSO on intracellular glutathione in *Pseudomonas aeruginosa, Alternaria spp.* and *Candida albicans* (n=3).





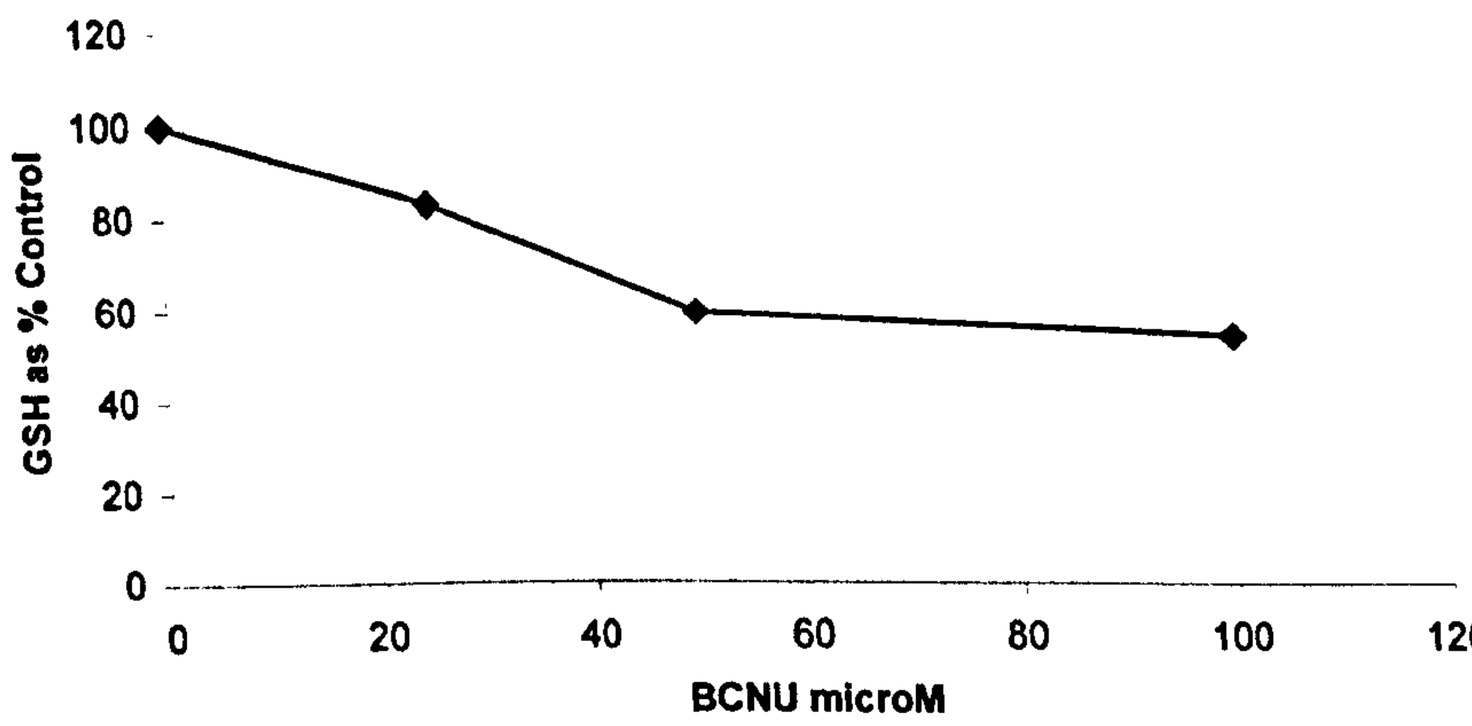


Figure 6.3. The effect of carmustine (BCNU) on intracellular glutathione in *Pseudomonas, Alternaria* and *Candida* (n=2).

The treatment of test organisms with a combination of both inhibitors to determine whether they had a synergistic effect was more successful, and a reduction in the intracellular glutathione levels in all 4 test organisms was observed (Figure 6.4). High concentrations of BCNU were still required to achieve the desired reduction in glutathione (50%) with *Alternaria* and *Pseudomonas*. However, cells were found to be viable even at these concentrations of inhibitors.

For the Purite™ dose response curves, a concentration of 2 mM BSO was used with the varying BCNU concentrations that gave approximately 50% depletion of glutathione, as indicated by the labelled points in Figure 6.4.

The concentrations of inhibitors found to deplete 50% of native glutathione were 2mM BSO + 5μM BCNU in RCE, 2mM BSO + 25μM BCNU in *Candida albicans,* 2mM BSO + 400μM BCNU in *Alternaria spp,* and 2mM BSO + 200μM BCNU in *Pseudomonas aeruginosa.*

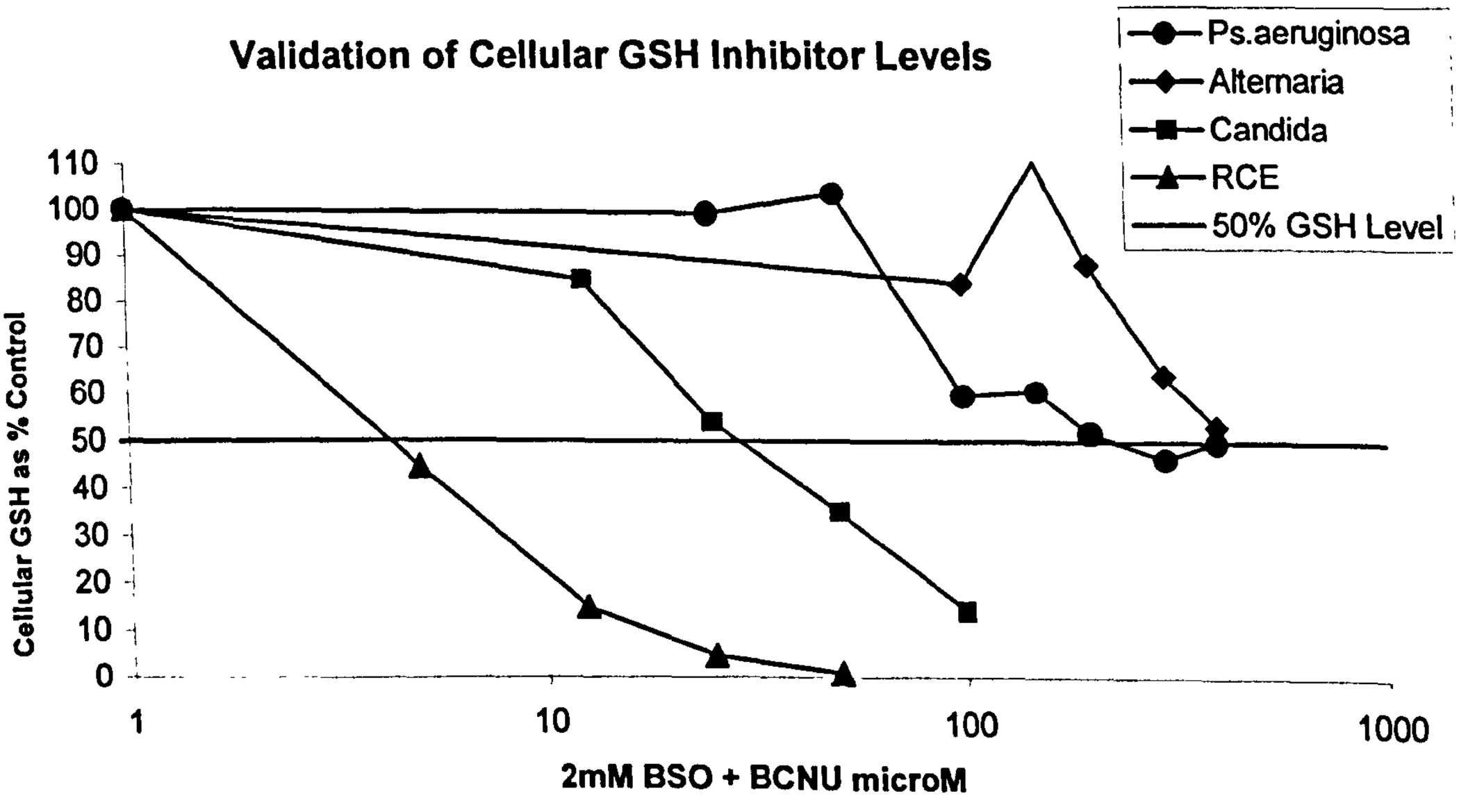


Figure 6.4. Establishment of inhibitor conditions required to give a 50% reduction in intracellular glutathione for various cell types (n=3).

Depletion of cellular catalase by aminotriazole.

The concentration of aminotriazole required to cause a 50% loss of viability when cells were simultaneously incubated with hydrogen peroxide was investigated in RCE, *Candida albicans, Alternaria spp,* and *Pseudomonas aeruginosa.* The results are shown in Figure 6.5. The concentration of hydrogen peroxide chosen was different for each cell type, at the commencement of cytotoxicity (10-25 % loss of viability) in order to give comparable stress levels. It can be seen that RCE cells were quite resistant to catalase inhibition, as 400 mM aminotriazole was required to reduce the viability to 50%. *Candida* was more sensitive to aminotriazole, with 50% loss of viability at 100 mM.

The final concentrations of aminotriazole inhibitor found to deplete 50% of native catalase were 400mM in RCE, 100mM in *Candida albicans,* 150mM in *Alternaria spp,* and 450mM in *Pseudomonas aeruginosa.*

