The thesis

Enhanced methods of microbial measurement and detection

Submitted for the degree of
Doctor of Philosophy (PhD)

By

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Abstract

This study demonstrates the effects of different peptones, made from different biological sources and produced by numerous manufacturers, on the growth dynamics and morphology of various bacteria. The effects these differing growth medium constituents have upon the outcome of microbiological procedures, from identification of bacterial species to public health diagnostics, is of great significance.

Peptones were assessed as a constituent of the pre-enrichment broth buffered peptone water. Generation times and yields at 24 h were measured using optical density techniques and were significantly affected by the type of peptone employed as nutrient source. Growth characteristics indicated that where peptones performed poorly, this was a result of poor nutrient quantities and not due to the presence of endogenous inhibitors.

It is shown that *Salmonella* exhibit morphological differences, including cultures which lack flagella and are consequently non-motile, dependent on the peptone constituents of the culture medium. Transfer of aflagellate *Salmonella* from nutritionally poor media into rich nutrient broth allowed flagella synthesis: indicating that the aflagellate form is still able to produce flagella. Amino acid sequencing of the peptones producing aflagellate organisms showed a relatively low tyrosine concentration: addition of tyrosine and glucose to these media produced flagellate *salmonellae*. Identification of the *Salmonella* serotypes is based on flagellar and somatic antigens; therefore the absence of flagella may consequently affect complete identification of the serotype.

Antibiotic susceptibility of the Enterobacteria was shown to be markedly affected by peptone, causing breakpoints to vary from sensitive to resistant.

Thus the inclusion of different, undefined ingredients in growth media has a considerable effect upon the ability of the medium to enumerate bacteria; furthermore these medium constituents affect the outcome of scientific research and should be carefully considered before work is commenced.

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My thanks go to Professor David Lloyd, from whom I have learnt antique methods of scientific research and the ability to deal with all types of "scientist". Most of all I would like to express my appreciation for instilling in me such passion and enthusiasm, that I am happy whenever I step foot inside the lab.

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Table of Contents

Table of Tables

Table of Figures

Abbreviations

°C	Degrees celsius
ACDP	Advisory Committee for Dangerous Pathogens
A/E	Attaching and Effacing lesion
ATCC	American Type Culture Collection
ATP	Adenosine-Tri-Phosphate
BG	Brilliant Green (agar)
BHI	Brain Heart Infusion
BPA	Buffered Peptone Agar
BPW	Buffered Peptone Water
BSA	Bovine Serum Albumin
BSAC	British Society for Antimicrobial Chemotherapy
CDC	Centre for Disease Control
CFU	Colony Forming Units
cGMP	Cyclic Guanine Mono Phosphate
CLED	Cystine Lactose Electrolyte Deficient
dH_2O	Distilled water
Ec	*Escherichia coli*
E. coli	*Escherichia coli*
EHEC	EnteroHaemorrhagic *Escherichia coli*
EIEC	EnteroInvasive *Escherichia coli*
EM	Electron Microscopy
EPEC	EnteroPathogenic *Escherichia coli*
ESI	ElectroSpray Ionisation
ETEC	EnteroToxigenic *Escherichia coli*
g	Gram
g	Generation time
h	Hour
HE	Hektoen Enteric (agar)
HPLC	High Performance Liquid Chromatography
I	Unscattered light
I_0	Incident light
ISA	Iso-Sensitest Agar
ISO	International Standards Organisation
IZD	Inhibition Zone Diameter
l	Litre
LB	Luria-Bertani
Log	Logarithmic
LPS	LipoPolySaccharide
LT	heat Labile Toxin
m	Metre
mm	Milli metre
µm	Micro metre
M	Molar
mM	Milli molar
µM	Micro molar
MIC	Minimum Inhibitory Concentration
min	Minute
mg	Milli gram

µg	Micro gram
ml	Milli litre
µl	Micro litre
MRSA	Methicillin Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
MW	Molecular Weight
NA	Nutrient Agar
NB	Nutrient Broth
nm	Nano metre
nM	Nano molar
NCTC	National Culture Type Collection
OD	Optical Density
OMP	Outer Membrane Protein
PBS	Phosphate Buffered Saline
PC	Principle Component
PCA	Principle Component Analysis
PEG	Poly Ethylene Glycol
PH_2O	Polished water
PMF	Proton Motive Force
psi	Pounds per square inch
QA	Quality Assurance
QC	Quality Control
RP	Reverse Phase
s	Seconds
Sa	*Staphylococcus aureus*
S. aureus	*Staphylococcus aureus*
SEC	Size Exclusion Chromatography
SMF	Sodium Motive Force
SP	*Salmonella enterica* subspecies *enterica* serovar Poona
***S*.ser. Poona**	*Salmonella enterica* subspecies *enterica* serovar Poona
ST	*Salmonella enterica* subspecies *enterica* serovar Typhimurium
ST	heat Stable Toxin
***S*.ser. Typhimurium**	*Salmonella enterica* subspecies *enterica* serovar Typhimurium
TEM	Transmission Electron Microscopy
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
TSS	Toxic Shock Syndrome
TSST-1	Toxic Shock Syndrome Toxin 1
UHP	Ultra High Purity
UPEC	Uro-Pathogenic *E. coli*
US	United States
USA	United States of America
UTI	Urinary Tract Infection
UV	Ultra Violet
V. cholerae	*Vibrio cholerae*
Vis	Visible
VTEC	VeroToxigenic *Escherichia coli*
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight
XLD	Xylose Lysine Desoxycholate

Publications and Presentations

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Gray, V.L., Müller, C.T., Watkins, I. D. & Lloyd, D. (2004). The effect of medium constituents upon morphology of *Salmonella* Typhimurium. Presented at the second year postgraduate poster evening.

Gray, V. L. (2004). The effect of medium constituents upon morphology of *Salmonella* Typhimurium. In *Abstracts from the ASM 104th General Meeting,* ISBN 1-555-81-315-1. Abstract P-068.

CHAPTER ONE

Introduction

1. Introduction

Bacterial culture methods are tools employed to recover, enumerate and store bacteria; they are essential basic techniques for microbial research. Various recipes allow many uses for this simple technique: from recovery of sub-lethally injured organisms to preliminary identification utilizing a selective differential medium. Assessment of a universally used component of growth medium and its performance in the *Salmonella* pre-enrichment medium, buffered peptone water (BPW), was performed in order to establish the effects of different commercially available peptones on the subsequent cultivation of various bacteria. It has already been recorded that commercially available dehydrated BPW obtained from different manufacturers produces variable growth of *Salmonella* species (Baylis *et al.*, 2000).

There is still, even today, a need to achieve a uniform, consistent composition of culture medium which ensures reproducible high quality growth; this was first noted by Frost (1910). Boyd (1917) went on to describe in detail the recipe for a bacteriological nutrient medium which included quality control. Inconsistency of a growth medium performance can be attributable to the many undefined ingredients present in many complex culture media; therefore this thesis investigates a medium whereby peptone is the only variable component.

1.1. Summary

The analyses of the numerous peptones were achieved using a wide variety of microbiological and chemical techniques which included:

- Analysis of 24 h growth curves using optical density (OD) measurements.

The dramatically different bacterial growth profiles which resulted from growth in the various peptones were the foundation for this work.

- Assessment of bacterial motility and morphology by transmission electron microscopy (TEM) emphasized the extent to which peptone as a source of nutrition affected growth of bacteria.
- Determination of resultant serological types using agglutination techniques allowed insight into the effect of medium components on essential identification methods.
- Antibiotic susceptibilities were performed by the disk diffusion method; again the outcome of this important diagnostic technique was greatly altered when variable peptones were used in the culture medium.
- Chemical analyses of the peptones included separation and molecular weight determination using high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS). These findings were then compared to the performances of each peptone with respect to their ability to support microbial growth.

1.2. Culture media

Growth of micro-organisms in an artificial environment is dependent on the presence of necessary nutrients and compounds needed to synthesize constituents of the cell. The requirements of different organisms vary greatly and this accounts for the large number of differing media available. Culture media are essential for medical, industrial and environmental microbiology. It is the foundation step in culturing, identifying and isolating micro-organisms, and remains so despite advances in molecular biology and biochemistry. For example growth media can

be used to obtain microbial cultures from clinical, environmental or industrial samples; these resultant cultures can then be maintained on media (agar plates/slopes or broth) for subsequent investigation or research. Culture media are a useful means of identification: colony size, morphology, colour, texture and general physical appearance are distinctive for many microbial species. An individual colony may then be picked in order to produce a pure culture which can then be subjected to further biochemical or molecular investigation. Certain media can be used for preliminary identification of species from mixed cultures, clinical, environmental or industrial samples based on the biochemical properties of the different species. For this purpose there are complex media recipes which target these differing properties in order to allow distinction between species; xylose-lysine-desoxycholate (XLD) medium is a selective medium able to distinguish between the *Salmonella* and *Shigella*. The XLD medium generates *Salmonella* colonies which are red in colour with central black zones (Figure 1.1) whereas, in contrast, *Shigella* is present as a red colony which does not exhibit a black centre. This occurrence of a black zone is indicative of H_2S production: *Shigella* is H_2S negative and so does not present the resultant black colour.

Performance is not the only consideration for commercial media manufacturers, requests for kosher, halal and peptones which are guaranteed to be free of bovine spongiform encephalopathy and/or genetically modified organisms are manufactured to meet consumer needs.

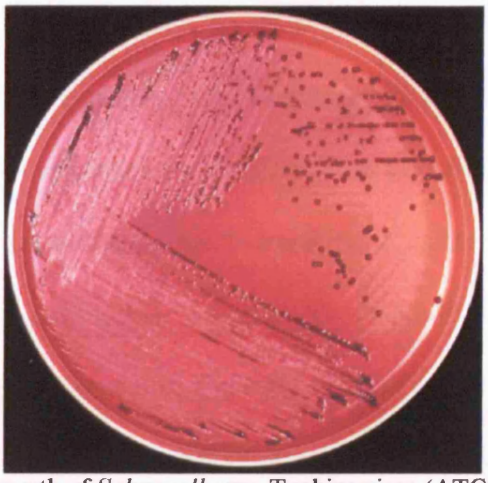

Figure 1.1. Growth of *Salmonella* ser. Typhimurium (ATCC 14028) on xylose lysine desoxycholate agar (Bridson, 1995).

1.2.1. History of bacterial culture media

In 1745 Needham recognised the requirement for nutrient fluids in microbial growth, and for a consistency in preparation. Pasteur (1860) studied nutrient media composition and produced the first recorded recipe for a culture medium: it was for the growth of *Penicillium* molds and contained sucrose, ammonium tartrate and bakery yeast ash. Pasteur also studied batch variation. Cohn (1870) developed Pasteur's work and published the formulation of "normal bacterial liquid". Nageli first described peptone in 1880, who noted that chemo-organotrophic organisms grew best in media containing a partially digested protein. Koch (1881) added sodium chloride and peptone to an aqueous meat extract and produced what is still used today as a basic culture medium. Heuppe (1882) explored the time-saving convenience of desiccated meat extract instead of Koch's fresh meat watery extract. By 1910 Frost had noted the need for commercial production of desiccated culture media of high quality and consistent composition. (This historical section was adapted from Bridson, 1995).

1.2.2. Buffered peptone water

Buffered peptone water is a pre-enrichment medium for recovery of sub-lethally injured organisms and subsequent enumeration (Van Leusden *et al.*, 1982). BPW was the medium of choice as it contains five components, of which peptone is the only complex ingredient. Defined as "small polypeptides that are intermediate products in the hydrolysis of proteins, the term is often used for any hydrolysate of protein" (Bender and Bender, 1995). Peptone provides the organism with essential nutrients and originates from various sources including animal, plant and microbial origins. Digestion of the protein material also varies, achieved either by acid hydrolysis or enzyme catalysis. Many proteases are used and so increase peptone variation. This large variability in peptone quality has a direct effect upon the performance of the growth medium; generation time (g), yields, motility and public health diagnostic tests all exhibit differences in performance when peptone is varied.

1.2.2.1. Peptones of differing biological source

The raw materials used and the manufacture of peptones is outlined below.

1.2.2.1.1. Peptones made from animal material

Meat peptones and extracts originate from a porcine, bovine or poultry origin; the tissue types in most common use are heart, lungs and oesophagus. The characteristic biochemical constituents of these tissue-types differ and consequently affect performance of the medium with respect to their support for microbial growth.

Gelatin is a relatively more defined substrate compared with other peptones investigated in this work: it is produced by boiling collagen (isolated from animal skin, bones and connective tissues) in water. The resulting heterogeneous mixture, colourless and odourless, is water soluble and contains proteins of high average molecular weight (Budavari, 2001).

Casein, the phosphoprotein component which makes up 3% of bovine milk, is considered a good nutritive source as it contains all the common amino acids and is high in essential amino acids. Casein is produced in mammary tissue and is precipitated out of skimmed milk by acidification; the commercial product is supplied as a white amorphous powder which is odourless and of low solubility in water (Budavari, 2001).

1.2.2.1.2. Peptones made from plant material

Soy peptones are the most commonly used plant peptone, they are an enzymatic digest obtained using *Carica papaya* that contains papain.

1.2.2.1.3. Extracts made from micro-organisms

Yeast extract is the water soluble extract obtained after aqueous extraction of autolysed *Saccharomyces cerevisiae*.

1.2.2.2. Digestion of protein material

The raw material used to produce peptone is digested before incorporation into growth media as it was observed that low molecular weight peptides or amino-acids promoted faster growth and higher yields (Bridson, 1995). It should be noted

that extracts are not digested, they are spray dried powders derived from infusions of meat or yeast. As they are not digested, certain growth factors which are lost during peptone digestion processes, for example vitamins and some amino acids, are available from these nutritive sources. Digestion can be enzymatic, using various enzymes, or chemical; this contributes to the variability of the peptone produced. The following methods are used to digest raw materials to form peptones (Berg *et al.*, 2002).

1.2.2.2.1. Enzymatic digestion using pancreatin

Pancreatin is a cocktail of enzymes secreted by the pancreas:

1. Amylase: an endoglycosidase which hydrolyses α (1-4) glucosidic linkages to yield maltose, maltotriose and α-dextrin.

2. Trypsin: this enzyme is initially secreted in the duodenum as the zymogen trypsinogen. Enteropeptidase hydrolyses lysine-isoleucine peptide bonds present in the trypsinogen; the trypsin produced this way then activates more trypsinogen, and other zymogens. Trypsin is a serine protease: its reaction mechanism involves the covalent substrate during catalysis. This results in two stages of hydrolysis; acylation (cleavage of peptide bond) and deacylation (acyl-enzyme hydrolysed by water). Trypsin acts on the carboxyl side of lysine and arginyl bonds, this is due to the presence of aspartate within the non-polar niche which attracts the positive side chains of trypsin. Trypsin also catalyses general ester and amide hydrolysis.

3. Triacylglycerol Lipase: also known as steapsin, this enzyme performs hydrolysis of a triacylglycerol to give a diacylglycerol and a carboxylate, or glycerol and fatty acids.

1.2.2.2.2. Enzymatic digestion using papain

A thiol protease which requires a free sulfhydryl group for activity, papain exhibits a similar mechanism to trypsin, with the substrate being covalently bonded to the enzyme during a two-stage hydrolysis reaction.

1.2.2.2.3. Acid digestion using hydrochloric acid (HCl)

An acid hydrolysate is prepared by the reaction of protein material with hydrochloric acid at high pressures and temperatures. The acid is then neutralized with sodium hydroxide.

10

1.2.2.3. Commercial peptone suppliers

Various companies today manufacture culture media or their components on a large scale, however it is acknowledged that quality and performance still vary from one batch to another (Hyde and Denton, 1987) and between companies (Baylis *et al.*, 2000). Inter-manufacturer differences remain substantial, probably due to the dissimilar production processes (Figure 1.2.) The companies supplying those peptones analysed in this study are listed below:

Merck KGaA; Darmstadt, Germany: records from 1892 document the first Merck
20 peptones for scientific purposes. Merck is a diverse company with expertise in pharmaceuticals, chemicals, bacterial culture media, diagnostics, supplements and kits for detection of bacterial contamination (Merck, 2002)

Oxoid; Basingstoke, England: in 1860, Justus Von Liebig devised concentrated meat extract that would provide essential nutrients for the poor of Europe; this did

not materialise without factories to produce the extract. In 1861, George Christian Giebert met with Liebig and together set up a factory in Uruguay. This partnership formed the Liebig Extract of Meat Company, later registered as LEMCO. In 1924 Oxoid branched out into culture media, developed Lab-Lemco, and investigated acid/enzyme hydrolysis to improve amino nitrogen content and flavour of meat cubes (OXO). 1957 Oxoid culture media became the main focus of the company. Oxoid currently supply culture media, bases, diagnostics, supplements and detection kits (Bridson, 1995)

Becton Dickinson; NJ, US: founded in 1895, Difco were producers of enzymes (pancreatin/pepsin), dehydrated tissue and glandular products. The acronym DIFCO represents Digestive Ferments Company. By 1913 Difco had expanded into bacterial culture media, and Bacto peptone was readily available. At present Difco supplies culture media, bases, diagnostics, supplements and detection kits. (Difco, 1985). The company is now Becton Dickinson (BD).

Solabia; Paris, France: supplier of peptones from plant extracts including soya based peptone.

Organotechnie; La Courneuve, France: supplier of many peptones and extracts including casein, vegetable, animal and yeast-based products.

Biospringer; Cedex, France: division of LeSaffre Group which supplies bio-nutrients and peptones.

HiMedia Laboratories Pvt. Ltd.; Mumbai, India: a general supplier of media and peptones, specializing in vegetable peptones.

DMV International bv; Veghel, The Netherlands: manufactures hydrolyzed proteins, peptones and bioactive peptides. In addition this company offers kosher-certified peptones.

Biotechnica International Inc; Cambridge, Massachusetts: Supplier of peptone for microbiological growth media.

1.2.2.4. Peptone production process

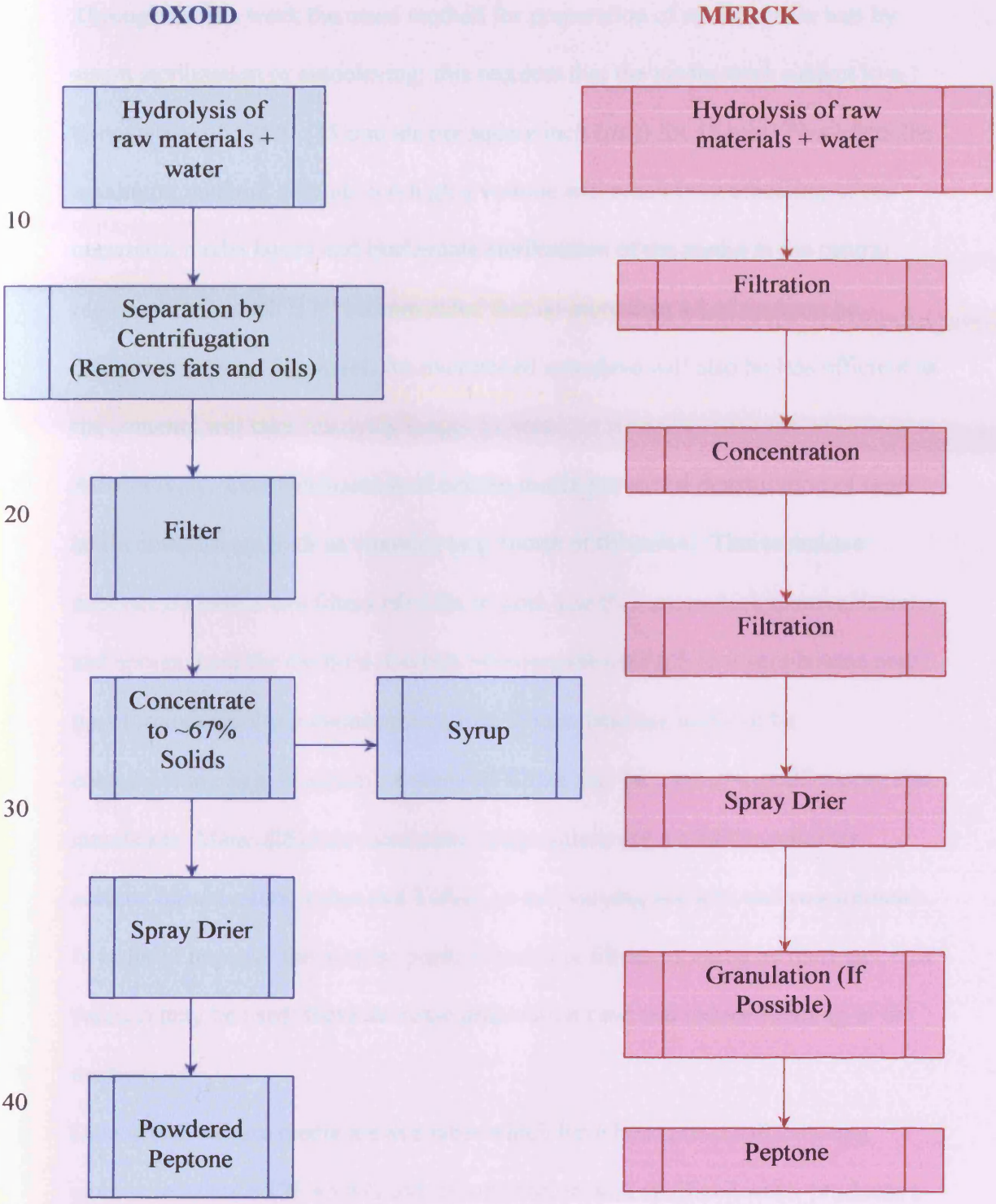

Figure 1.2. Peptone manufacture flow-chart, taken from the respective company manuals.

1.2.3. Sterilization of culture medium

Throughout this work the usual method for preparation of sterile media was by steam sterilization or autoclaving; this requires that the media were subject to a temperature of 121°C (15 pounds per square inch (psi)) for 15 min. This limits the maximum medium volume: too high a volume will result in overheating of the outermost media layers and inadequate sterilization of the media in the central region of the vessel. It is recommended that no more than 1 l of medium be contained within a 2 l vessel. An overloaded autoclave will also be less efficient as the contents will take relatively longer to heat.

Alternatively, filter sterilization of culture media prevented denaturation of heat-labile components such as vitamins (e.g. biotin or thiamine). This technique depends on membrane filters of uniform pore size (0.2 μm) which remove bacteria and spores from the medium. Certain microorganisms such as mycoplasmas may pass through a 0.2 μm membrane and so, where these are likely to be contaminating (e.g. in serum), a series of filters may be used or a smaller pore size membrane. Many different membrane compositions are available, cellulose acetate, nitrate esters, nylon and Teflon, to suit varying solvents and requirements. In order to improve the process positive-pressure filtration, using an inert gas, or a vacuum may be used: these decrease preparation time and reduce foaming of the medium.

Dehydrated culture media are available which have been pre-sterilized using gamma irradiation (25-45 KGray); reconstitution with sterilized water produces a sterile culture media. Gamma irradiated culture media are of a more consistent standard to those sterilized by other methods (Kim *et al.*, 2004).

1.3. Bacterial species

Several species of bacteria were used throughout this work in order to show that the effects of peptone on subsequently achieved bacterial growth were not limited to one strain or species. These bacteria encompass different serovars (*Salmonella* serovars Typhimurium and Poona), different conformations (rod and coccoid shapes) and different cell wall structures (Gram positive and negative).

1.3.1. *Salmonella*

A Gram-negative rod of 0.3-1.0 x 1.0-6.0μm dimensions, motile by means of peritrichous flagella, *Salmonella* is facultatively anaerobic with optimal growth at 37°C. Biochemical characteristics indicative of *Salmonella* in comparison with other Enterobacteriaceae include the production of H_2S and a negative indole reaction. On the selective differential medium, xylose lysine desoxycholate (XLD) agar, *Salmonella* colonies are red with black centres and are of 3-5 mm in diameter; a complete guide to media for this species is provided by Busse (1995). *Salmonella* are important in both medical and industrial environments, with differing serovars producing different disease: *Salmonella typhi* causes a severe systemic disease in humans known as typhoid or enteric fever whereas *Salmonella* Ser. Typhimurium is a food-borne organism causing localized gastroenteritis (food-infection).

1.3.1.1. *Salmonella* nomenclature

Salmonella nomenclature is complex and scientists use different systems with reference to this genus. The full lineage of the *Salmonella* sp. used throughout this work is:

Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Salmonella; Salmonella enterica; Salmonella enterica* subspecies (subsp.) *enterica; Salmonella enterica* subsp. *enterica* serovars (ser.) Typhimurium (Le Minor and Popoff) and Poona (Dunbar).

Modifications were proposed in response to phylogenetic species definition (Brenner *et al.*, 2000; Yabuuchi and Ezaki, 2000; Tindall *et al.*, 2005); confusion occurs between species and serovars of identical epithet, and serovars only designated due to medical importance (e.g. *Salmonella typhi* and *paratyphi*). Consequently several epithets are in use, to include *Salmonella choleraesuis* subsp. *choleraesuis.*

1.3.1.2. Identification of *Salmonella*

Salmonella are distinguished by serological methods which target their highly variable antigenic components. Antigens are presented as lipopolysaccharides (LPS) on the surface of the organism (O antigens) or within the protein-based motility organelle, the flagellum (H antigens), as shown in Figure 1.3. Some serovars, for example *S. typhi*, also present a V (capsular) antigen. The presence of flagella specific antigens was first recognized in *Salmonella choleraesuis* by Smith and Reagh (1903). The symbols O and H were respectively given to aflagellate and flagellate organisms by Weil and Felix (1918 a and b, 1920) after organisms which were able to spread (Hauch) and those unable to spread (Ohne Hauch). These have now been extended to indicate the somatic (O) and flagellar (H) antigens (Iino, 1969).

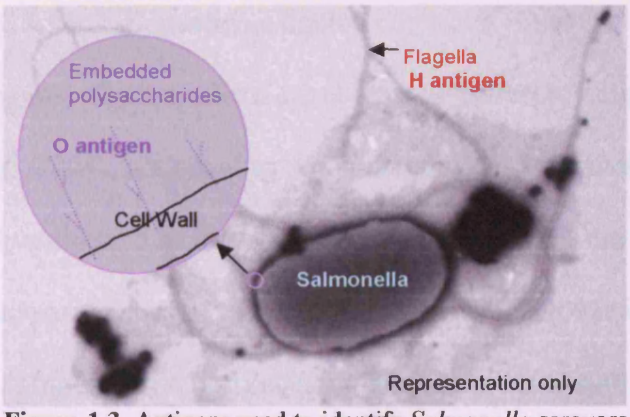

Figure 1.3. Antigens used to identify *Salmonella* serovars

Commercial sera containing specific antibodies to these antigens are available, thus contact between a matching antibody (sera) and antigen (*Salmonella*) results in visible agglutination or precipitation (Figure 1.4). The serovars are then identified according to their antigenic formulae: 2250 were documented by Le Minor and Popoff (1988), and this high variability is a result of the species being able to present more than one O or H antigen.

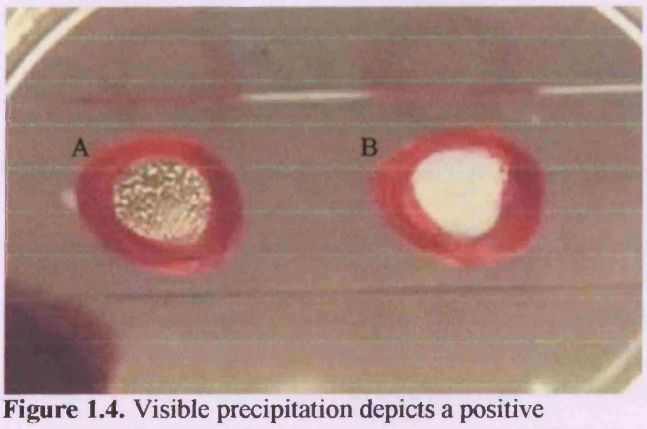

Figure 1.4. Visible precipitation depicts a positive antibody-antigen match (A) whereas an even suspension indicates a negative match (B).

Tables of antigenic formulae are available for identification purposes and these are named Kauffmann-White after their primary contributors Kauffmann (1966, 1978) and White (1926).