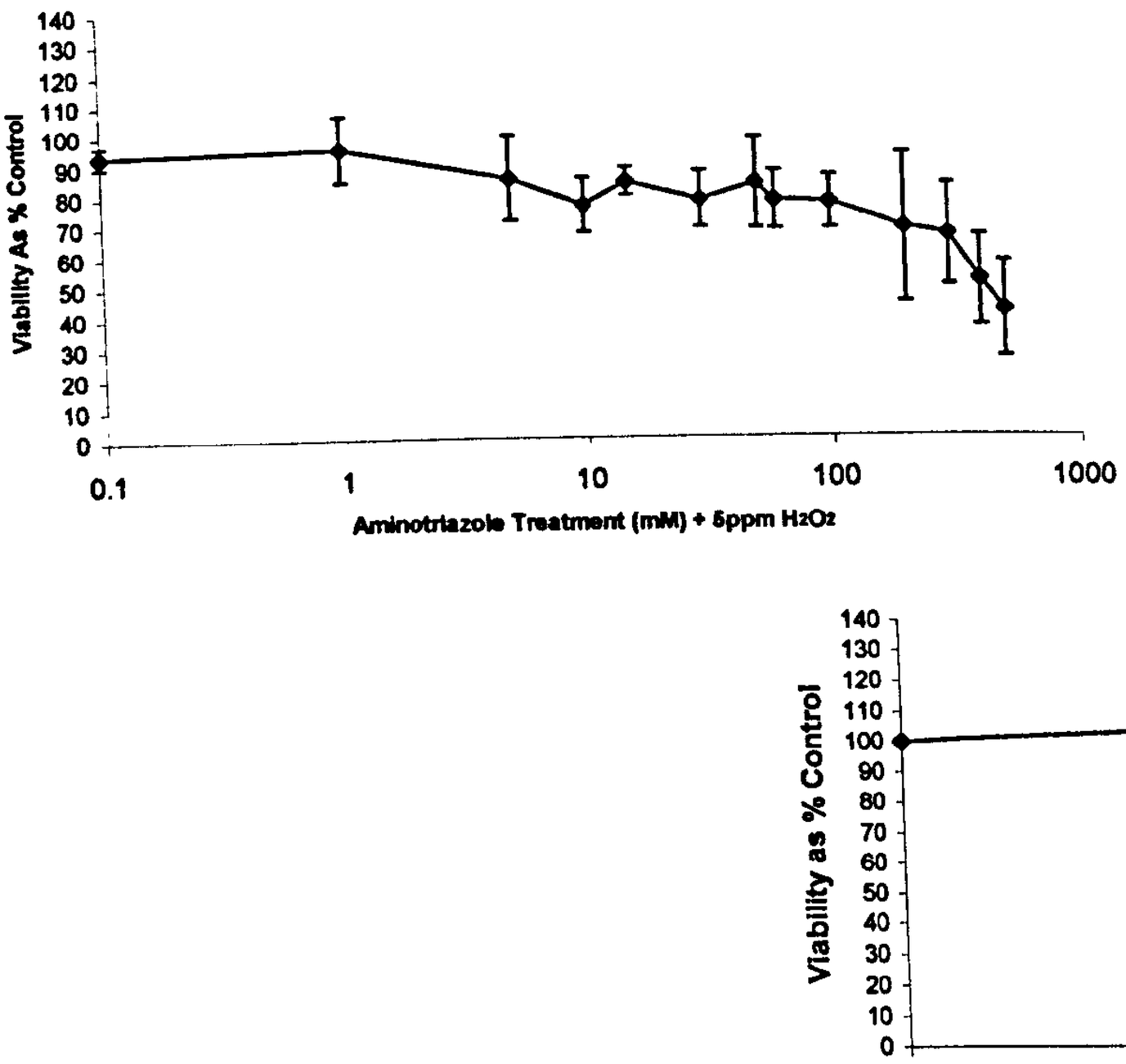


Figure 6.5. The effect of aminotriazole and hydrogen peroxide on cell viability (n=3 +/-SEM)

Cytotoxicity dose response of Purite™ in the presence of inhibitors.

Purite™ Dose Response In The Presence Of Glutathione Inhibitors.

The dose response curves for Purite™ treatment of cells with depleted glutathione are shown in Figure 6.6. As two ATP/cell standard curves were performed for each experiment, with and without inhibitors present, it is possible to conclude that the effect of the inhibitors alone on viability was negligible. It can be seen with all of the cell types tested that the presence of glutathione metabolism inhibitors caused a shift to the left in the response curves, i.e. the viability decreased at lower Purite™ concentrations than in the absence of inhibitors. With RCE and *Candida* cells the inhibitor treatment actually appeared to cause some stimulation of growth or increased ATP levels in the presence of low concentrations of Purite™, but this is not a significant effect (Figure 6.10). One possible reason for the increased viability found in low Purite™ concentrations of inhibited RCE and *Candida* may be that the metabolism of these cells was increased in response to growth arrest. Thus, cytosolic ATP levels may have increased above the uninhibited control levels in the cells undergoing treatment with sub-lethal Purite™. This rise in ATP would explain the apparent lack of toxicity of intermediate concentrations of Purite™ leading to a cross-over of uninhibited and glutathione inhibited viability dose response curves of RCE and *Alternaia* at concentrations causing significantly different levels of cell death. At higher Purite™ concentrations it was apparent that the loss of viability was exacerbated by the inhibitors in all cell types, compared to uninhibited controls. A general trend observed in all of the organisms, except *Pseudomonas*, was that the viability of glutathione inhibited cells exposed to low levels of Purite™ were similar to controls. *Pseudomonas* treated with glutathione inhibitors showed increased toxicity to Purite™ than uninhibited controls even at low Purite™ concentrations. In the other organisms, concentrations of Purite™ over 100ppm were found to generate an increased level of sensitivity in all of the glutathione inhibited cells compared to their controls. These differences were statistically significant (Figure 6.10). Together, the results provide evidence that intracellular glutathione is important in the

resistance of cells to Purite™, and, therefore, it acts as an antioxidant to prevent oxidative damage to the cells, most probably in the cytosol.

During these experiments, the consumption of Purite™ from the medium was also monitored, in order to investigate the role of glutathione in Purite™ disappearance. The results are shown in Figure 6.7. With all of the organisms tested, the Purite™ consumption was decreased by depletion of glutathione from the cells during treatments, although a small amount of Purite™ consumption did still occur with RCE cells and *Pseudomonas* at the lower concentrations tested. The most outstanding difference was observed with *Candida*, which under control conditions consumed a large proportion of the Purite™ present, but which demonstrates almost no consumption following depletion of glutathione. This observation provides further evidence to link the role of native glutathione in the detoxification or metabolism of Purite™.

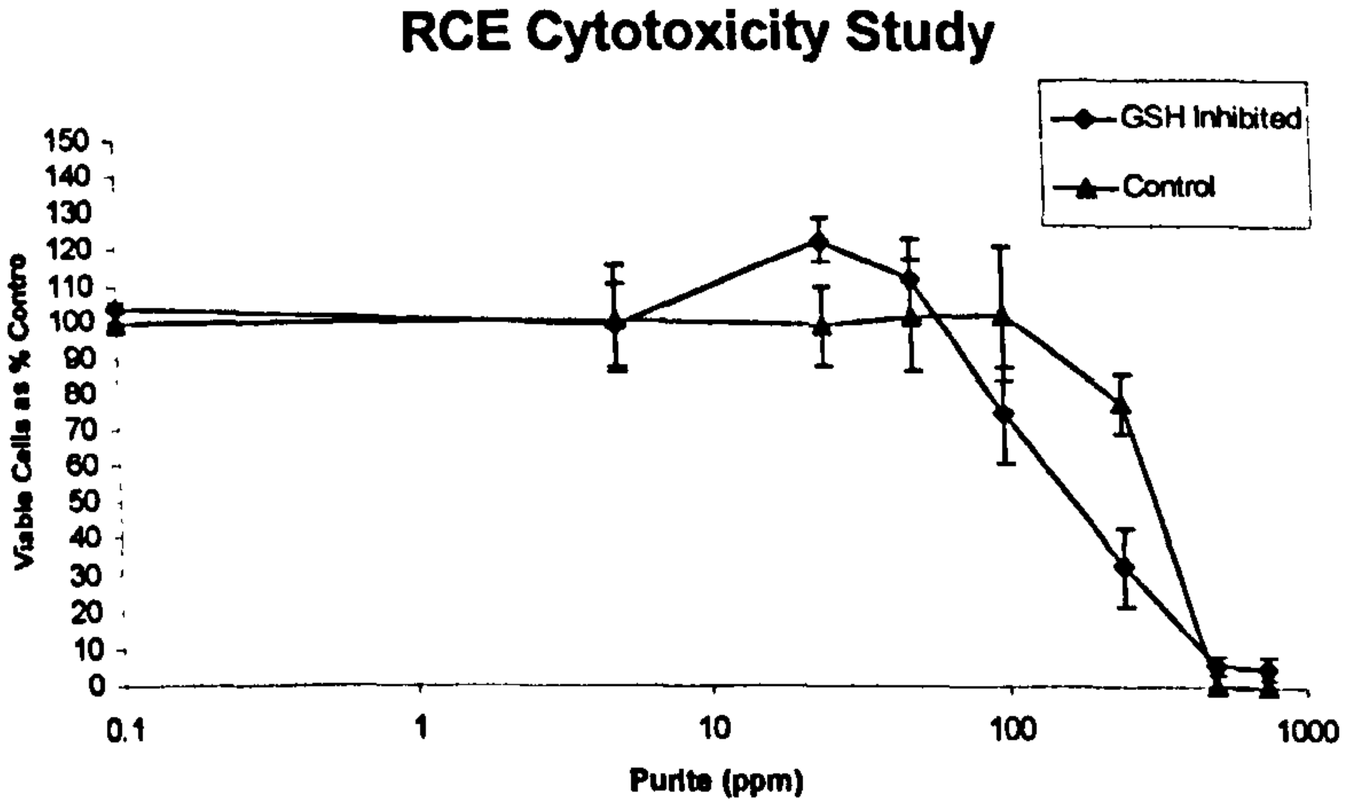


Figure 6.6. The effect of intracellular glutathione depletion on viability dose response curves of cells treated with Purite™. The concentrations of inhibitors were 2mM BSO + 5μM BCNU in RCE, 2mM BSO + 25μM BCNU in *Candida albicans*, (n=3 +/-SEM)

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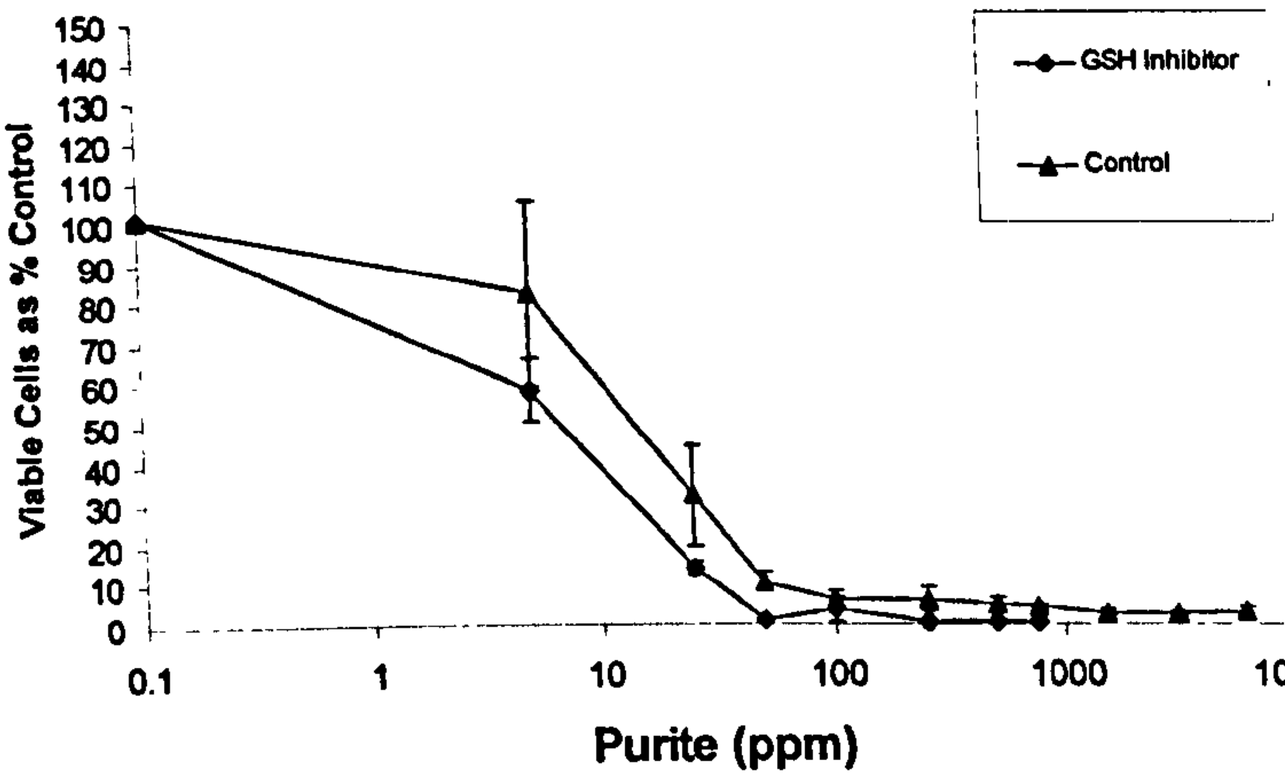