1.3.1.2.1. Somatic antigens

Somatic or O antigens, of which there are at least 70 different antigenic variations (Wildschutte *et al.*, 2004), are noted as Arabic numerals; *Salmonella* are divided into groups based on these. The more common serovars are in the first seven groups (A-G) which are recorded as upper case letters. A serovar is able to present more than one O antigen, and it is these antigens which determine host specificity. Many *Salmonella* are host specific, e.g. *S.* serovars Gallinarum, Typhisuis and Abortusovis infect avian, porcine and ovine species respectively. It is thought that this host specificity is based on the protozoan predators present in the host gut, as protozoan predators recognize antigenically diverse *Salmonella* with varying efficiencies based solely on the O antigen (Wildschutte *et al.*, 2004). However, complete identification of individual serovars requires that the flagellar or H antigens are also determined.

1.3.1.2.2. Flagellar antigens

There are three distinct flagellar antigens, which differ in antigenic specificity, and any *Salmonella* culture may possess only one kind or more, but will only express one type at any time. The differing antigen types are termed phases; some *Salmonella*, for example *S.* ser. Enteritidis, produce flagella which always have the same antigenic specificity and these are termed monophasic. Serovars which possess the ability to present two phases are diphasic (Andrewes, 1925); rarely three phases (triphasic), or more (complex organisms), can be expressed. These phases can then be switched alternately, for instance *S.* ser. Typhimurium generate flagella consisting of either i or 1, 2 antigen. A clone of an i producing organism would also produce i antigenic flagella; however, at a frequency of 10^{-3}-10^{-5},

daughter cells with 1, 2 flagella antigen would appear in the culture. As this organism is motile, inoculation onto semi-solid agar would produce growth throughout the medium; if i specific antiserum were added to the medium then those organisms expressing i antigen would become non-motile. The cells expressing the alternative phase (1, 2) would remain motile, and therefore would still spread throughout the agar. The opposite of this (adding 1, 2 specific antiserum to the medium) would result in *Salmonella* currently expressing the i antigen being motile and those presenting the 1, 2 antigen being non-motile. This is known as the H antigen phase-inversion method and is outlined, along with all serological methods for *Salmonella* identification, by Gruenewald *et al.*, (1990). It allows selection of organisms whereby their flagella phase does not correspond to the antiserum used and is widely accepted as the method used for complete identification of *Salmonella* serovars.

The antigens in phase 1 are presented as lower case letters from a-z; once z was reached, H antigens discovered since have been denoted as z_1, z_2 z_3 etc. Antigens z_2 and z_3 are as different as e and d. Antigens in phase 2 are given as Arabic numerals from 1-7, however a few lettered antigens also occur in phase 2: this inconvenience is the result of historical development of knowledge.

Flagellin, the monomeric unit of the flagellum within which the antigen properties are expressed, is a major pro-inflammatory determinant (Zeng *et al.*, 2003); the H antigens presented on the flagellum differ between the varying serovars due to a difference in the amino acid composition of flagellin (McDonough, 1965; Ada *et al.*, 1963), with the outer regions of the flagellin molecule being relatively more conserved throughout serovars. In contrast there is a great deal of variation in the central portion of flagellin (Ada *et al.*, 1963).

The antigenic formula for *Salmonella* ser. Typhimurium, a B group serovar, is:

1, 4, 5, 12 :i: 1, 2

whereas the antigenic formula for *Salmonella* ser. Poona, a G group serovar, is:

1, 13, 22 :z: 1, 6

- O antigens: **1, 4, 5, 12** for *S.*T. and **1, 13, 22** for *S.*P.
- H antigens- phase 1: **i** and **z** for *S.*T. and *S.*P. respectively
- H antigens- phase 2: **1, 2** and **1, 6** for *S.*T. and *S.*P. respectively.

1.3.1.3. *Salmonella* ser. Typhimurium

This organism is a ubiquitous enteric pathogen causing gastro-enteritis in humans and typhoid fever in mice; thus is a good model for research without using *Salmonella typhi,* an ACDP level 3 organism. Infection is usually the result of ingestion of contaminated foods such as meats, in significant numbers; a few organisms survive the stomach's acid environment to proceed into the distal ileum and colon. Here *S.* ser. Typhimurium adheres to the apical surface of the host epithelial cells, causing ruffling of the cell membrane and endocytosis of the organism within a vesicle. Fusion of the vesicle with a phagocytic vacuole is inhibited by S*almonella* as it secretes ammonium chloride; the organisms then go on to multiply within the un-fused vesicle and re-seed the intestine. In addition, Salmonella produces a thermo-labile entero-toxin (similar to *V. cholerae* toxin) which causes water secretion (Finkelstein *et al.*, 1983) and a cytotoxin which inhibits protein synthesis (Ashkenazi *et al.*, 1988); both of these toxins contribute towards diarrhoeal symptoms. This response protects the host, removing the pathogen quickly, but also allows vast numbers of *Salmonella* to re-enter the environment. *S.* ser. Typhimurium remains a localised infection except in the

young or elderly and those who are immuno-suppressed or immuno-compromised. Chemotherapy with cephalosporins constitutes effective treatment. The infection can be transmitted by the faecal-oral route; however basic hygiene standards will prevent transmission. *Salmonella* is designated as Advisory Committee for Dangerous Pathogens (ACDP) level 2, as human infection can be treated by chemotheraputic methods, and transmission is unlikely.

Historic and other given names for this species are:

- *Salmonella typhimurium* (Loeffler, 1892)
- *Bacillus typhimurium* (Loeffler, 1892)
- *Salmonella choleraesuis* subsp. *choleraesuis* ser. Typhimurium (Le Minor *et al*, 1982, 1986).
- *Salmonella* Typhimurium (Le Minor and Popoff, 1987)
- *S.* Typhimurium (Le Minor and Popoff, 1987)

Identifying strain numbers for different companies for this particular organism are:

- American type culture collection (ATCC) 14028
- Centre for disease control (CDC) 6516-60
- National culture type collection (NCTC) 12023

1.3.1.4. *Salmonella* ser. Poona

Salmonella ser. Poona presents in an identical manner to *S.* ser. Typhimurium: biochemically, phenotypically and the symptoms caused by the organism as a human pathogen. It differs only in serological composition and in the type of food-stuffs which it contaminates. This serovar is in group G and has the serological formula:

1, 13, 22 :z: 1, 6

The serovar reference number is NCTC 4840, which was deposited after isolation from an infant with enteritis. Most outbreaks in the US are associated with infected outer rind of cantaloupe melon imported from Mexico; but *S.* ser. Poona has also been known to contaminate poultry, other meats, eggs and dairy products. It is relatively rare, in comparison with *S.* serovars Typhimurium or Enteritidis, for *S.* ser. Poona to cause food borne disease (http://www.fehd.gov.hk).

1.3.2. *Escherichia coli*

The taxonomy of the *Escherichia coli* species used throughout this thesis is as follows:

Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Escherichia coli* (Castellani and Chambers).

The organism may also be referred to as *E. coli* and is supplied by the ATCC, strain number 10536; and the NCTC, strain number 10418. This particular strain is a control strain for the disk diffusion method of antibiotic susceptibility as laid out by the British Society for Antimicrobial Chemotherapy (BSAC); it is sensitive to most common use anti-microbials.

E. coli is one of the most commonly used bacterial species for research and hence its molecular biology, biochemistry and physiology are well documented. The species is a Gram negative rod, facultatively aerobic and is motile with peritrichous flagella (Figure 1.5). It characteristically produces indole at 37 °C and 44 °C and identification is, amongst others, by serological techniques to establish the O and H antigens (similar to identification of *Salmonella* spp). Growth on MacConkey agar, which distinguishes many coliforms, generates small red colonies which are non-mucoid.

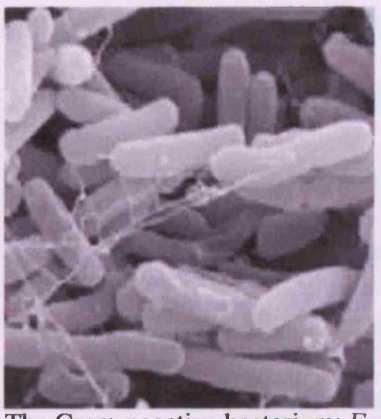

Figure 1.5. The Gram-negative bacterium: *Escherichia coli*.

E. coli is naturally present in the intestines of humans and most warm-blooded animals as a commensal organism, may even aid host nutrition by synthesis of vitamins (such as vitamin K). Certain serovars are, however, also pathogenic: *E. coli* spp. are the most common cause of human urinary tract infections (UTI), and infection may also cause suppurative lesions, neonatal septicaemias and meningitis. Animal infections include mastitis, pyometria in bitches and white scours in calves.

Generally, pathogenic *E. coli* species infect the gastrointestinal system; different serovars have different mechanisms of infection and thus symptoms vary in their severity.

- Entero-pathogenic *E. coli* (EPEC): these strains tend to cause infantile and traveller's diarrhoea; a non-invasive pathogen which predominantly affects the small intestine. The organism attaches and forms a pedestal like "attaching and effacing" (A/E) lesion (Knutton *et al.*, 1987). This is an intimate adherence (i.e. host cell and pathogenic organism in direct contact) which is in contrast to a non-intimate adherence, whereby adhesins project from the organism to the host cell, such as that observed during infection of enterocytes with *Vibrio*

cholerae. Once attached EPEC then triggers a signalling cascade which, by an unknown mechanism, triggers diarrhoea (Mims *et al.*, 2001).

- Entero-toxigenic *E. coli* (ETEC): infection results in gastroenteritis in children and adults caused by consumption of contaminated water or food (such as uncooked or undercooked meat). ETEC produces entero-toxins which vary in that some are heat stable toxins (ST) and others are heat labile (LT). ST's act on the cell membranes of enterocytes, binding to a receptor which activates guanylate cyclase, this in turn generates a signal to the enterocytes causing it to elevate internal concentrations of cGMP. Increasing cGMP results in a higher rate of ion efflux by the enterocytes, and consequently water, which produces diarrhoea. ETEC remains in the lumen of the host cell, it is non-invasive.
- Entero-invasive *E. coli* (EIEC): causes a disease similar to *Shigella* displaying classic bacillary dysentery. The intestinal epithelium is invaded through the baso-lateral membrane and intra-cytoplasmic replication occurs. Again toxins are also secreted similar to ETEC however this strain is invasive.
- Vero-toxigenic *E. coli* (VTEC): also known as entero-haemorrhagic *E. coli* (EHEC) is thought to cause haemolytic-uraemic syndrome and, in contrast to EPEC, colonises mainly the colon and is able to invade sub-epithelial tissues. Serotype O157:H7 has caused several serious outbreaks which have caused fatalities. This is due to the exo-toxin causing both haemorrhagic diarrhoea and kidney failure.
- Uro-pathogenic *E. coli* (UPEC): these organisms invade the uro-genital system by means of a pilus (fine hair) projecting from the cell surface. These

pili adhere to uroepithelial cells which prevents the bacteria being flushed away during urination.

(Neidhardt, 1987; Madigan *et al.*, 2000; Mims *et al.*, 2001; Collins and Lyne, 2004).

1.3.3. *Staphylococcus aureus*

The taxonomy of the staphylococcal species used in this work is as follows: Firmicutes; Bacilli; Bacillales; Staphylococcaceae; *Staphylococcus; Staphylococcus aureus; Staphylococcus aureus* subsp. *aureus* Rosenbach (also known as the Oxford strain).

The organism may also be referred to as *S. aureus*; and is supplied by the ATCC, strain number 9144, and the NCTC, strain number 6571. This particular strain is a control strain for the disk diffusion method of antibiotic susceptibility as laid out by the British Society for Antimicrobial Chemotherapy (BSAC); it is sensitive to most common use anti-microbials.

S. aureus is a Gram positive, facultatively anaerobic bacterium of coccoid conformation which is non-motile (Figure 1.6). The species is distinguished as being coagulase and phosphatase positive, producing a golden yellow pigment and, under microscopic examination, growth in clusters which resemble a bunch of grapes. On Baird-Parker medium (a yellow, opaque medium) *S. aureus* colonies are convex, shiny and black measuring ~1-1.5 mm in diameter. These colonies are surrounded by a zone of clearing which is between 2-5 mm.

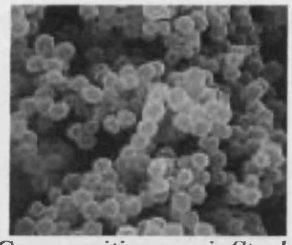

Figure 1.6. The Gram-positive cocci: *Staphylococcus aureus*

This species is a common skin commensal and is present within the nasal cavity, on skin and in the hair of a large proportion of the population. Infection generates many different diseases including abscesses, septicaemia, food poisoning and impetigo.

Strains of *S. aureus* which are common pathogens secrete extra-cellular enzymes (e.g. haemolysins) or toxins (e.g. entero-toxins); the ability to produce coagulase, which clots fibrin, allows the organism to seal itself off providing protection from an immune response. The secretion of leukocidin destroys any leukocytes able to reach *S. aureus*; together these defences result in the ability to escape phagocytosis. Human illness as a result of food poisoning occurs after the ingestion of 10^4-10^5 cells, onset of symptoms begins 1-7 hours after ingestion and lasts for up to 2 days. The resulting symptoms are due to the secreted staphylococcal entero-toxins (A, B, C, D and E, which are super-antigens and act in the intestine only) which give rise to severe diarrhoea, vomiting and shock; stimulation of the vagus nerve by these toxins causes the sudden onset of sickness. Contamination of the food is caused by human error: food stuffs mainly affected are fish, prawns and cream products being processed and stored at inappropriate temperatures (Mims *et al.*, 2001).

Some staphylococci strains cause toxic shock syndrome (TSS) which is a systemic disease. Previously it was most frequently seen in menstruating women wearing tampons which supported growth of the staphylococci. The TSST-1 (toxic shock syndrome toxin 1) is able to traverse the vaginal-mucosal barrier and cause onset

of fever, vomiting, diarrhoea, erythematous rash followed by peeling skin, distributive shock leading to impairment of renal and hepatic functions and sometimes death. TSST-1 is only found in menstruating women but other TSS toxins, and similar toxins produced by streptococci, cause similar problems (Madigan *et al.*, 2000; Mims *et al.*, 2001; Collins and Lyne, 2004).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a growing concern for today's clinicians, particularly with respect to nosocomial infections; these strains often exhibit resistance to multiple antibiotics and consequently are difficult to treat.

1.4. Microbial growth

The term microbial growth refers to an increase in the biomass of a population and possibly, for most bacteria, an increase in numbers. For many prokaryotes, including the bacteria studied in this work, an individual organism continues to grow and elongate until a partition begins to form, resulting in separation of the bacterium into two new daughter cells; this process is called binary fission (Figure 1.7). Each new daughter cell then has the potential to repeat this process which gives rise to an exponential increase in cell numbers.

The ability of microbiologists to assess accurately the growth of bacteria without great expenditure of time and non-invasive procedures is limited, with our inability to culture many species highlighted by techniques such as molecular probes. Molecular biology demonstrates a wealth of new bacteria which are un-culturable using standard resuscitation procedures: these organisms are viable, able to increase in numbers given favourable conditions, yet remain undetected by culture techniques as media in common use do not support growth. Viable but not-

culturable organisms are discussed by Bloomfield *et al.*, (1998) who clearly state that these bacteria, in particular the entero-pathogens, are not un-culturable: "we are simply failing to provide appropriate conditions to support culture". Lloyd and Hayes (1995) demonstrate new methods to estimate microbial numbers, such as flow cytometry and confocal laser scanning microscopy, which allows non-invasive data for those cells which are vital but not viable.

Naturally the content of the growth medium is critical for resuscitation and subsequent enumeration of species which are difficult to culture; this again indicates the need to re-consider these methods which have remained largely unchanged. The sensitivity of bacterial growth to environmental stimuli allows considerable insight into the effect that experimentally controlled variables have upon the population; this information is invaluable to the researcher and thus has led to multiple methods of monitoring microbial growth to have been developed.

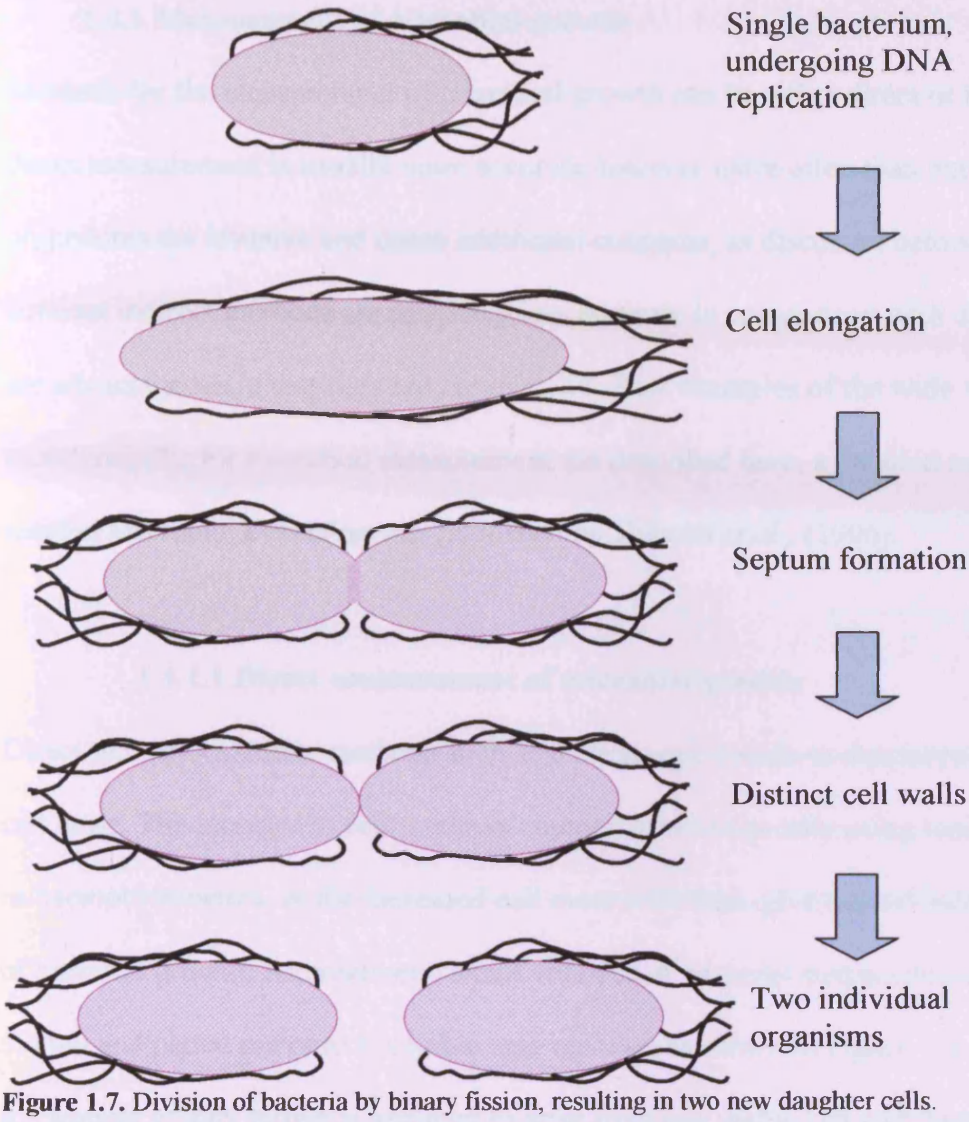

Figure 1.7. Division of bacteria by binary fission, resulting in two new daughter cells.

1.4.1 Measurement of microbial growth

Methods for the measurement of microbial growth can be either direct or indirect; direct measurement is usually more accurate however more often than not these procedures are invasive and cause additional concerns, as discussed below. In contrast indirect methods are relatively less accurate in comparison with direct, yet are advantageous in that they are not invasive. Few examples of the wide variety of tools available for microbial measurement are described here; a detailed review entitled Microbial Detection was published by Hobson *et al.*, (1996).

1.4.1.1 Direct measurement of microbial growth

Direct techniques utilize methods such as microscopic counts or measurement of cell mass. The increase in cell numbers counted microscopically using tools such as haemocytometers, or the increased cell mass with time, give a good indication of bacterial growth. Alternatively, small volumes of bacterial suspension may be diluted and plated out onto a suitable agar medium as shown in Figure 1.8; each subsequent colony grown is assumed to arise from one viable cell and therefore the unit of measurement is termed colony forming units (CFU). This technique is useful as it distinguishes between viable cells and dead cells: microscopic and mass methods count all cells. A calculation to include dilutions and sample volume plated out onto the agar give the units CFU ml^{-1}. For microscopic methods a similar calculation accounts for the dimensions of the counting device (e.g. haemocytometer). Flow cytometry and confocal laser scanning are also useful tools for measurement of cell numbers, with the addition of dyes distinction between living and dead cells can also be achieved (Lloyd and Hayes, 1995).

Sequential measurements over time depict the increase in cell numbers or mass and can be plotted to produce a growth curve, this can be used to analyse growth dynamics and is discussed below.

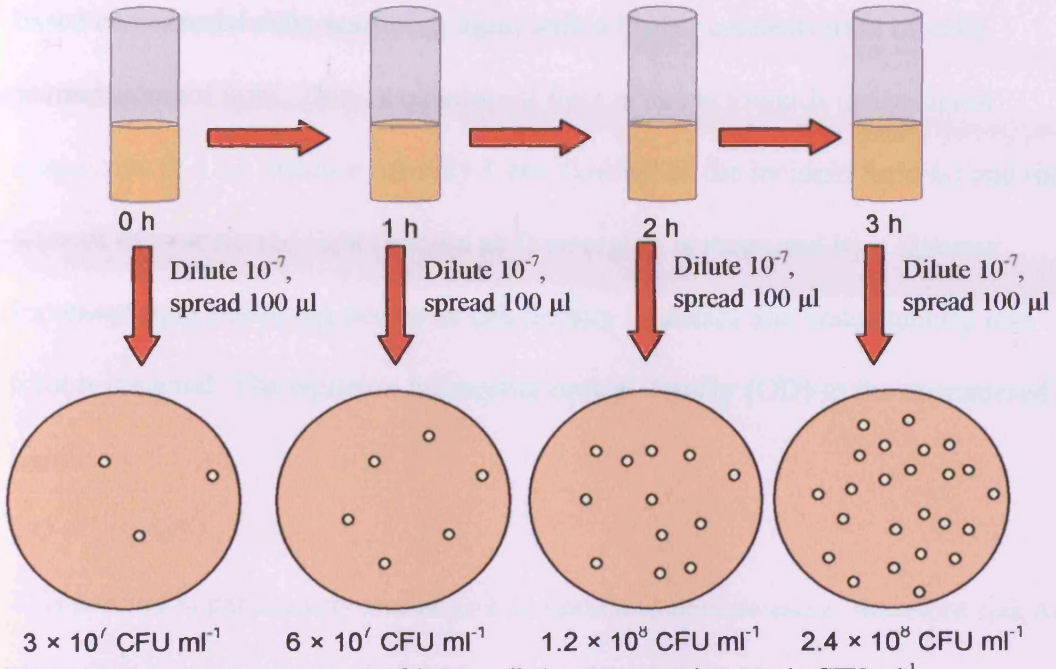

Figure 1.8. Growth measurements of *Salmonella* bacteria as an increase in CFU ml^{-1}

The disadvantage of these methods is that they all require a sample to be removed from the experimental culture at every time point (where growth kinetics are being measured). This invasive procedure decreases the culture volume which may affect growth kinetics between time points. For example, initially there would have been a greater volume of media, yet as sampling continues, the volume of bacterial suspension within the growth vessel will decrease; oxygen will diffuse more easily through a higher surface area to volume ratio and thus as more samples are removed, oxygen will become more readily available. This increase in oxygen availability will affect growth kinetics and is difficult to compensate for, thus skewing experimental proceedings. In addition, with each sample removal, risk of contamination is greater.

1.4.1.2 Indirect measurement of microbial growth

Turbidimetric measurements are a more convenient way of estimating cell numbers in a given population, and are achieved using a spectrophotometer. This method is based on bacterial cells scattering light, with a higher concentration of cells scattering more light. Thus, a quantity of light is shone towards the bacterial suspension at a set distance, usually 1 cm, (known as the incident light I_0) and the amount of unscattered light (known as I) emerging is measured by a detector. Increased light scattering occurs as cell density increases and consequently less light is detected. The equation measuring optical density (OD) to the unscattered light is:

$$OD = \text{Log } I_0/I$$

This method is particularly useful as it is rapid and non-invasive, therefore risk of contamination is significantly lower and artefacts attributable to sample volume removal do not result in unwanted variation of growth conditions. Again, OD measurements do not distinguish between viable and non-viable cells and, as an indirect method, error may occur when calculating cell numbers from the OD readings. Calculation of numbers from OD is performed using a calibration curve, as shown in Figure 1.9. The construction of a calibration curve is performed by diluting an overnight culture (diluent should be the relevant sterile growth medium) and measuring the OD of each dilution. These dilutions are then plated out onto a suitable agar (e.g. nutrient agar) and plate counts performed after 24 h. Cell numbers can then be plotted against OD, this graph can then be used to convert OD readings into cell numbers. The curve is specific to each spectrophotometer and species of bacteria, each machine differs and a coccus will scatter light differently to a vibrio.

The use of automated growth analysers which record optical density allow many growth curves to be produced at one time (Stephens *et al.*, 1997; Johnston, 1998), again this increases the rapid achievement of results. The methods used in this thesis are described in detail during Chapter 2.

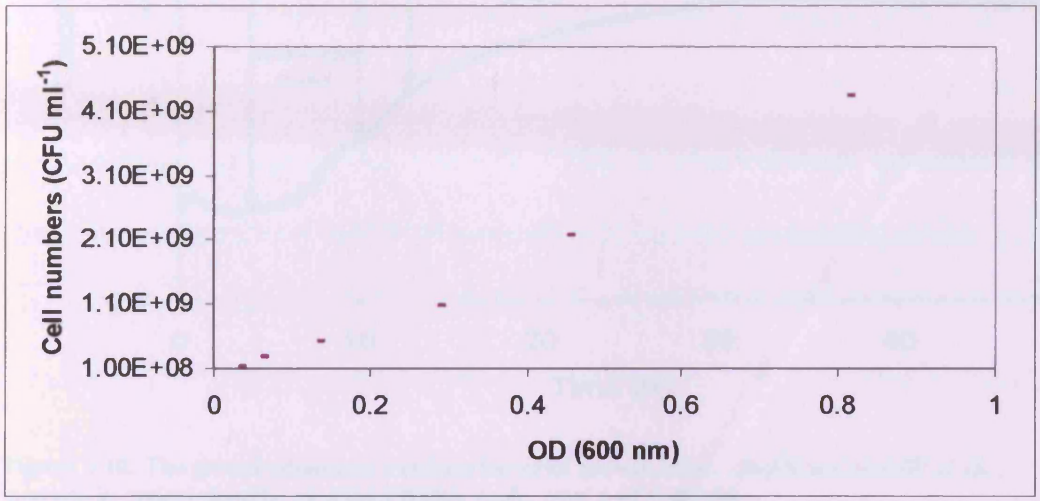

Figure 1.9. Calibration curve relating optical density to cell numbers for *Salmonella* ser. Typhimurium grown in S5 peptone-based BPW, performed on an automated growth analyser.

1.4.2 Analysis of microbial growth

Growth measurements over time, obtained using the techniques described above, give data which are usually plotted on a semi-logarithmic graph, with the logarithm of OD on the y-axis and time plotted arithmetically on the x-axis (Figure 1.10). Historically, Ward (1895) first presented his growth observations in this manner and went on to formulate basic concepts about microbial kinetics; his work included the effects caused by different environmental variables, e.g. temperature, illumination and nutrient concentration. Concurrently, Müller (1895) established the existence of distinct phases of growth: lag, logarithmic (log) or exponential, stationary and death phase which are clearly labelled in Figure 1.10.