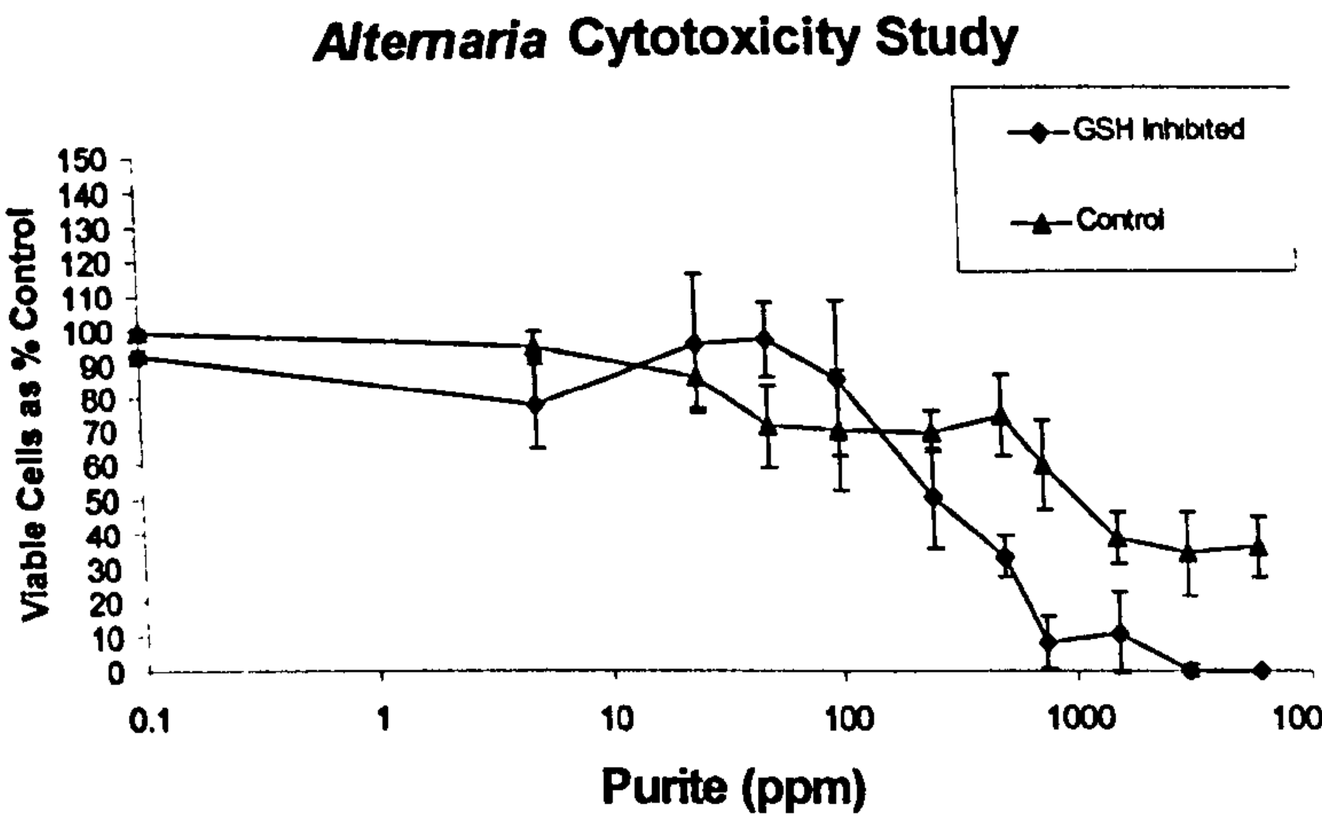
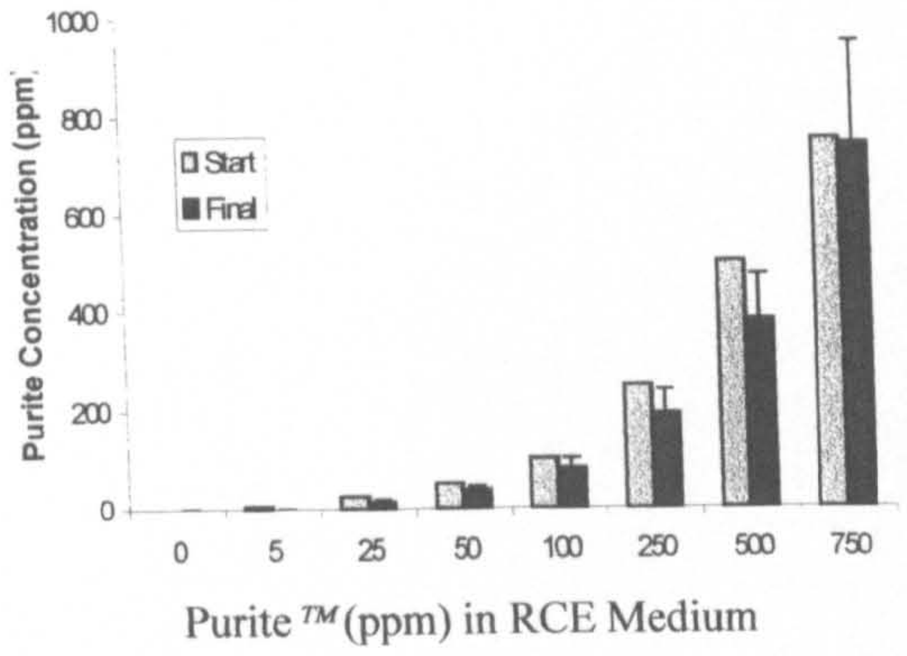
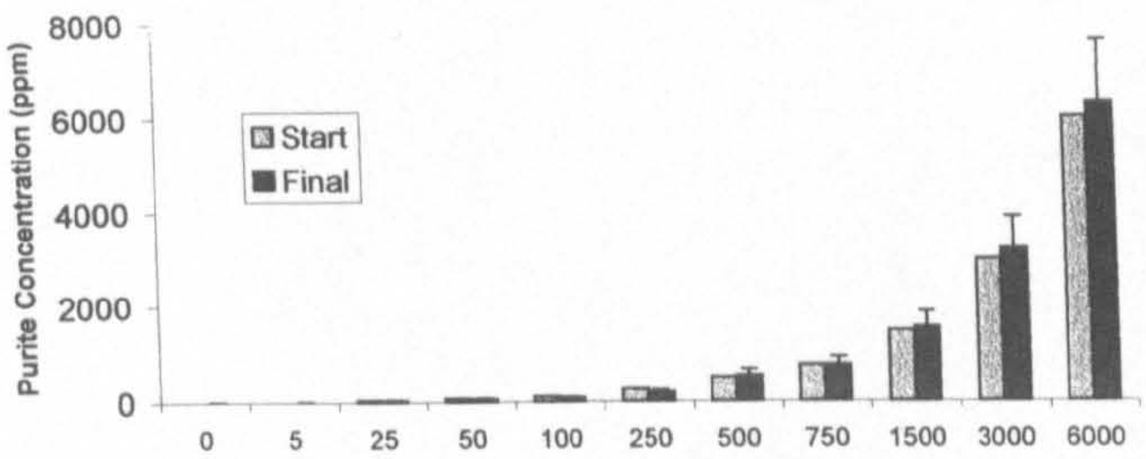


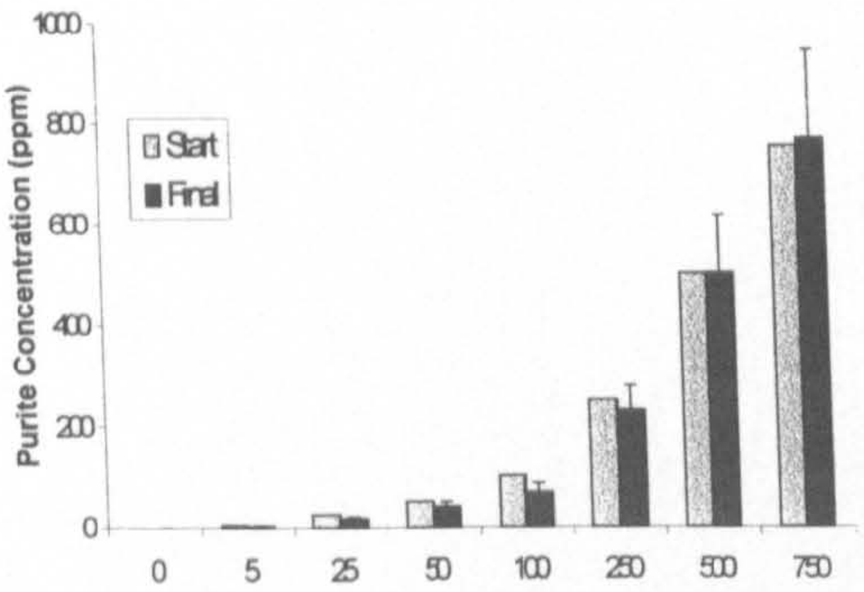
Figure 6.6. The effect of intracellular glutathione depletion on viability dose response curves of cells treated with Purite™. The concentrations of inhibitors were 2mM BSO + 200μM BCNU in *Pseudomonas aeruginosa* and 2mM BSO + 400μM BCNU in *Alternaria spp*,(n=3 +/-SEM)