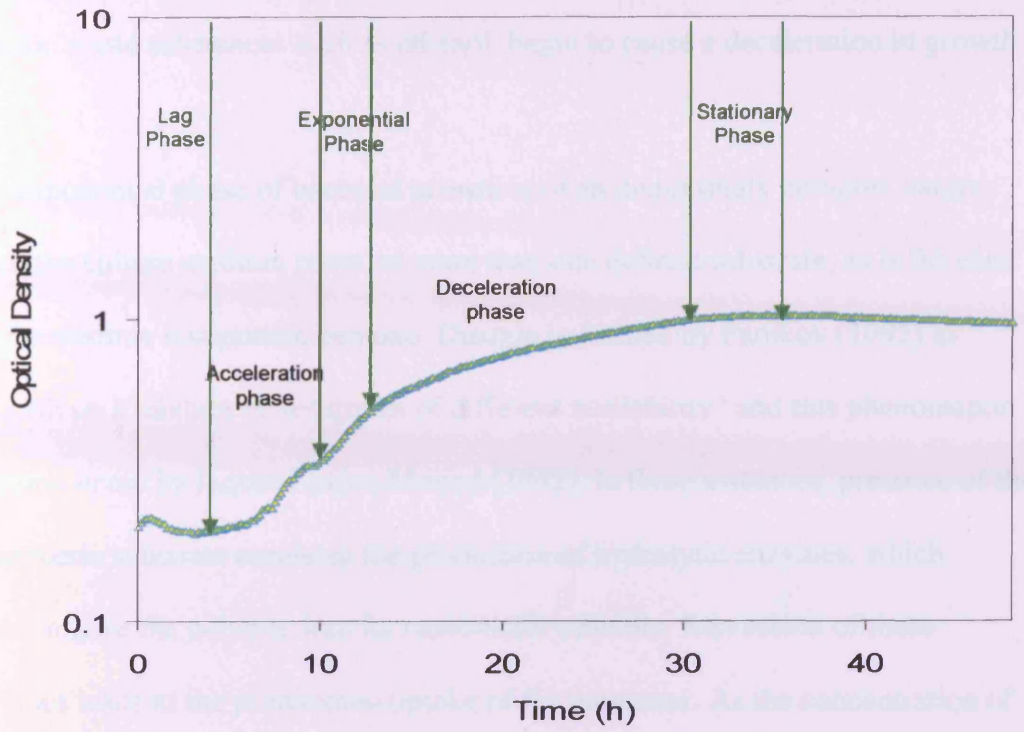

Figure 1.10. The growth phases of a classic bacterial growth curve: displaying growth as an increase in optical density, on a logarithmic scale, over a 48 h period.

Lag phase: this period of growth is a prolonged latency which is due to 1) the time taken to synthesize some intermediate metabolite or an enzyme 2) the need to alter the environment in order that it is suitable for subsequent growth (for example the production of CO_2) 3) recovery from altered environment and resultant stresses 4) cell inertia (Penfold, 1914).

Exponential phase: this refers to the point in which the population is approximately doubling with every unit of time, resulting in a straight line where data is plotted on a logarithmic axis. The increase in cell numbers (or cell mass, OD) per unit time is termed the growth rate, whereas the time taken for an for an organism to reproduce is known as the mean generation time (culture doubling time in reference to the population). Exponential growth occurs for a limited period in

batch culture as other factors, for example nutrient/oxygen availability or build up of toxic waste substances such as ethanol, begin to cause a deceleration in growth.

The exponential phase of bacterial growth is of an increasingly complex nature when the culture medium contains more than one defined substrate; as is the case for the medium component peptone. Diauxie is defined by Panikov (1995) as "growth on a mixture of substrates of different availability" and this phenomenon was first noted by Jaques Lucien Monod (1942). In these instances, presence of the monomeric substrate represses the production of hydrolytic enzymes, which depolymerise the polymer into its monomeric subunits. Repression of these enzymes leads to the preferential uptake of the monomer. As the concentration of the monomeric substrate decreases, the hydrolytic enzymes are no longer repressed and depolymerization resumes; thus leading to a second latent growth period whilst the polymer is depolymerized.

Diauxie may also be exhibited when two different substrates are present in the medium and preferential uptake of one substrate occurs, similarly resulting in a second lag phase as the organism re-adjusts to the new substrate. Graphically this phenomenon gives the growth curve more than one exponential phase, as shown in Figure 1.11.

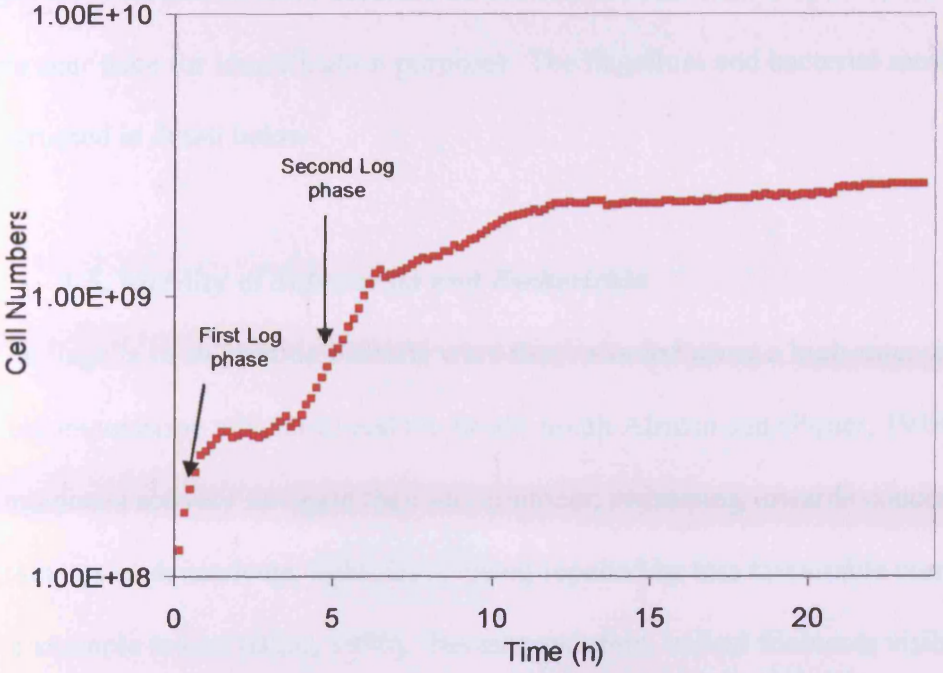

Figure 1.11. A diauxic growth curve, depicting cell numbers over a 24 h time period.

Stationary phase: as the culture continues to decelerate it will eventually reach stationary phase, this is where there is no net increase or decrease in cell number. Death phase: following stationary phase the bacterial culture may begin to die, leading to the death phase where a marked decrease in cell number is observed.

This thesis continues to explore the effect of peptone upon the morphology of Enterobacteria with particular note to the flagellum. The nutrient content of the growth medium and its subsequent effect upon motility has been shown in previous works: *Salmonella* ser. Pullorum is noted under the Kauffman-White identification system as being non-motile and aflagellate however, upon addition of maltose, the organism displays motility (Harshey and Matsuyama, 1994; Guard-Petter, 1997). The opposite effect, i.e. suppressing motility, has even been commercially exploited; for example cystine-lactose-electrolyte deficient (CLED) agar is, as its name suggests, deficient in electrolytes in order to prevent swarming of *Proteus*

species. This allows more accurate distinction between mixed-species colonies on the agar plate for identification purposes. The flagellum and bacterial motility are discussed in detail below.

1.5. Motility of *Salmonella* and *Escherichia*

The flagella of swimming bacteria were first recorded using a high-intensity dark-field microscopy which utilized the bright South African sun (Pijper, 1948). Organisms actively navigate their environment; swimming towards concentration gradients such nutrients, light, O_2 or being repelled by less favourable compounds, for example toxins (Blair, 1995). The external, thin, helical filaments visible by electron or light microscopy make up only a portion of the motility organelle which is collectively known as the flagellum. These filaments are arranged peritrichously (all around the organism) with wild-type *Salmonella* ser. Typhimurium usually possessing between 5-10 flagellar filaments (Aizawa & Kubori, 1998; Manson *et al.*, 1998). Varying flagellar arrangements are noted in prokaryotes, for example the flagella may be attached at the ends of the organism (polar) or in small tufts (termed lophotrichous). Propulsion through semi-solid or liquid medium is by rotation of these filaments which are driven by a motor at the filament base. The filament is joined to a segment called the hook, which is itself then connected to a series of rods and rings known as the basal body (Figure 1.12).

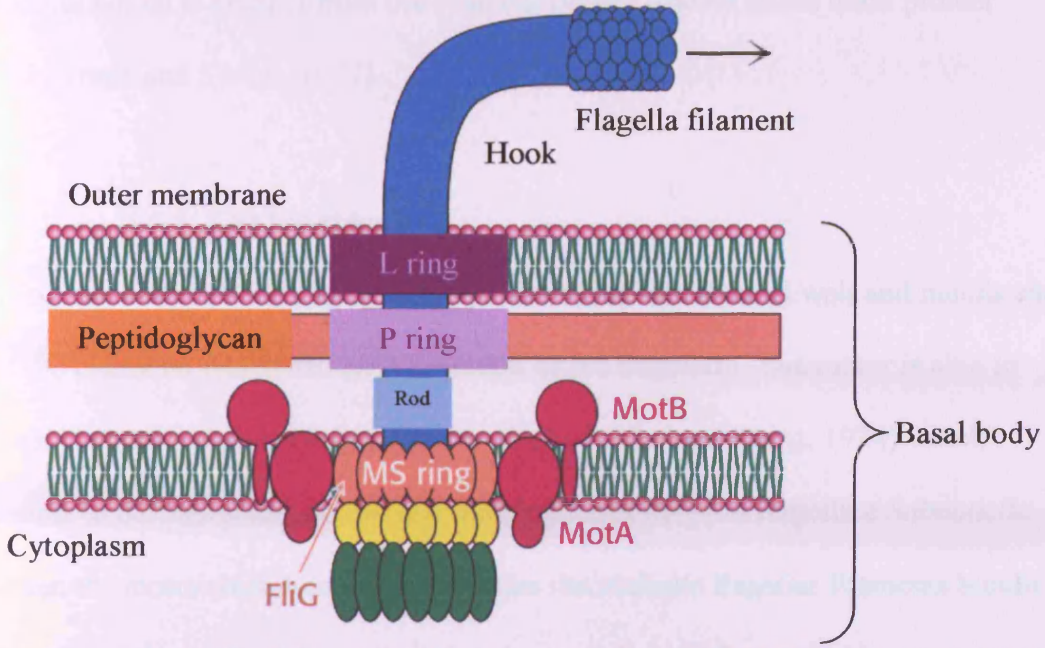

Figure 1.12. Pictorial representation of the flagellar apparatus

1.5.1. The flagellar filament

The filament is helically arranged and typically of a length between 5 to 10 µm, diameter of ca. 20 nm with a wavelength of 2 to 2.5 µm (Macnab, 1987). As previously mentioned above it is made up of a single protein subunit called flagellin which is also responsible for the variation in H antigens; this monomeric subunit has a molecular weight of around 55000 (Khondoh and Hotani, 1974).

10 Presence of flagella may indicate motility and is a useful tool for identification; consequently many methods for staining and subsequent visualization, predominantly by microscopic methods, are recorded in the literature (Leifson, 1951; Leifson *et al.*, 1955; Hodaka *et al.*, 1982; Grossart *et al.*, 2000).

1.5.2. The hook

The filament is connected to the main body of the organism by the hook, this acts as a flexible joint between the filament and the bacterial cell. The hook contains a

subunit which is distinct from the filament protein known as the hook protein (Silverman and Simon, 1977).

1.5.3. The basal body

The basal body is a structural complex embedded into the cell wall and membrane of the organism which drives the rotation of the flagellum. The motor is able to rotate in both a clockwise and counter clockwise fashion (Berg, 1974) which results in the characteristic run and tumble phases noted in flagellate *Salmonella*. When the motor rotates counter-clockwise the multiple flagellar filaments bundle together at the back of the organism and propel the cell forwards. However, once the motor switches to a clockwise rotation the individual filaments are pushed outwards in different directions, causing a tumble (Figure 1.13).

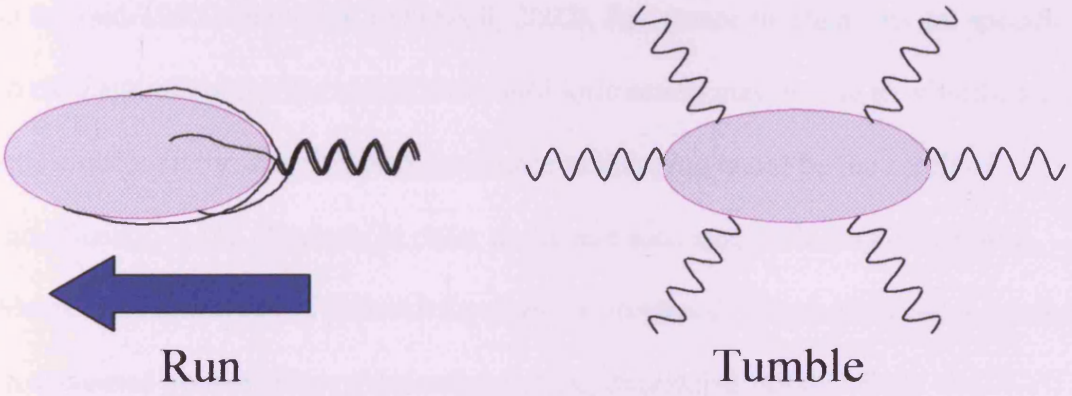

Figure 1.13. Peritrichously arranged flagella bundle at one end and propel the organism forwards. In contrast, when the flagella are splayed outwards in individual directions a tumble occurs.

Energy to drive the motor is derived from a proton motive force (PMF) (Manson *et al.*, 1977; Khan and Macnab, 1980) which is powered by the flux of ions across the cytoplasmic membrane. However a PMF is not the only source of energy able to drive the prokaryotic flagellar motor: a sodium motive force (SMF) has also shown to be capable (Hirota and Imae, 1983). In addition lithium ions are able to support

flagellar rotation using the Na+ motors (Liu et al., 1990) with deuterium performing a similar function utilizing the H+ motors (Chen and Berg, 2000).

Phenotype was also noted to alter dependent on the constituents of the culture medium and this thesis explores the effect that variation of peptone has on antibiotic susceptibility testing.

1.6. Antibiotic susceptibility testing

Zoonotic pathogenic bacteria such as *Salmonella* ser. Typhimurium are known to be resistant to commonly used antibiotics, and these resistant organisms are now widespread. *Salmonella* with multiple antibiotic resistances, where the bacterium exhibits resistance to four or more antimicrobial agents, were found in this serovar in the mid-1960's in the UK (Threlfall, 2002). Resistance mechanisms are specific to each antimicrobial; for instance the antibiotic action may be due to inhibition of ribosomal activity, and therefore resistance to this drug could be the result of modification to the ribosome in order to prevent subsequent binding of the drug. Mechanical resistance of *Salmonella derby* to streptomycin was observed to be due to decreased permeability of the cell envelope, decreasing uptake of the antimicrobial, as well as modification of the ribosomes (Ktsonian *et al.*, 1988). As classes of antimicrobial have different sites of activity, and consequently resistance mechanisms of the organisms differ, each class will be detailed separately.

1.6.1. Antibiotic classes

Antibiotics are categorized based on their structure and mode of action, some compounds being analogs of amino acids whereas others contain carbohydrates, to name a few examples.

1.6.1.1. Aminoglycosides

The aminoglycosides (amikacin, gentamicin and streptomycin are used in this thesis) are carbohydrate containing compounds which generate a bactericidal effect from two sites: the membrane and the ribosomes. Binding of the antibiotic to the outer membrane protein (OMP) creates holes in the membrane, causing the cytoplasm to leak into the external environment; the reduced integrity of the membrane serves to increase antibiotic uptake, thus making the drug increasingly effective. Additionally aminoglycosides act by inhibiting protein synthesis at the 30 s subunit of the ribosome.

Gentamicin is produced by *Micromonospora* species, the compound is basic and water soluble with a broad spectrum of activity. Aminoglycosides are used therapeutically for many infections, from septicaemia to pneumonia. They are all bactericidal and are active against some Gram-positive and many Gram-negative organisms. Streptomycin is active against *Mycobacterium tuberculosis* and is now reserved for this purpose only; amikacin is usually only prescribed for gentamicin-resistant Gram-negative bacilli (Longmore *et al.*, 2001).

Resistance to the aminoglycosides is usually due to modification of the ribosome subunits or OMP.

1.6.1.2. β-Lactam antibiotics

The group of antibiotics which contain the β-lactam ring structure are of great medical importance, the group encompasses the penicillins, cephalosporins and the cephamycins. In this work only the penicillin and cephalosporin classes are investigated, which together account for over half of the available antibiotics, and therefore the cephamycins are not discussed further.

Ampicillin, a semi-synthetic penicillin, inhibits cell wall synthesis by binding to the penicillin binding proteins preventing the cross linking of peptidoglycan. This effect generates a very weak cell wall which then lyses under osmotic pressure (Madigan *et al.*, 2000). Ampicillin is active against some Gram-positive and – negative organisms however it is rendered inactive by any species which produce the enzyme penicillinase. Species capable of this include *Staphylococcus aureus*, 50% of *Escherichia coli* strains and 15% of *Haemophilus influenzae* strains (British National Formulary, 2006). Inactivation of the drug, reduced cell permeability, alteration of binding proteins and the production of penicillinases contribute to resistance mechanisms found in β-lactam antibiotics.

Cephalosporins are derived from cephalosporin C which is produced by the fungus *Cephalosporium acremonium* (Kavanagh, 1972). Mode of action is similar to ampicillin and cephalothin (the cephalosporin investigated in this thesis) is a broad spectrum, semi-synthetic cephalosporin produced from the reaction of thiophene-2-acetyl chloride with 7-aminocephalosporanic acid. *In vivo*, cephalothin is partially degraded to deacetylcephalothin which is a biologically active metabolite displaying a similar antimicrobial spectrum to cephalothin. Cephalosporins are used in the treatment of many infections to include urinary tract infections.

1.6.1.3. Polymyxins

Colistin sulphate is a polymyxin with activity against the Gram-negative organisms to include *Pseudomonas aeruginosa*, however it is highly toxic and therefore is rarely used therapeutically. The mode of action is against the cytoplasmic membrane structure and resistance can be due to the binding of LPS to the polymyxin, preventing access to the organism.

1.6.1.4. Quinolones

Ciprofloxacin is member of the synthetic quinolone family, a derivative of nalidixic acid. The quinolones target the DNA gyrase of the bacterium, thus preventing supercoiling of the DNA. Ciprofloxacin is active against both Gram-positive and –negative however it is particularly useful for the treatment of *Salmonella, Shigella, Neisseria, Pseudomonas* and *Campylobacter*; where the affected area is the respiratory or urinary tract, or the gastro-intestinal system. Alteration of the target DNA gyrase site results in resistance.

CHAPTER TWO

Materials and Methods

2. Materials and methods

2.1. Culture media

The reproducibility of a consistent culture medium allowing good growth is essential to both research and industry. Often, undefined and complex ingredients are the cause of variation in growth media (Hyde and Denton, 1987). One such ingredient is peptone and the analysis of different peptones based upon microbiological performance and chemical constitution forms the foundation for this work.

Peptone is the only undefined ingredient in buffered peptone water (BPW), the simple enrichment broth containing only NaCl and phosphate buffer in addition to peptone. Thus this medium was varied by making up many different peptones in order to analyse microbiological performance of each peptone in turn as the sole substrate.

BPW was made up with 10 g l^{-1} peptone, using material from various sources as described in Table 2.1., together with 5 g l^{-1} NaCl (BDH), 3.5 g l^{-1} di-sodium hydrogen phosphate (BDH) and 1.5 g l^{-1} potassium di-hydrogen phosphate (BDH). A comprehensive list of peptones, from which each was selected, is given in Appendix 1, Table 1.1.

Other culture or selective-differential media used throughout this work, and the details of procedure by which they are routinely prepared in accordance with manufacturer's instructions, are detailed in Appendix 1, Section 1.2. Nutrient Agar (NA), Nutrient Broth (NB), Tryptone Soya Agar (TSA) and Tryptone Soya Broth (TSB), all manufactured by Oxoid, were employed as non-selective growth media, principally for inoculum preparation or culture storage.

Table 2.1. Peptones selected and investigated throughout this work. Where identical product numbers are listed the materials originate from different batches.

Code	Product Name	Source	Digest	Manufacturer	Product Number
C1	Casein	Casein	Pancreatin	Merck	1.07213
C2	Casein I		Pancreatin	HiMedia	RM014
C3	Casein: Technical		Acid HCl	HiMedia	RM013
C4	Casein: Vitamin Free		Acid HCl	Merck	1.02238
C5	Tryptone		Enzymatic	Organotechnie	19553
C6	Casein		Pancreatin	Merck	1.07213
C7	Casein		Acid HCl	DMV	CE 90 ML
C8	Casein		Acid HCl	DMV	CE 90 ML
G1	Gelatin	Gelatin	Pancreatin	Merck	1.07284
G2	Gelatine		Pancreatin	HiMedia	RM020
G3	Gelatin		Pancreatin	Merck	1.07284
G4	Gelatin		Pancreatin	Merck	1.07284
G5	Gelatone		Pancreatin	Becton-Dickinson	265710
M1	Meat Extract	Meat	Proteolysis	Merck	1.03979
M2	Meat Peptone (Bovine)		Pancreatin	Biotecnica	102
M3	Meat Peptone		Pancreatin	Merck	1.07214
M4	Meat Peptone (Porcine)		Pancreatin	Biotecnica	307
M5	Meat Peptone (Porcine)		Pancreatin	Biotecnica	307
M6	Liver Powder		No digestion	Merck	1.05347
S1	Peptone: Soymeal	Soy	Papain	Solabia	A1601
S2	Soytone Peptone		Papain	Becton-Dickinson	243620
S3	Soy		Papain	Biospringer	HSP-A
S4	Neutralised Soya		Papain	Oxoid	L44
S5	Peptone: Soymeal		Papain	Solabia	A1601
Y1	Yeast Extract	Yeast	Autolysed Extract	Merck	1.03753
Y2	Bacto-Yeast Extract		Autolysed Extract	Becton-Dickinson	212750
Y3	Yeast Extract		Autolysed Extract	Merck	1.03753
Y4	Yeast Extract		Autolysed Extract	Merck	1.03753

The chemically-defined recipe for M9 medium (Neidhardt *et al.* 1974), containing no complex ingredients, acted as a control medium. Selective differential media for *Salmonella* used throughout to ensure culture purity were: Xylose Lysine Desoxycholate (XLD), Brilliant Green modified (BG) and Hektoen Enteric (HE) agars, all manufactured by Oxoid. Colony morphology on the respective agars is detailed in Appendix 1, Section 1.2. For antibiotic susceptibility testing, ISO sensitest agar (Oxoid) was used as appropriate to that specific application.

2.1.1. Sterilization of culture media

All media, unless otherwise specified, were sterilized by autoclaving (Astell) for 15 min at 121°C.

Where indicated, filter sterilization was employed, using a disposable 32 mm, 0.2 µm Supor membrane (Pall Acrodisc®).

The subsequently employed methods applied during the course of this work fall into two categories: those for the assessment of microbiological performance of each medium, and those used to analyse chemical and physical properties.

2.2. Microbiological methods

The methods in this section are used for investigation of microbiological performance of the medium; that is to say the ability to recover, enumerate and sustain growth of bacterial species. Other factors which may have affected bacterial growth, for example NaCl, pH, temperature and atmospheric pressure (Van Schothorst *et al.*, 1977; Thayer *et al.*, 1987) were unaltered for all studies.

2.2.1. Bacterial species

Several bacterial species were employed to assess performance of the different BPW media in order to confirm that the effects observed were general and not merely limited to a single strain or species.

2.2.1.1. *Escherichia coli*

The *E. coli* strain used during this work was ATCC 10418, kindly supplied by Gareth Williams, Cardiff School of Biosciences.

2.2.1.2. *Salmonella* ser. Poona

Salmonella enterica subspecies *enterica* Serovar Poona (*SP*) NCTC 4840, initially cultivated from a Twister® culture (Prolab), ensured that results obtained were not an unusual trait of the *ST* Serovar alone.

2.2.1.3. *Salmonella* ser. Typhimurium

Salmonella enterica subspecies *enterica* Serovar Typhimurium (*ST*) ATCC 14028, initially cultivated from a Twister® culture (Prolab), was the predominant Serovar utilized in all research described in this thesis.

2.2.1.4. *Staphylococcus aureus*

Staphylococcus aureus subspecies *aureus* (*Sa*) was the Oxford strain ATCC 9144, kindly supplied by Paul Seaman, Cardiff School of Biosciences.

2.2.2. Maintenance and storage of cultures

Cultures were maintained on NA (Oxoid) slopes at 4°C, and sub-cultured every 14 days. Freezer stocks on beads (Prolab) were kept at -20 °C.

2.2.3. Preparation of inocula

Experimental inocula were prepared by streaking out the desired bacterium from a NA slope onto a NA plate and incubating overnight at 37 °C. A single colony was then selected and transferred to 100 ml BPW (Merck) in a 500 ml culture flask. This was incubated overnight in a reciprocating water bath (Gallenkamp) at 37°C, 100 strokes min^{-1}. Aliquots (5 ml) of the suspension were dispensed into sterile containers and kept at -20 °C, ensuring similar inocula for all related experiments; cultures were thawed at ambient temperature prior to use. Purity of culture was ensured by streaking out the inoculum onto XLD, HE and BG: the plates were then incubated as detailed previously and examined for appropriate colony morphology.

2.2.3.1. Optimized inocula

Experimental media were inoculated with the prepared inocula as described in 2.2.3.

2.2.3.2. Heat-shocked inocula

Prepared inoculum (1.0 ml), as described in 2.2.3, was transferred to a 1.5 ml eppendorf tube and incubated for 15 min at 60 °C in a water bath. This heat-shocked culture was then immediately used to inoculate experimental media.

2.2.4. Analysis of bacterial growth

Growth of bacterial species was assessed by direct plate counts and growth curves.

2.2.4.1. Plate counts

Plate counts were performed to determine the number of organisms per ml of a given culture. The bacterial suspension was diluted with sterile PBS (Oxoid) by serial decimal dilution down to 10^{-8} using aseptic technique. The "drop count" method devised by Miles and Misra (1938) was then performed: three drops of 10 µl per dilution were expelled onto a NA plate using a pipette, as shown in Figure 2.1.

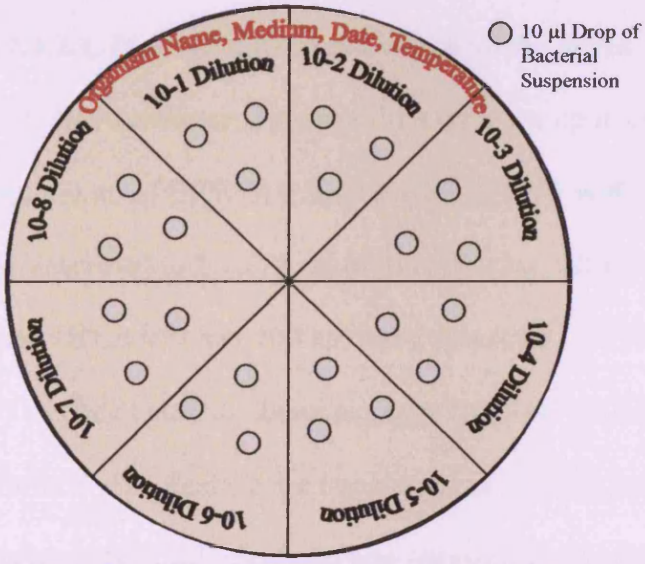

Figure 2.1. Miles and Misra drop count technique

The droplets were allowed to dry, the plate was then incubated at 37 °C for 24 h; subsequently developed colonies were counted and the initial CFU ml^{-1} calculated using:

CFU ml^{-1} = Number of colonies counted × 100 (10 µl to ml) × Dilution Factor

2.2.4.2. Growth curves

Manual and automated growth curves both utilized spectrophotometric techniques. Calibration curves to determine the relationship between OD and CFU ml^{-1} were achieved by diluting a bacterial suspension of known concentration and recording its corresponding OD. These calibration curves were performed for each bacterial species in each of the media tested. An equation was derived from these plots using linear regression analysis and these equations were used to convert OD measurements into cell numbers (CFU ml^{-1}). This data also provided the limits of linearity for the spectrophotometer. Generation times for all species were read directly from OD versus time semi-logarithmic plots.