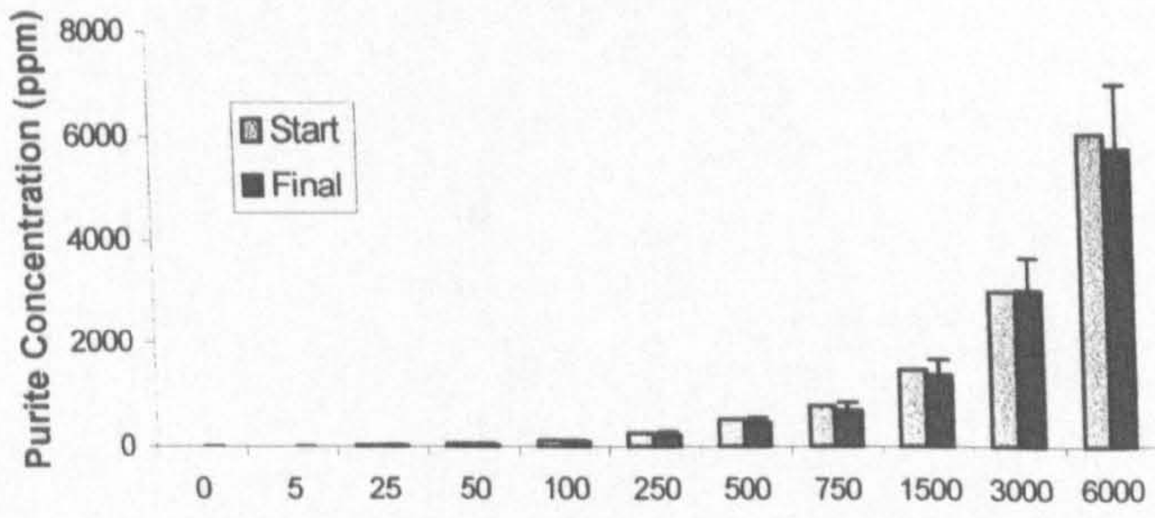
Purite ™ (ppm) in RCE Medium



Purite ™ (ppm) in *Candida* Medium



Purite ™ (ppm) in *Pseudomonas* Medium



Purite ™ (ppm) in *Alternaria* Medium

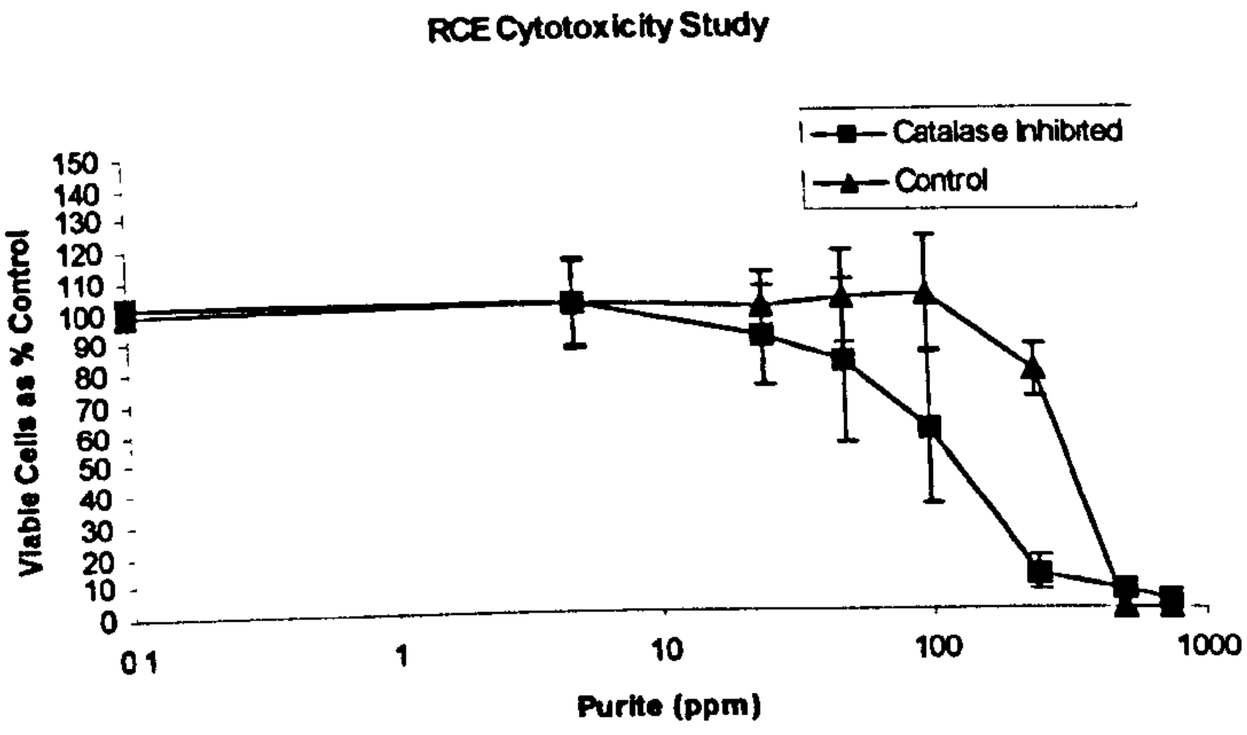
Figure 6.7. Changes in Media Purite ™ Levels Upon Treatment of Glutathione Depleted Cells for Six Hours. Controls are untreated cells (0ppm). Graphs show changes in Purite ™ from Start to Final. (n=3 + SEM).

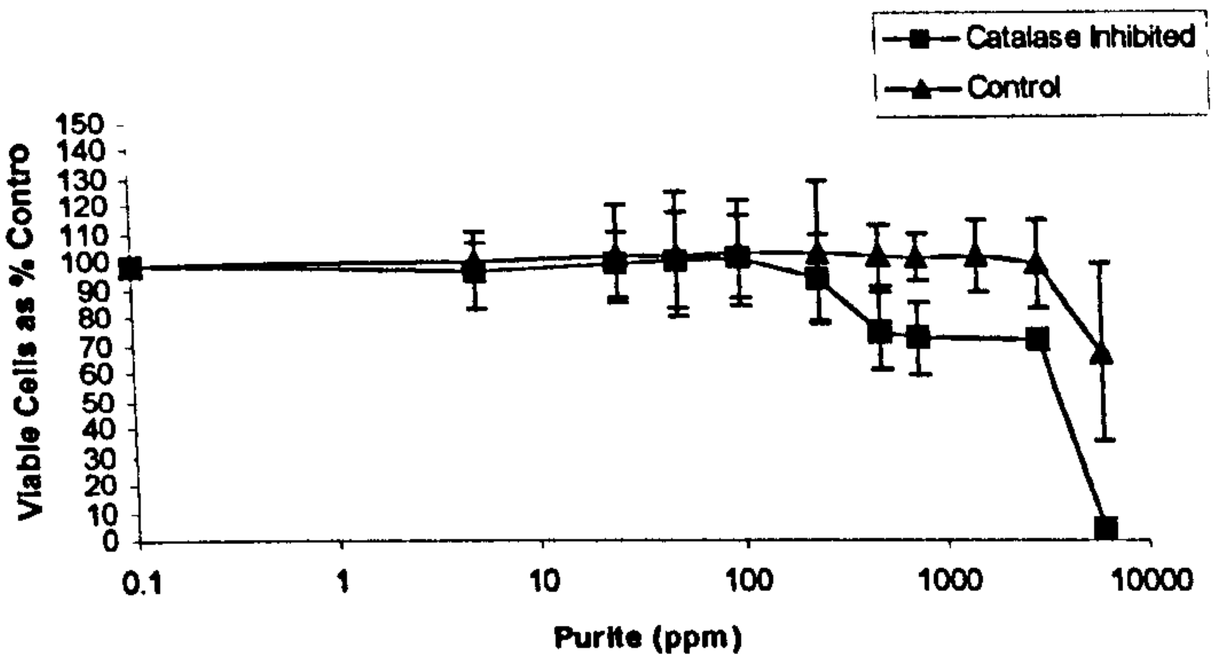
Purite™ Dose Response In The Presence Of Catalase Inhibitors

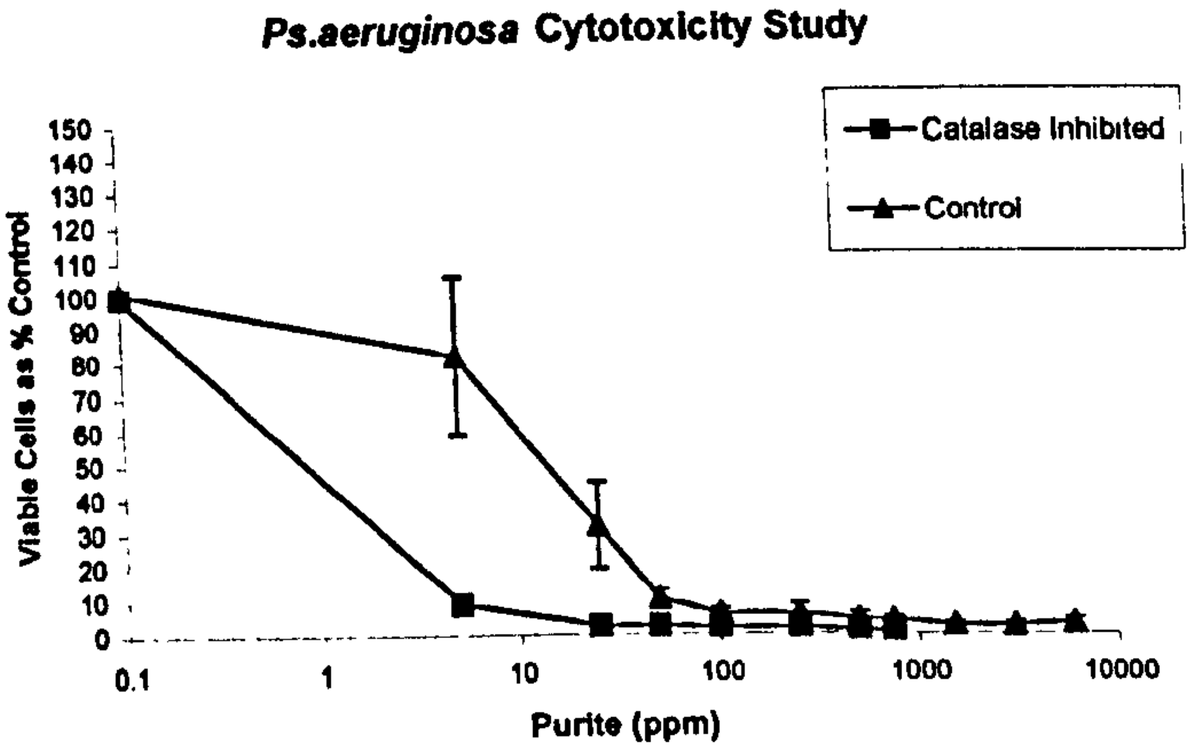
The dose response curves for Purite™ treatment of cells with depleted catalase are shown in Figure 6.8. Again, two ATP/cell standard curves were performed for each experiment, with and without inhibitor present, it was possible to conclude that the effect of the inhibitors alone on viability was negligible. It can be seen with all of the cell types tested that the presence of catalase inhibitors caused a shift to the left in the response curves. This indicates that the viability decreased at lower Purite™ concentrations than in the cells without depletion of catalase.

The inhibition of catalase significantly increased the sensitivity of RCE and *Pseudomonas* cells to Purite™ treatments as the catalase inhibited cell viability can be seen to decrease at lower Purite™ concentrations. Similar results were obtained with *Candida*, although the sensitising effect was perhaps not so clear. However, overall it can be concluded that catalase was also able to offer protection against Purite™, thus reflecting the oxidative nature of Purite™'s action. Inhibition of catalase in *Alternaria* showed little effect on viability and the increased sensitivity of catalase inhibited cells was only significant at 6000ppm.

Again, during these experiments the consumption of Purite™ from the medium was also monitored, in order to investigate the role of catalase in Purite™ disappearance, and the results are shown in Figure 6.9. With all of the organisms tested, the Purite™ consumption was not inhibited by depletion of catalase from the cells during treatment. This observation provided evidence to suggest that catalase did not play a role in the detoxification or metabolism of Purite™ and supported the idea of a more specific role for glutathione.