2.2.4.2.1. Manual optical density growth curves

Duplicate cultures of *Salmonella* ser. Typhimurium were set up in experimental BPW by inoculating 100 ml of BPW in a 500 ml conical flask with 1 ml of prepared inocula, as described in 2.2.3. 1 ml of the resulting cultures were removed immediately and their OD at 600 and 400 nm were measured (UV Vis 4000 spectrophotometer). A plate count to obtain accurate CFU ml^{-1} at the initial time point was also performed. The flasks were transferred to a 37 °C incubator (Gallenkamp). Subsequently, 1 ml of culture was removed from each flask every 30 min and the OD at 400 and 600 nm measured. Cultures were agitated for 5 s before sampling; this was continued for 8 h. A plate count was also performed on the final 8 h culture.

2.2.4.2.2. Automated optical density growth curves

Labsystems Bioscreen C measures the OD of up to 200 wells at a time, with each honeycomb well having a capacity of 400 µl. Uses for the automated growth analyser varied from quality control of growth media (Johnston, 1998) to recovery times of sub-lethally injured bacteria (Stephens *et al.*, 1997). Growth medium (280 µl) and prepared inocula (20 µl), as described in 2.2.3 and diluted 1:100 with PBS, were placed in each well giving a final volume of 300 µl. Bioscreen was programmed with the following settings unless otherwise specified:

Pre-heating time to reach incubation temperature of 37 °C was 10 min. Optical density was measured every 10 min for 24 h at a wavelength of 600 nm; the cultures were shaken for 5 s min^{-1}. Data were transformed from Ascii into Microsoft Excel® format and processed into growth curves.

2.2.4.2.3. Comparison of bioscreen and manual growth curve methods.

Figure 2.2. shows growth curves of *Salmonella* cultured under similar conditions produced by the two different methods of growth analysis used in this work, automated and manual, described previously. Consistent methodology throughout included: identical medium, temperature, shaking frequency, inoculum preparation and measurement intervals. Variables employed were culture volume and culture surface area to volume ratio.

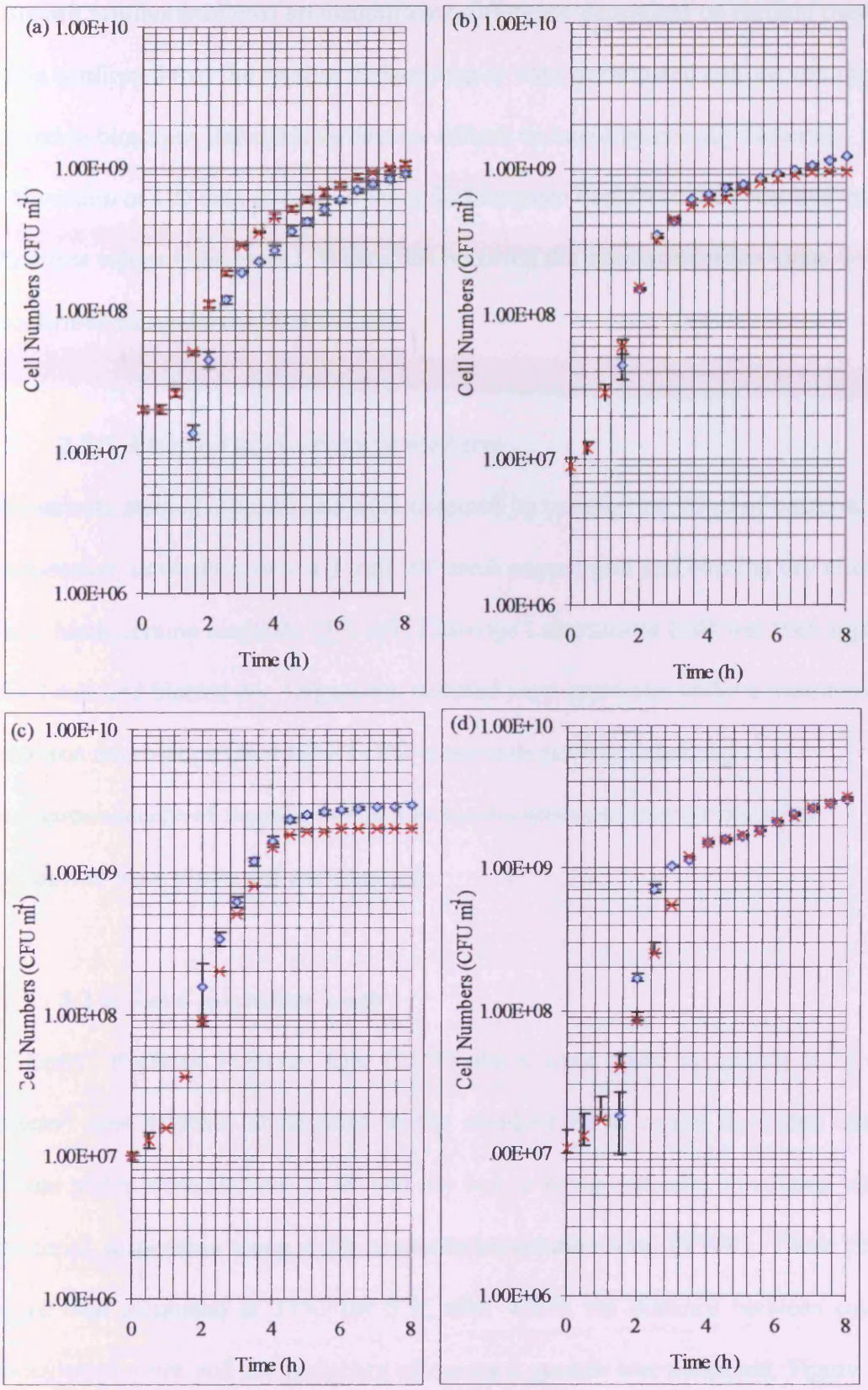

Figure 2.2. Growth profiles of *Salmonella* ser. Typhimurium in (a) C1, (b) M2, (c) S2 and (d) Y1 peptone based-BPW incubated under identical conditions using different methods: ◊ represent bioscreen obtained data and × represent manually obtained data.

Growth profiles exhibited an insignificant difference dependent on method used. This confirmed that the smaller surface area to volume ratio and culture volume found in bioscreen did not have adverse effects on bacterial growth. However, conversion of OD data to cell numbers for bioscreen data frequently resulted in the first few values to be absent. Where this occurred the generation times were confirmed using the original OD data.

2.2.5. Electron microscopy procedures

Negatively stained preparations were obtained by transferring 10 µl of bacterial suspension, unwashed, onto a 3 mm 200 mesh copper grid and blotting dry after 1 min. Methylamine tungstate (3% w/v, Emscope Laboratories Ltd) was then applied for 1 min and blotted dry. Organisms cultured were examined under a transmission electron microscope (Jeol JEM-1210); observations were noted, e.g. presence/absence of flagella, and representative electron micrographs taken. Negatives were processed and scanned.

2.2.6. Swim migration assay

"Sloppy" Buffered Peptone Agar (BPA) plates were made by adding 0.3% w/v Bacto® agar (Becton Dickinson) to the standard BPW recipe described above. These plates were allowed to set and dry before being centrally inoculated with a bacterial suspension using a 10 µl sterile inoculation loop (VWR). These plates were then incubated at 37°C for 5 h, after which the distance between central inoculation point and the periphery of outward growth was measured, Figure 2.3. Plates were read over a dark background. The control was 0.3% agar with NB and TSB; the same inocula were used and incubated identically along with the test

plates. The experiment was performed in triplicate and the average speed of migration from inoculation point (mm h^{-1}) calculated.

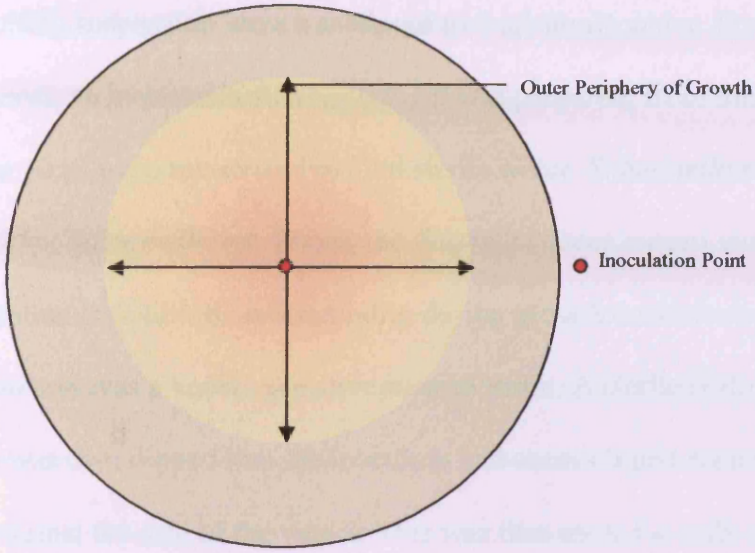

Figure 2.3. Measurement of a typical swim plate

2.2.6.1. Effect of different agars on swim migration assay

Table 2.2. shows the effect of different agars, obtained from the companies Becton-Dickinson and Sigma, on swim rate of *Salmonella* ser. Typhimurium and *Escherichia coli* in 0.3 % NA (Oxoid). Becton-Dickinson's Bacto-Agar was used for all swim migration assays in this work as this product allowed increased migration in comparison with Sigma agar. This can be attributed to Bacto-agar creating a more porous matrix which allowed the bacteria to swim outward more easily.

Table 2.2. Swim rates of *Salmonella* ser. Typhimurium and *Escherichia coli* in 0.3 % "sloppy" nutrient agar where the agar was of variable source. Values are the mean of triplicate data ± standard deviation.

Agar Source	Swim Rate (mm h^{-1})	
	S. Typhimurium	*E. coli*
Becton-Dickinson	8.07 ± 0.07	7.92 ± 0.33
Sigma	3.11 ± 0.00	3.22 ± 0.11

2.2.7. Antibiotic susceptibility determination

The inocula for the Enterobacteriaceae were produced by taking prepared inoculum, as described in 2.2.3, and diluting 1:100 to OD_{500} 0.01-0.05, then 250 µl of the resulting suspension were transferred to 5 ml sterile water. For the *Staphylococci*, an inoculum of OD_{500} 0.6-1.0 was prepared; from this stock suspension 10 µl were transferred to 5 ml sterile water. *Salmonella* ser. Typhimurium, *Salmonella* ser. Poona and *Staphylococcus aureus* were used to assess variation in antibiotic susceptibility during growth on various peptones, *Escherichia coli* was a known sensitive control strain. A sterile cotton-wool swab (Guilbert) was then dipped into the inoculum and excess liquid removed by turning the swab against the side of the vessel. This was then spread evenly over the media in three directions. Experimental media were Buffered Peptone Agar (BPA) plates prepared by adding 1.5% (w/v) Bacto® agar (Becton Dickinson) to the standard BPW recipe described above. Control medium was Iso-Sensitest Agar (Oxoid). Antibiotic disks (Oxoid) for the following antimicrobials were then placed upon the inoculated plate: amikacin (30 µg), ampicillin (10 µg), cephalothin (30 µg), ciprofloxacin (1 µg), colistin Sulphate (25 µg), gentamicin (10 µg), streptomycin (10 µg). The plates were incubated at 37°C for 18 h and then examined. Zones of inhibition were measured (in mm) and compared with BSAC (British Society for Antimicrobial Chemotherapy) standards which allow interpretation of sensitivity or resistance. This method was taken from Andrews (2004).

2.2.8. Serotyping of *Salmonella* species

Serotyping of *Salmonella* ser. Typhimurium (**1, 4, 5, 12 *:i: 1, 2***) and *Salmonella* ser. Poona (**1, 13, 22 *:Z_{59}: 1, 6***) was performed in order to assess if the presence of

the somatic O antigens or the *flagellar H antigens* was variable after growth on different peptone substrates. Prepared inocula (10 µl) (Section 2.2.3) were dispensed into 10 ml of media in 20 ml culture tubes which were then incubated for 18 h at 35°C. After this time, an equal volume of formalin solution was added (0.6% formalin (Sigma), 0.85% NaCl (BDH)), and the tubes incubated for a further 18 h at room temperature. Formalinised cultures (10 µl) were then mixed with 10 µl of specific antiserum (Rapid 1-H *Salmonella* flagella: Biotec Laboratories. Hz, Hi, H_2 and H_6, O_1, O_4, O_5, O_{12}, O_{13}, O_{22} *Salmonella* agglutination: Pro-Lab Diagnostics) on a polished microscope slide. Slides were rotated for up to 5 min, then the degree of agglutination was recorded. Tubes containing un-inoculated media were also set up as controls. This method was adapted from Gruenwald *et al.*, 1990; the original agglutination procedure was outlined by Craigie (1931).

2.3. Chemical methods

The methods detailed below provide data with respect to the chemical composition of the peptones analysed.

2.3.1. Bradford method for protein determination

A calibration of absorbance values for known concentrations of Bovine Serum Albumin (BSA) was performed on an UV-Vis spectrophotometer (Unicam SP1800). Standards were made from a 2 mg ml^{-1} stock of BSA (Sigma) in ultra high purity (UHP) water and dilutions tested ranged from 0-1.4 mg ml^{-1}. Protein standard or sample (0.1 ml) was added to 3 ml of the Bradford reagent (Sigma) (Bradford, 1976). Bradford reagent is a mixture of Brilliant blue G in phosphoric acid and methanol which is stored at 4 °C and allowed to reach room temperature before use. Gentle whirlimixing ensured a homogenous solution, samples were then incubated at room temperature for 45 min and finally transferred to cuvettes (Hughes and Hughes). Absorbance was measured at 595 nm.

It was found that this method did not give accurate results for the estimation of protein, with most values generated being below 2 % w/w. The Bio-Rad protein assay instruction manual (1984) indicates that incompatible substances for this assay include gelatin, which shows a low dye response. This appeared to be the case for all the peptones investigated in this thesis.