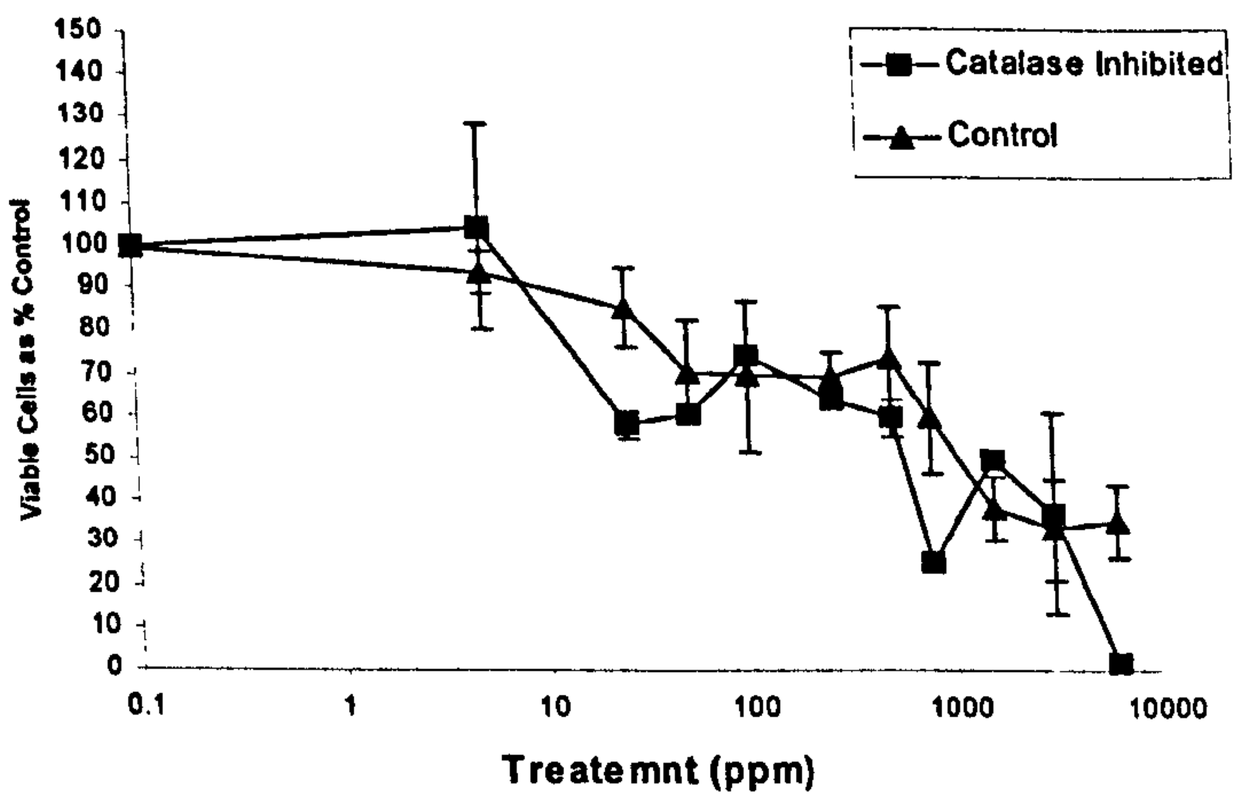
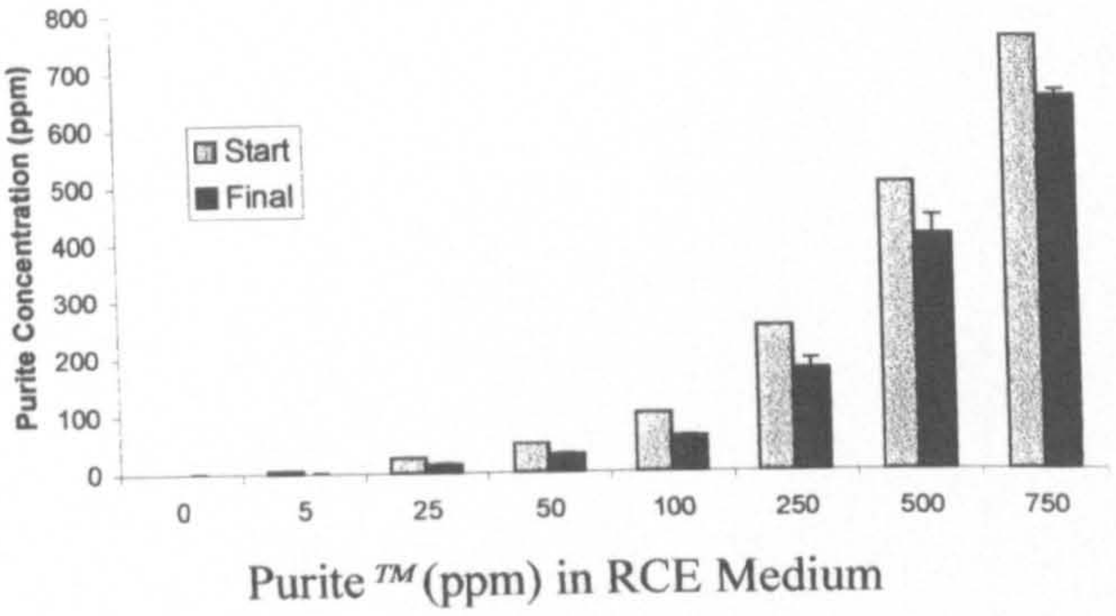
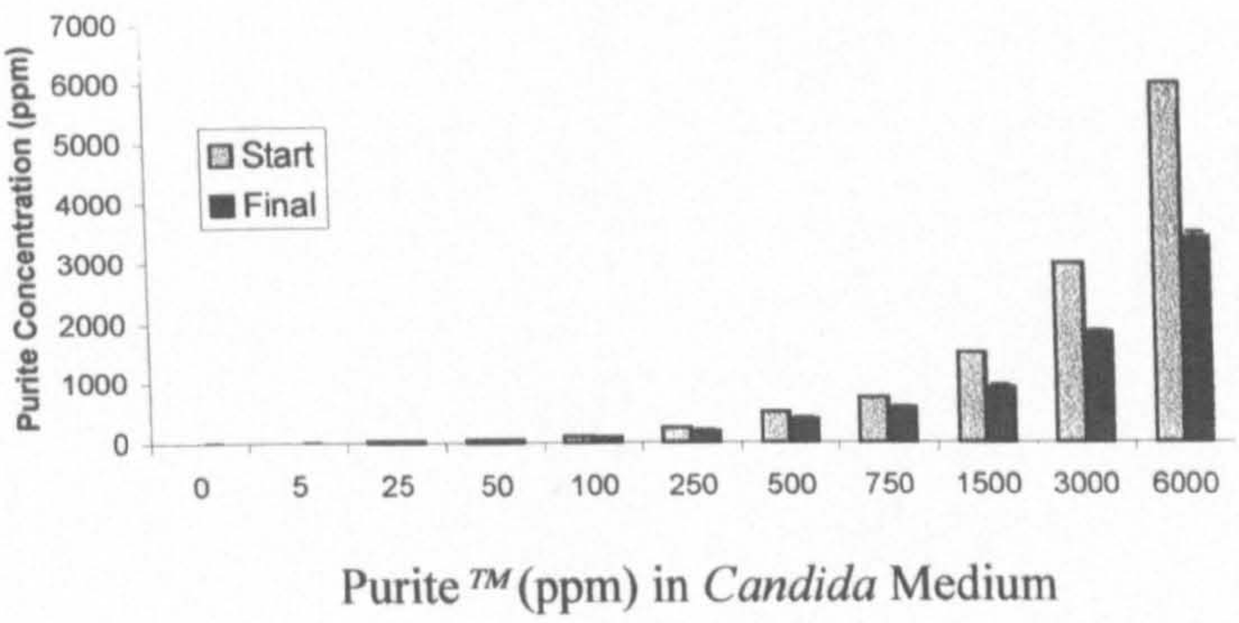
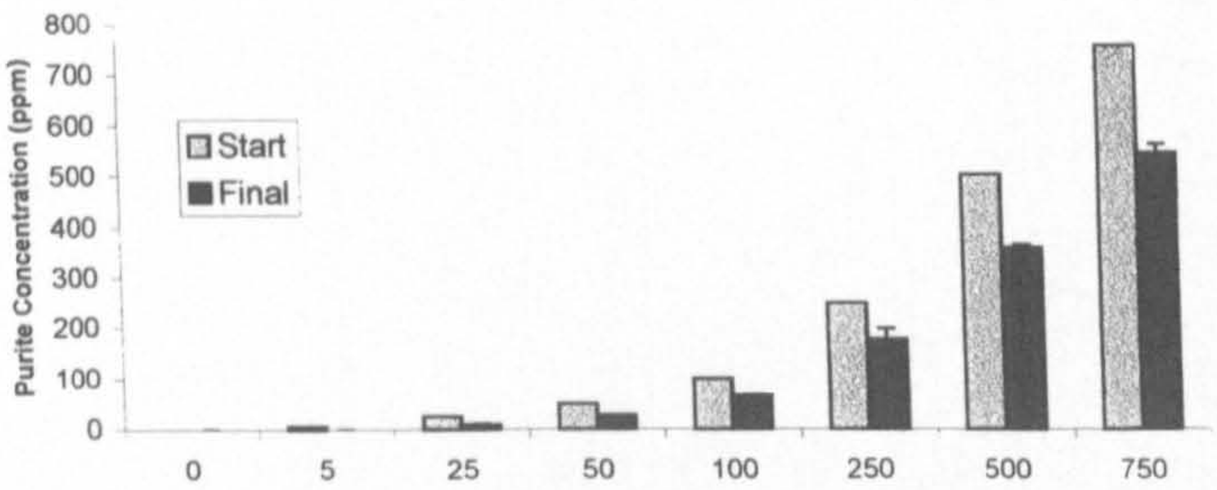


Figure 6.8. The effect of intracellular catalase depletion on viability dose response curves of cells treated with Purite™. The concentrations of inhibitors were 400mM in RCE, 100mM in *Candida albicans*, 450mM in *Pseudomonas aeruginosa* and 150mM in *Alternaria spp.*







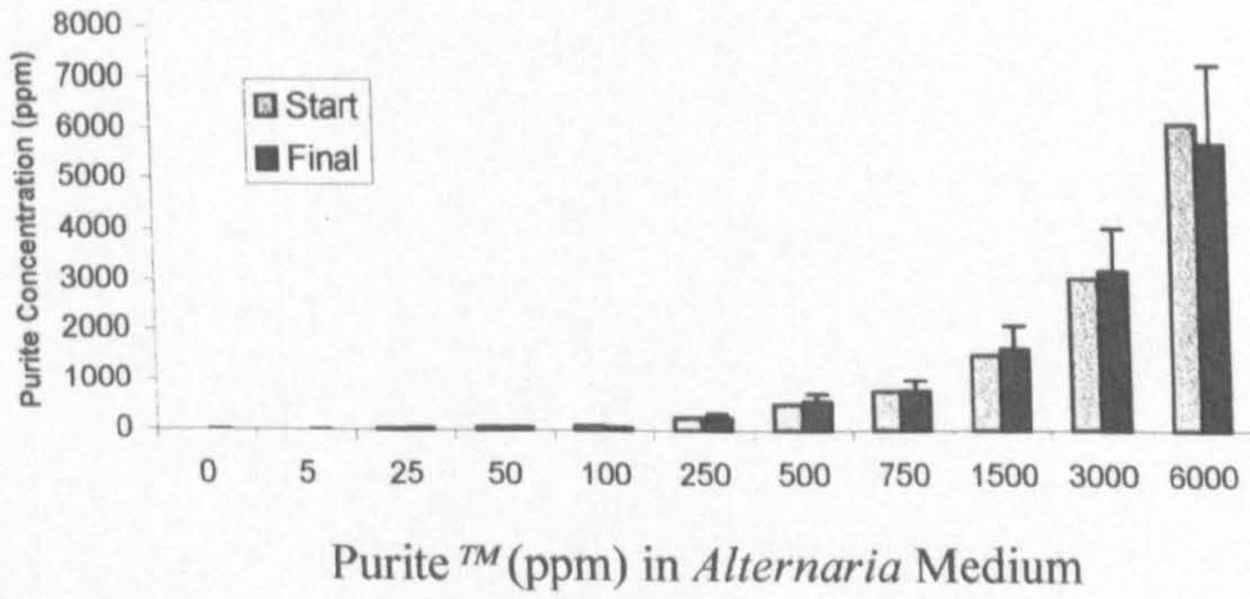


Figure 6.9. Changes in Media Purite ™ Levels Upon Treatment of Catalase Glutathione Depleted Cells for Six Hours. Controls are untreated cells (0ppm). Graphs show changes in Purite ™ from Start to Final. (n=3 + SEM).

Statisitical Analysis of Inhibitor Dose Response Data.

Mann Whitney Test.

ATP Response: Significance at P≥0.05

Cell Type	'GSH Inhibited' to 'Control' Purite™ Response
RCE	500, 750ppm
Candida	25ppm
Alternaria	750, 6000ppm
Ps.aeruginosa	25, 50, 250ppm

ATP Response: Significance at P≥0.05

Cell Type	'Catalase Inhibited' to 'Control' Purite™ Response
RCE	100, 250ppm
Candida	250, 500, 750, 1500 3000ppm
Alternaria	6000ppm
Ps.aeruginosa	5, 25, 50, 500, 750ppm

Figure 6.10. Statistical analysis results of inhibitor viability Purite™ dose response curves compared to control.

Discussion

The characterisation of the responses of organisms to Purite™ over a range of concentrations provided a powerful tool for investigating the mechanism of action of the preservatives in the presence of antioxidant inhibitors.

The major aim of this investigation was to obtain information about the mechanism of action of Purite™ as a preservative, particularly in relation to the role of glutathione. Previous results reported in this thesis have shown that Purite™ reacts readily with glutathione *in vitro*, thus supporting the concept that glutathione may be able to detoxify Purite™ *in vivo*. A comparison of glutathione dose response curves and ATP dose response curves provides evidence that glutathione is important in the mechanism of action of Purite™. In the majority of the cell types studied, the depletion of glutathione preceded the loss of viability, thus indicating a possible protective role. Many other homeostatic and messenger pathways contain thiol-based motifs and their state of oxido-reduction controls their activity. The enhanced effect of Purite™ in thiol-containing GSH depleted cells may well be due to the increase in oxidation of these ubiquitous protein-thiols. This supports the findings of other researchers studying the effects of $NaClO_2$ on erythrocyte glutathione (Abdel-Rahman *et al.*, 1985).

In order to assess the correctness of the above hypothesis the level of glutathione in the cells was depleted, by the use of inhibitors of glutathione synthesis and in the redox cycle. The susceptibility of all the cells to Purite™ was then assessed. Unexpectedly, micro-organisms were highly resistant to the depletion of glutathione by inhibitors of glutamate cysteine lyase and glutathione reductase over the incubation periods used here. Single inhibitor studies indicated that test organisms were able to maintain high levels of glutathione over six hours. This was probably due to the lack of metabolic and oxidative stress in the test organisms over this short incubation period. Thus, in the absence of an exogenous stress the test organisms were able to maintain their native glutathione pool by either synthesising more glutathione *de novo* or recycling GSSG to GSH in the glutathione redox cycle to overcome a single pathway inhibitor. However, combining both inhibitors to block

both glutathione systems resulted in a 50% reduction of native glutathione in the test organisms with full viability. However, the presence of the inhibitors during the Purite™ treatments may have generated some synergistic potentiation of Purite™ and these toxic inhibitor compounds during the incubation. Due to logistical/time restrictions, it was not possible to pre-treat cells with inhibitors and then incubate with Purite™. It may have been possible to grow the cultures in inhibitor containing media and other researchers have successfully utilised these inhibitors singularly over longer incubations and used treatments with lower inhibitor concentrations during cell culture/growth. Tokutake *et al.* (1998) investigated the effect of BSO on *E.coli.* They found that 55μM BSO depleted native glutathione levels to below 50%. Starke *et al.* (1985) found that rat hepatocytes, high in native glutathione, required 300μM BCNU to reduce the level by 85% over 24 hours. Lee *et al.* (2001) depleted native glutathione levels of glioma cells by 56% using 200μM BSO over 24 hours. In this study, the control values of native glutathione in specific test organisms were taken after six hours of incubation with and without Purite™ treatment (Chapter 5); and so validation of cellular depletion of glutathione had to be evaluated at this point for comparison to these uninhibited controls. There are no comparable reports in the literature of similar inhibitor test conditions as those employed in this study.

The lowered concentration of Purite™ required to increase the cytotoxicity is observed in Figure 6.6. Depletion of 50% of the native intracellular glutathione also decreased the Purite™ consumption by cells during the treatment. This suggests that glutathione may play a vital role in protecting cells against the oxidative action of Purite™, and was probably involved in detoxifying it. Depletion of intracellular glutathione increased the sensitivity of all cells to Purite™ induced cytotoxicity. However, the degree of increased susceptibility was not as high as expected. Depletion of 50% of native glutathione in RCE and *Candida* would have rendered these organisms with approximately the same glutathione concentration as that found in non-inhibited cultures of *Alternaria.* Previous studies in this thesis have found a comparatively high resistance to Purite™ in non-inhibited *Alternaria* cultures (Chapters 4 & 5) and so other factors must play a role in *Atlernaria* resistance. Two possible mechanisms of enhanced Purite™ toxicity may be occurring. First, as an

indirect effect, depletion of the protective cellular glutathione meant that Purite™ was not removed from the medium. So lower treatment concentrations of Purite™ now remain in a higher cytosolic concentration to have a greater affect on other sensitive thiol-containing cellular systems. Alternatively, the depletion of cytosolic glutathione has made the cells more susceptible to the same Purite™ concentration, thus, directly correlating resistance to glutathione presence.

Catalase also protected against Purite™ induced cytotoxicity in most cells. The lack of effect of catalase inhibition in *Alternaria* was probably due to low levels of native catalase and a general dependence in this organism on other anti-oxidants. This is further supported by the observation that, compared to the other test organisms, *Alternaria* required the lowest level of aminotriazole to generate 50% catalase depletion. Aminotriazole may also inhibit cellular peroxidases to effect cellular resistance to oxidants (Diemann *et al.,* 1984). Thus, it is also possible that peroxidase inhibition was responsible for the enhanced cytotoxic effects of Purite™ found here. Generally, this indicates that the cytotoxicity of Purite™ in these experiments depended on oxidative damage. The results obtained with dose response curves in the presence of a catalase inhibitor showed that catalase is also important in protecting cells against Purite™ effects. Thus, it appears that antioxidant defence in general is important, rather than specifically glutathione. Presumably, Purite™ is not detoxified directly by catalase, as this is thought to be specific for hydrogen peroxide (Gutteridge & Halliwell, 1991).

However, Purite™ solutions may contain other reactive/toxic species, possibly chlorine dioxide or sodium chlorate, which could generate superoxides that may lead to hydrogen peroxide formation and toxicity in catalase depleted cells. The lack of Purite™ consumption in glutathione inhibited organisms indicated that it probably was not sodium chlorate or chlorine dioxide driven toxicity. If sodium chlorate or chlorine dioxide were driving the toxic reaction they would have been consumed from the media. Purite™ would have also still been consumed to maintain the equilibrium of Purite™ to other, possibly toxic, products. Purite™ consumption did not follow this trend and so it is unlikely that the other minor components in Purite™ solutions were generating the toxicity. In any case, it is clear that Purite™

must act largely via an oxidative mechanism of attack, and that cellular antioxidant defence is important in resistance to toxicity.

Observations on the consumption of Purite™ by cells during catalase inhibited treatments also strengthen the argument in favour of its detoxification by glutathione. Depletion of glutathione from the cells almost abolished the consumption of Purite™, but remained normal in aminotriazole treated cells. This implies that a major part of the Purite™ that disappears during cell treatment is removed by reaction with glutathione. This agrees with our previous finding that Purite™ and glutathione react readily *in vitro*. Thus, overall there is considerable evidence in favour of an oxidative damage mechanism of Purite™, involving interaction with glutathione, and with various cellular antioxidants contributing to defence and resistance.

Chapter 7:

General Discussion.

The aim of the investigations described in the previous experimental chapters was to gain an understanding of the mechanism of action of the stabilised oxy-chloro compound, Purite™, in comparison to other FDA approved ocular preservatives. Purite™ is formulated in the final products between 5-100ppm (0.005-0.01%), depending upon the drug being used, at near physiological pH in opaque containers. The major component of Purite™ formulated in these conditions is sodium chlorite ($NaClO_2$).

It was necessary to perform the work from Chapters 2, 3, and 4 simultaneously to confirm that the effects of the same concentrations of oxidants and preservatives used in the treatments of bio-molecules and cellular lipids were high enough to show cytotoxic effects. Therefore, it was possible to declare that the test solutions were at relevant *in vitro* concentrations as the same concentrations were also found to cause cell death in the test organisms within the time course cytotoxicity study of Chapter 3.

In the early chapters NaOCl and t-BHP were used, as positive control oxidant treatments in test incubations, for the production of chlorohydrin and hydroperoxides, respectively. The control oxidants were then compared to experimental treatments that included Purite™ and BAK.

Synthetic phospholipid vesicles were treated with positive control oxidants and test preservatives. Oxidative modifications were monitored using the sensitive HPLC-ESMS technique. Initially over short time courses of up to four hours, no major oxidation products were identified with Purite™ treatments compared to controls. Interestingly, the effect of lowering the pH of the Purite™s formulation generated increasing levels of lipid hydroperoxides; which had only previously been observed in Purite™ incubations over 24 and 48 hours. Initially, the formation of hydroperoxides in Purite™ treated vesicles was surprising given the chemistry of Purite™ ($NaClO_2$). No lipid-chlorohydrins were detected in Purite™ treatments unlike the hypochlorous acid (NaOCl/HOCl) positive control results. The NaOCl and

t-BHP observations agree with the findings of Panasenko *et al. (1997) and Spickett et al.* (1998). In addition, the formation of chlorohydrins, at native lipid m/z+52Da; and hydroperoxides, at native lipid m/z+32Da agrees with Schaur *et al* (1998), Spickett *et al.* (1998) and Jerlich *et al.* (1999). The effect of pH altering the reactivity of Purite™ with the phospholipid vesicles to generate hydroperoxides may be due to the formation of chlorine dioxide from sodium chlorite in the presence of hydrogen ions (Abdel-Rahman and Scatina, 1984). Chlorine dioxide is known to be a very toxic and reactive oxidant used for killing microbes during water treatment (Zanetti *et al*, 1996).

^{1}H-NMR was shown to be a sensitive *in vitro* method for detecting glutathione oxido/reduction state (Reglinski *et al.*, 1991). In summary Purite™, at preservative doses, was found to have relatively low reactivity with phospholipid vesicles and tryptophan, but it was very oxidative to GSH *in vitro*. When compared to hydrogen peroxide and hypochlorite, Purite™ was found to be the most highly oxidative compound of those tested. Interestingly, Purite™ did not form further oxidation products of GSSG when incubated with GSH. Upon reflection, this may be important *in vivo* as adequate levels of cellular glutathione reductase to reduce GSSG back to GSH within the glutathione redox-cycle may be responsible for generating innate cellular resistance to Purite™ induced oxidative stress.

There appears to be a fundamental difference between the way Purite™ ($NaClO_2$) acts compared to the other oxidants. Comparing the positive controls of t-BHP, for hydro-peroxidation and NaOCl for chlorohydrination, it was observed that Purite™ did not clearly react with the same efficacy as t-BHP and NaOCl in terms of depletion of vesicle native PC's with unsaturated acyl-chains. Oxidative modification of PCs in vesicles by Purite™ only occurred at higher oxidant concentrations and at a lower pH than those required to affect cell viability. Given that the phospholipid membrane is a primary site of interaction of all treatments this suggests that lipid damage was not the primary site at which Purite™ elicits its anti-microbial activity. However, the situation *in vivo* may be different due to presence of other components that affect the membrane and exacerbate the effects. Divalent metal ions have been shown to affect the rate of haemolysis of erythrocytes induced by hypochlorite (Ginsburg *et al.*, 2002).

To confirm that membrane lipid oxidation was not a mechanism of Purite™ action, the incubation studies, analysing lipid alterations, of Chapter 4, were performed. This work also provided new information on the native phospholipid profiles of the organisms used, i.e. the Gram-negative bacteria, *Pseudomonas aeruginosa*, the Gram-positive bacteria, *Staphylococcus aureus*, the yeast, *Candida albicans*, the filamentous fungi, *Alternaria spp.* and the two mammalian ocular epithelial cell lines RCE and WKD. Table 7.1 allows comparison of the lipid analysis results between all the test organisms.

The outcomes of Chapter 4 were twofold: firstly the native lipid profiles of the organisms showed that specific membrane phospholipid composition did not correlate to organism-specific resistance to Purite™. Secondly, that Purite™ had little oxidative effect upon cellular phospholipids. Test organisms with native membrane lipid profiles containing a higher proportion of unsaturated acyl-chains may have had a predicted susceptibility to oxidation and, thus, toxicity by control and experimental oxidant treatments. This study could be extended to investigate the effect of a full dose response curve of Purite™ treatments upon membrane lipids. This would generate a better understanding of the results for comparison.

However, Chapter 3 confirmed the relative toxicities of the treatments used in the experimental Chapters 2 and 4 to the panel of test organisms and showed that the presence of unsaturated acyl-chains in cell lipids did not correlate to resistance. A summary of the number of unsaturated phospholipids for each organism can be seen in Table 7.1. Results suggested that maintaining the cellular antioxidant glutathione levels may play a vital role in organism-specific Purite™ protection.

	RCE	WKD	*C.albicans*	*Alternaria spp*	*Ps.aeruginosa*	*Staph.aureus*
No. of lipids with ≥ 2 double bonds.	1	1	5	8	6	1
No. of Purite™ induced oxidised lipids.	0	0	0	0	0	3
Native GSH: GSSG µmol/g	2.6	1.65	3.53	0.33	1.73	0
Purite™ conc. ≥90% cell- kill.	500ppm	250ppm	>5000ppm	>5000ppm	50ppm	250ppm
Purite™ conc. To start GSH: GSSG depletion	50ppm	50ppm	750ppm	750ppm	25ppm	Not Determined (ND)
%GSH: GSSG left at ≥90% cell kill Purite™ dose	0%	< 5%	20%	< 5%	< 5%	ND
BCNU conc. (a)	5µM	ND	25µM	400µM	200µM	ND
Purite™ conc. (b)	500ppm	ND	>5000ppm	750ppm	25ppm	ND
Aminotriazole conc. (c)	400mM	ND	100mM	150mM	450mM	ND
Purite™ conc. (d)	250ppm	ND	5000ppm	5000ppm	5ppm	ND

Table 7.1 Comparison of the test organism observations from the Purite™ investigations of this thesis. Notes:

(a) to deplete 50% GSH:GSSG + 2mM BSO.

(b) to cause ≥90% cell- kill with 50% of native GSH:GSSG.

(c) to cause 50% cytotoxicity + H_2O_2.

(d) to cause ≥90% cell- kill with 50% native catalase.

Chapter 3 employed a number of classical test methods for counting the cells of specific organism types. Mammalian cell viability was measured by trypan blue exclusion, unicellular microbial cell killing was measured by dilutional plating of cultures for CFU's and the viability of the filamentous fungi was assayed by changes in dry mass. The type of techniques used to assess cell killing in the different organisms meant it was not easy to compare the effects of the treatments between all of the organisms. Also, mammalian cell results were found to be misleading by the trypan blue cell exclusion technique as BAK and t-BHP treatments destroyed the entire cell structure leaving nothing to count as viable or dead. This noted, it was possible to assess the relative toxicity of control and preservative treatments within a given organism. Purite™ was not as toxic to the mammalian cells as BAK, t-BHP or HOCl treatments over 24 hours.

Chapter 3 also evaluated the amounts of native glutathione in the panel of organisms and measured the effect of 4 hour treatments upon cytosolic glutathione concentration. Table 7.1 shows the varying concentration of glutathione in the different test organisms. The relative concentration of glutathione approximately reflected overall resistance to oxidant treatments observed in Chapter 3.

Critically, the ability to maintain cytosolic glutathione in Purite™ treated *Candida* and *Alternaria* appeared to be associated with the relative resistance found in this organism. Table 7.1 shows a comparison between test organisms and the doses of the Purite™ required to initiate glutathione depletion. The theory of a possible adaptive response of some organisms due to an initial decline and then re-growth may not be justified. The treatments were in a non-growth supporting medium (PBS). The amount of treatment may also not have been enough for the large cell numbers used so that there were sufficient cells for glutathione analysis in the assays. Thus, only a proportion of the test inoculation may have been killed and a proportion remained viable over the longer time-course. Also, the presence of organic matter carried over from the incubation of cells in media has been discussed and this may have been a factor in the resistance of some organisms in these experiments.

Further valuable work beyond this study could evaluate the oxidation of other antioxidant molecules that Purite™ may affect directly or by interaction with

(Arner *et al.*, 2000) and those involved in the Ascorbate-GSH Cycle (Vitamin E and Acsorbate) shown in Figure 7.1. These molecules have been shown to play a role in cellular protection in a wider range of cell types exposed to oxidants.

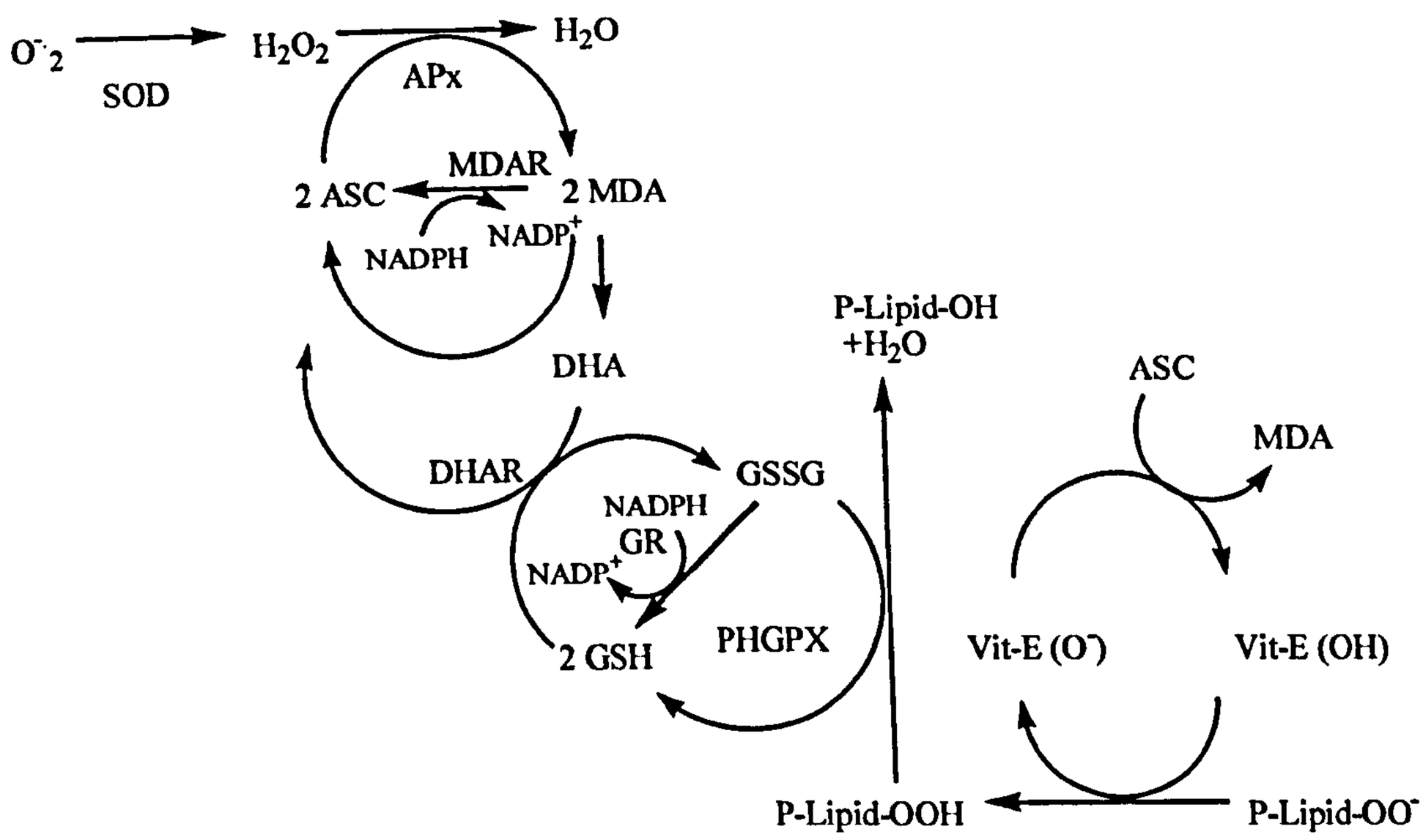


Figure 7.1. Ascorbate-glutathione cycle (adapted from Mullineaux et al., 2000). Superoxide ($O^+_2{}^-$); Tocopherol (vitamin E) (VIT-E OH); Hydrogen peroxide (H_2O_2); Superoxide dismutases (SOD); Reduced glutathione (GSH); Oxidized glutathione (GSSG); Ascorbate (ASC); Ascorbate peroxidase (Apx); Monodehydroascorbate (MDA); Dehydroascorbate reductase (DHAR); Chromanoxyl radical (VIT-E O^+); Glutathione reductase (GR); Phospholipid hydroperoxide-dependent-glutathione peroxidase (PHGPX); Phospholipid alcohol (P-LIPID-OH); Phospholipid peroxy radical (P-LIPID-OO^+); Phospholipid peroxide (P-LIPID-OOH); Dehydroascorbate (DHA).

It may also be enlightening to investigate real-time changes in *in vivo* GSH:GSSG ratios during treatment, using NMR. Quantitative investigations of intact cells in both time-course and dose response studies would only be possible using ^{1}H-NMR if the problems associated with the large water signal could be overcome. Other researchers have studied *in vivo* GSH/GSSG ratios by analysing cells pre-loaded with ^{13}C-GSH using electron paramagnetic resonance (EPR) detection. This technique was used by Kuppusamy *et al.* (2002) to compare the GSH composition of hypoxic solid tumors and the surrounding normal tissue, *in vivo,* to understand the tumor growth and therapy with glutathione depleting drugs. However, members of

staff with anti-oxidant research interests within Strathclyde University do have a desire to develop this methodology and perform *in-vivo* GSH:GSSG analysis. This technique may also give further information about the disappearance of glutathione observed in Chapter 3. In this study, the final levels of glutathione in the cytosol and media did not match the control (native) glutathione level. Willis *et al.* (1996) have used ^{13}C-GSH NMR analysis to investigate t-BHP effects upon GSH/GSSG in the eye *in vivo.* This study suggests that protein-GSH adduct formation may function as a mechanism for modulating the cytosolic glutathione redox buffer under condtions of oxidative stress in ocular tissue. If such GSH-protein adducts were formed in Chapter 3 investigations this may explain the loss of GSH activity in the cytosolic/media compartments for all cell types. The reasons for these differences may also be important in understanding the interaction of Purite™ with glutathione and other thiol compounds in the cells.

In Chapter 5, the organic load in the final treatment of the dose response experiments was minimised wherever possible. The characterisation of the responses of organisms to the preservatives of interest over a range of concentrations provided a powerful tool for investigating the mechanism of action of the preservatives, and for testing approaches to improve their antimicrobial activity.

For the viability dose response curve, cell viability was assessed by measurement of the intracellular ATP concentration, using a bioluminescent assay. This method has been used extensively for cytotoxicity assays in mammalian cells (Geerling *et al,* 2001, Alongi *et al,* 1998, Crouch *et al,* 1993). Although it is less common in microbial cell research, it has been validated against conventional CFU counting. Use of this method has the advantage that all the cells tested were assayed by the same technique, thus allowing cytotoxicity comparisons between Purite™ treated organisms as seen in Table 7.1.

A comparison of the glutathione dose response curves and the ATP dose response curves provided evidence that glutathione is important in the mechanism of action of Purite™. As shown in Table 7.1, in the majority of cell types studied, the depletion of glutathione preceded the loss of viability, thus indicating a possible role in the eventual cytotoxicity. However, from the *in vitro* observations, glutathione-thiols may preferentially react with Purite™ before reacting with thiols of the bio-molecules in other vital pathways. For example Thioredoxins have been shown to control the activity of numerous transcription factors including NHκB and AP1 gene products (Arner *et al.*, 2000). Furthermore, Kim *et al*,(2002) have shown that transcription factor OxyR may be oxidised at its thiol group into different several stable species. These oxidised OxyR species were then shown to generate distinctly different transcriptional responses. Other thiol attacking, anti-protazoal, drugs include the antimonials and arsenicals. These have been shown to lower cytosolic GSH but in fact DNA protein cross-linkage has been shown as their probable mechanism of action (Gebel, 1997).

It is also clear that BAK does not have a mechanism of action that involves glutathione. In general the loss of viability with this preservative occurred earlier in the dose response curve than the loss of glutathione. This indicates that the latter was a non-specific effect resulting from the loss of membrane integrity and leakage of cellular contents. As the ATP luciferin/luciferase methodology allows direct comparison between organisms it may be useful extend these studies to culture cells in sub-lethal Purite™ doses and then determine the new required killing dose of Purite™ to determine if resistance can be acquired. Further studies into acquired resistance could include determining the glutathione levels and oxido/reduction state.

Observations on the consumption of Purite™ by cells during treatments also strengthens the argument in favour of its detoxification by glutathione, as depletion of glutathione from the cells almost abolished the consumption of Purite™. This implies that a major part of the Purite™ that disappears during treatment is removed by reaction with glutathione. This also agrees with our previous finding that Purite™ and glutathione react together readily *in vitro*. Thus, overall, studies with Purite™ have shown there is considerable evidence in favour of an oxidative damage mechanism, involving the interaction with glutathione, and potentially with various

cellular antioxidants contributing to cellular defence and protection from reactive oxygen species produced.

One initial aim of the project was to obtain information about the mechanism of action of Purite™ in comparison to other preservatives, and secondly, to determine the effects of Purite™ at higher concentrations than presently used in preservative formulations. The viability dose response curves showed clearly that Purite™ had considerable advantages as a preservative for ocular formulations. It was significantly less toxic to corneal (RCE) and conjunctival (WKD) cells than hydrogen peroxide or benzalkonium chloride (BAK), whilst having antibacterial activity. However, it was a poor anti-fungal, which was not as good as BAK, although no worse than hydrogen peroxide. Thus, when assessing all the desirable characteristics for an ocular preservative, Purite™ may well be the best preservative option. The results from the glutathione dose response curve also support this view.

When the cellular levels of glutathione were depleted by the use of specific inhibitors, targeting glutathione synthesis or glutathione redox cycle, an increased susceptibility of all cell types to Purite™ was observed. This also supports the hypothesis that glutathione is important in the protection of cells against damage by Purite™. However, the degree of increased susceptibility was not as high as had been expected. The results obtained with dose response curves in the presence of a catalase inhibitor show that catalase is also important in protecting cells against Purite™. Therefore, it appears that antioxidant defence in general is important, rather than specifically glutathione. Presumably, Purite™ is not detoxified directly by catalase, as this is more specific for hydrogen peroxide (Switala *et al.*, 2002). Purite™ interaction in the cell may cause the production of other reactive species, such as superoxide, which can lead to hydrogen peroxide formation (Halliwell and Getteridge, 1985). The inhibitor study also found a lack of Purite™ consumption from the media in glutathione-depleted cells, but near normal Purite™ consumption in catalase inhibited cells. It was proposed that this observation may indicate that it is $NaClO_2$ having an effect on glutathione levels and not the by-products of Purite™ metabolism. However, it is clear that Purite™ must act largely via an oxidative mechanism of attack, and that cellular antioxidant defence is important in resistance to toxicity.

Further work that would generate lots of new insights would be the use of the proteomics and confocal-microscopy tools to investigate the cellular responses to Purite™. Confocal-microscopy has been used to investigate the morphological changes that arise when WKD cells are exposed to tear substitutes *in vitro*. Debbasch *et al.* (2002) studied the effects of two major tear substitutes, hyaluronic acid and a widely used carbomer, with and without the BAK preservative. These two preserved ophthalmic hydrogels were found to cause no cytotoxicity in WKD. They tended to reduce the toxic effects of the BAK preservative control. These results may allow the use of hydrogels, not only in dry eye, but also in ocular surface disorders involving oxidative stress and in ophthalmic drug therapy to improve ocular tolerance.

From the proteomic investigations of Lin *et al* (2002), studies of specific thiol-containing proteins are now possible in cells exposed to agents known to cause oxidative stress. In mammalian cells, mitochondria play a central role in redox-linked processes through mechanisms that are thought to involve modification of specific protein thiols. Previously the redox state of mitochondrial proteins this has proved difficult to assess. In particular, specific labelling and quantification of mitochondrial protein cysteine residues have not been achieved due to the lack of reagents available that can be applied to the intact organelle or cell. This novel proteomics approach enables measurement of the thiol redox state of individual mitochondrial proteins during oxidative stress and cell death.

Proteomics may also help to determine the organism specific role of the glutathione redox-cycle and how other enzymes play in protecting cellular constituents from Purite™'s oxidising potential. Furthermore, the use of microbial strains lacking specific biochemical pathways and bio-molecules could be utilised to determine the effect of specific molecules to the innate and acquired resistance possible to Purite™. For example, Ochsner *et al* (2000) have previously described the oxidative stress response in genetic mutants of catalse in *Pseudomonas aeruginosa* evaluating the genetic response upon exposure to H_2O_2, paraquat and organic peroxides. Mutants or gene-knockouts have been described for SOD (Hunter *et al.*, 2002), catalase (Ochsner *et al.*, 2000), thioredoxin, (Norgaard *et al.*, 2001) and many more within cell culture collections. Studies of the effect of Purite™ in specific gene knockouts would give an insight into the specific role of cell constituents when

compared to wild-type microbial strains. Proteomic investigations using lethal and sub-lethal doses of Purite™ in the resistant and gene knockout organisms may give a better understanding of the reactive oxygen species that are generated and specific thiol-containing targets Purite™ damages and how the cells respond to overcome the stress induced.

An interesting question arising from this study is whether it would be possible to increase the antimicrobial efficiency of Purite™, without significantly increasing its toxicity to mammalian cells, by using an adjuvant in the formulation e.g. inhibitors of glutathione metabolism. However, this study shows that the micro-organisms seem to have a very high resistance to them (Table 7.1), and depletion of glutathione could only be achieved with difficulty although toxicity was not induced in mammalian cells. Moreover, there was no evidence of a differential response between the mammalian cells and micro-organisms which would allow the adjuvant to increase their antimicrobial activity without compromising the viability of the ocular cells. There are many reports that high doses of BSO and BCNU are toxic to the ocular surface during cancer chemotherapy (Cruciani *et al.*, 1994). Aminotriazole induces cataract formation in animal models (Bhuyan and Bhuyan 1984 and Costarides *et al* 1991). Since ocular solutions applied to the tear film are cleared within minutes and the exposure of the eye to the formulation would be much lower than that of microbes contaminating the bottle, this may not be a critical problem. Nevertheless, the high concentrations of inhibitors that would be required, and their cost, may prohibit their use as adjuvants with Purite™. On the other hand, the major weakness of Purite™ lies in its anti-fungal activity, which may explain the problems that have been encountered with species like *Alternaria* and *Aspergillus*. It is clear from the viability dose response curves that BAK has a high effectiveness against *Alternaria*, and may, therefore, make a good adjuvant for Purite™ should they be chemically compatible with each other. The resistance of *Alternaria* may correlate to the presence of the black coloured pigment, melanin, which acts as a cellular anti-oxidant. Jacobson *et al,* (1995) studied the effects of permanganate, HOCl, and H_2O_2 upon albino, and black melanin-positive, *Alternaria,* viability. They found that melanin albino strains were more sensitive to these oxidants than the black form. Throughout the studies performed in this thesis the *Alternaria* was grown in the

unstressed albino form since the melaninic forms are difficult to maintain *in vitro*. This was also reported by Jacobson *et al* (1995) and, after attempting other methods, resorted to dry weight analysis to determine *Alternaria* resistance. Further work could be performed using the luminescence methodology developed in Chapter 5 to determine the specific role of melanin in Purite™ resistance. Assessing the cytotoxicity levels Purite™ induces in fungal samples removed from an ageing *Alternaria* culture would evaluate the cells as they start to produce melanin. However, our results suggest that since the other resistant organisms did not contain significant levels of melanin it is probably not the primary mechanism of Purite™ resistance in most organisms.

More specific anti-fungal agents with low mammalian toxicity could be investigated as adjuvants. Such a system, containing both Purite™ and a fatty acid biosynthesis inhibitor (e.g. Triclosan like agents, Heath *et al,* 2002) may offer good combined antibacterial and anti-fungal activity without significant effects on mammalian cells. These products are already licensed for use as biocides in healthcare products such as mouth-rinses. Furthermore, these products have been evaluated as antimicrobials against *Plasmodium falciparum* and *Toxoplasma gondii* (M^{c}Leod *et al.*, 2001). Thus they may also provide a good therapy in ocular toxoplasmosis and microbial keratitis. Amphotericin B is a broad spectrum anti-biotic that has been shown to have efficacy during ocular infections of *Asperillus spp.* (Boldrey, 1981).

One other system for consideration is the use of high dose Purite™ and neutralisation upon delivery into the eye. As discussed previously, metal salts have been used in water treatment to remove sodium chlorite (Katz *et al.*, 2001). The redesign of the dropper bottle to include a metallic coil or element when depositing solutions from the bottle may offer an effective way of lowering high Purite™ concentration to a safe, low dose, instillation into the eye.

In conclusion, this thesis has investigated the interaction of Purite™ with phospholipids and glutathione within *in vitro* model systems. It has compared the effect of preservatives and oxidants in a range of cells from the eye and micro-organisms. Compared to other preservatives Purite™ was found to have good differential toxicity allowing safe use at preservative doses. Purite™ was found to react well with thiol-containing anti-oxidant glutathione and at increasing doses depleted the cellular pool generating oxidative stress and death. Microbial resistance correlated to maintaining the cellular glutathione pool. This pool may protect other sensitive thiol-proteins from Purite™ induced oxidation. The results of anti-oxidant inhibitor studies indicated that Purite™ resistance may also be due to other anti-oxidant pathways in specific cell types. The work presented here provides a platform for future research into Purite™ resistance mechanisms of the specific microbes and the specific target molecules glutathione may protect.

Chapter 8:

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