Therefore, protein content of peptones (% w/w) was established as the summation of amino acids determined by amino acid analysis (Chapter 2.3.2). This gave the total amount of protein based material in each peptone.

2.3.2. Amino acid analysis

Peptone samples (50 µl UHP water containing 0.5 g l^{-1} peptone) incorporating an internal standard of 15 nM norleucine were hydrolysed with 6 M HCl at 110°C for 24 h. Samples were then dried under vacuum, filtered using a 0.2 µm membrane

(Millipore) and re-suspended in 150 µl 0.20 M sodium citrate loading buffer at pH 2.20 (Biochrom). This (100 µl) was loaded into an amino acid analyzer which utilised ion-exchange high performance liquid chromatography (Biochrom 20, Pharmacia Biotech) and measured spectrophotometrically. These samples were analysed in triplicate and resultant values adjusted relative to the internal standard. Tyrosine concentrations for Nutrient Broth NB (Oxoid) and Tryptone Soya Broth TSB (Oxoid) were calculated using data provided in the Oxoid Manual (Bridson, 1995).

2.3.3. Phenol sulphuric acid colorimetric method of carbohydrate determination

Standard, sample or control solution (200 µl) was added to 200 µl phenol reagent (5% w/v), then 1 ml of concentrated sulphuric acid (Fisher) was rapidly and directly added to the solution surface. Solutions were left for 10 min, whirlimixed, and then incubated for a further 30 min. Absorbance was measured at 490 nm on an UV spectrophotometer (Unicam SP1800 UV Spectrophotometer).

Sample solutions were 0.5 g l^{-1} peptone in polished water and had been autoclaved. Control solutions consisted of all reagents except peptone or glucose, these being replaced with 200 µl polished water. Standards ranged from 1-60 µg of glucose (Fisher) per 200 µl UHP water. Phenol reagent (5% w/v) was prepared by adding 1 g phenol (Sigma) to 20 ml polished water. Calibration curves of absorbance against known concentrations of glucose were then performed. This method was adapted from Dubois *et al.*, (1956).

2.3.4. Size exclusion high performance liquid chromatography

The High Performance Liquid Chromatography (HPLC) system used was a Thermo Finnigan Spectra System consisting of a Spectra System P4000 pump, Spectra Series AS300 auto-sampler and a Spectra System UV6000LP detector; the SEC column was a Bio-Sep 2000 (Phenomenex) of size 300 × 7.8 mm. The interface connecting the system to the Xcalibur software (Thermo-Finnigan) was a Spectra System TSP SN4000.

Peptone (10 µl), made up in polished water and autoclaved, at a concentration of 0.25 g l^{-1} was injected onto the column at a flow rate of 1 ml min^{-1} in 2% v/v acetonitrile (Fisher). The temperature was maintained at 25 °C throughout, and the column pressure remained constant, the sequence allowing 2 min of equilibration time before injection of the sample. A 400 µl flush of solvent was instigated after each 30 min sample run, and a broad detection wavelength range (total scan: 190-800 nm) was employed due to the unknown nature of the sample. Each sample was run in triplicate.

Calibration of the column was carried out using a molecular weight marker kit for gel filtration chromatography (Sigma: MW-GF-70), containing protein standards of known molecular weights as follows: albumin (bovine) 66000, cytochrome *c* 12000, carbonic anhydrase 29000, and aprotinin 6500. Blue dextran of molecular weight 200 0000 was used to calculate column volume.

Certain parameters were tested in order to determine optimum conditions and settings for this method, these included

1. Different concentrations of peptone; 0.25 g l^{-1}, 0.5 g l^{-1} and 1 g l^{-1}, were all assessed with 0.25 g l^{-1} giving the sharpest peaks. Less than 0.25 g l^{-1} resulted in smaller peaks failing to appear on the spectrum.

2. Varying run temperatures at 20 °C, 25 °C and 30 °C increased the width of the peak, therefore 20 °C was selected as lower temperatures were difficult to maintain.

2.3.5. Electrospray ionisation quadrupole mass spectrometry

Peptone sample (1 ml) was transferred to a precision 1 ml syringe of diameter 4.61 mm (SGE) and injected into the Navigator Quadropole Mass Spectrometer (MS) (Finnigan) for Electrospray Ionisation (ESI). The sample was injected using a syringe pump (Model 100, KD Scientific) at a flow rate of 70 µl per min. The source voltages used were +50 and +70 V. Samples were run for 2 min each and repeated in triplicate. A full-scan spectrum from 2 amu to 1402 amu was recorded. The data were processed into chromatograms using Xcalibur software (ThermoFinnigan). The process of ESI MS is demonstrated by Figure 2.4.

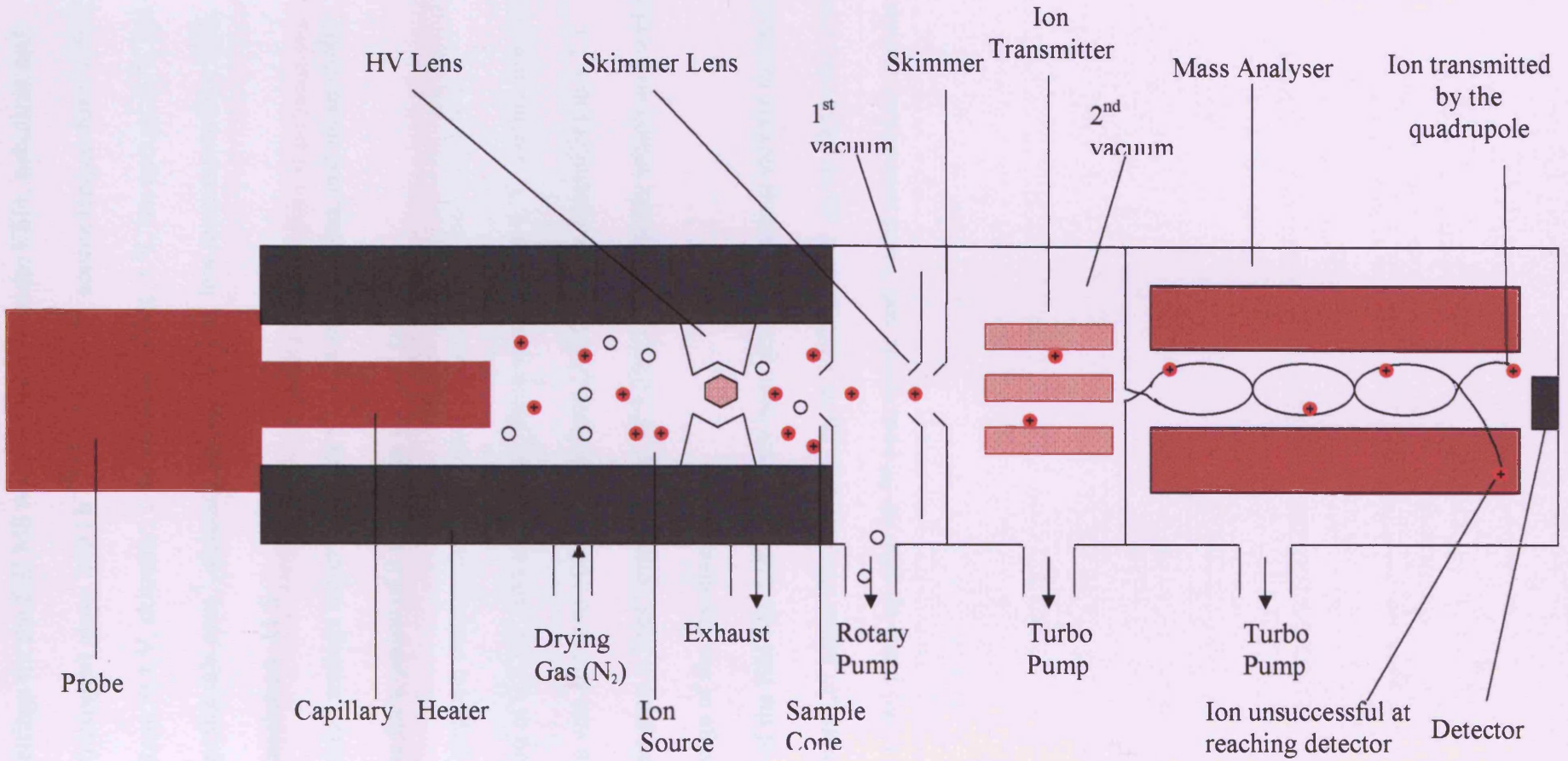

Figure 2.4. Overall process of ESI MS

2. Materials and methods

The Tune settings for the ESI MS were: ionization mode: ESI+, nebulizer and drying gas (nitrogen) glow: 200 l h^{-1}, capillary: 4 kV, source temperature: 110°C, RF lens voltage: 0.1 V, skimmer: 1 V, skimmer offset: 5 V, ion energy: 2 V, ion energy ramp: 0.2 mV amu^{-1}, detector voltage: 800 V, low mass resolution: 12.5, high mass resolution: 11.5.

The Acquisition settings were: 2.5 scans s^{-1}, start mass: 2 amu, end mass: 1402 amu, acquire for a period of 2 min, peak format: centroid.

Samples of peptone were made up by being diluted in polished water to a concentration of 0.5 g l^{-1} and autoclaved; these were kept at 4 °C. Before use, 500 µl of sample was added to 500 µl acetonitrile (ARG, Fisher Scientific) in a 1:1 dilution. This gave a final concentration of 0.25 g l^{-1} of peptone, which was within the linear range of the machine.

Calibration of the ESI MS had previously been performed using various molecular weight markers (25 µg/ml PEG 200, 50 µg/ml PEG 400, 75 µg/ml PEG 600, 250 µg/ml PEG 1000) made up with 50:50 acetonitrile and 2 mM ammonium acetate (Sigma).

CHAPTER THREE

Growth of bacteria in a liquid medium

3. Growth of bacteria in liquid culture media

3.1. Summary

The definition of the performance of a medium can take many forms: ability to recover healthy organisms or injured organisms, capacity to support fast growth resulting in high cell numbers of healthy organisms, media which allow growth of target organism yet inhibit other members of the microbial flora; the numerous possible definitions are certainly not exhausted here. However the purpose of this work is to show the differences in growth properties that result from use of a medium component with a broad category title: peptone. Here, I focus on (i) the diversity of commercially available peptones, (ii) their varying suitabilities for the demands made of culture media in science today, and (iii) the effects of the various chosen media on the experimental results subsequently obtained.

3.2. Introduction

Salmonellosis is caused by, amongst other strains, *Salmonella enterica* serovar Typhimurium, with clinical symptoms including watery and sometimes bloody diarrhoea, abdominal pain, headache, nausea, vomiting and fever. Transmission of this serotype occurs by ingestion of contaminated food, mainly meats, or via the faecal-oral route from an infected individual.

Pre-enrichment of sub-lethally injured *Salmonella* present in low numbers on food-stuffs, before selective enrichment, has been recognised as a critical stage in positive identification (Andrews, 1986; Fricker, 1987); this is of importance as even low cell numbers (e.g. 100 CFU) can result in infection (Havelaar *et al.*, 2001).

3. Growth of bacteria in liquid culture media

Salmonella enumeration and recovery is usually conducted in the pre-enrichment medium Buffered Peptone Water (BPW), comprising salts, phosphate buffers and the complex, undefined ingredient, peptone (Van Leusden, 1982). With peptone as the solitary complex ingredient, this medium allows study of the effect various peptones have on bacterial growth. Performances of commercial preparations of BPW have previously been compared (Baylis *et al.* 2000) and it was established that the capability of BPW manufactured by different companies to sustain recovery of injured organisms varied. The capacity of different peptones, from varying sources and manufacturers, to produce cell numbers which were significantly different was shown to correlate with the digestive process being either acid hydrolysis or enzymatic (Brãnes *et al.*, 1981). In this study it was found that enzymatic digests produced higher cell numbers.

Many recipes (Hoorfar and Baggesen, 1998) and supplements (Reissbrodt *et al.*, 1996) have been devised to achieve optimum recovery, and factors affecting percentage recovery of injured organisms such as pre-enrichment time, temperature, volume of sample:media ratio, pH and anaerobic or aerobic conditions have all been studied in detail (Andrews, 1986; Ferreira and Lund, 1987).

Selective enrichments (Sharma and Packer, 1969; Moriñigo *et al.*, 1990; 1993) and selective differential plating media (Busse, 1995) have also been studied intensely in order to improve recovery.

Despite the problem of unknown complex ingredients (e.g. peptone) being recognised as a significant factor in media performance (van Schothorst and Renaud, 1983; Hyde and Denton, 1987) very little work has been done on performance of different sources and batches of peptones. One such study was

Stephens *at al.*, 2000 describing how peptones, as a constituent of BPW, differ in production of harmful reactive oxygen species: this correlates with performance of the media. Instead a large emphasis has been placed on quality assurance from manufacturers (Nagel and Kunz, 1973) and quality control in laboratories (Johnston, 1998). The present study shows how variation between the classes of casein, gelatin, meat, soy and yeast peptones, and between manufacturer and batch of an identical peptone class, affect growth of bacteria.

Another important factor in media performance is preparation technique, particularly the sterilization process. Autoclaving is known to cause auto-oxidation of sugars in phosphate buffers (Baumgartner, 1938) producing toxic oxygen species within the medium (Mackey and Derrick, 1986a). Thus growth of *Salmonella* was assessed in BPW which was prepared by either filter sterilization or autoclaving.

3.3. Materials and methods

Of great importance to this work was consistency in preparation of media and bacterial inocula. Both variables would have a pronounced effect upon the performance of the media, thus these methods are given in detail below. All methods not given are set out in chapter 2.

3.3.1. Media preparation

BPW media to be used for each experiment were synthesized using a common background solution containing the salt and phosphate buffers. This removed inaccuracy in weighing these components and ensured distilled water was of

identical source (in case of contamination). Peptone at a concentration of 10 g l^{-1} was weighed out to the nearest ± 0.001 g.

Where media were sterilized by autoclaving, 250 ml of each medium was placed in a 500 ml Duran bottle with the screw caps loosely fitted; this ensured that media:container volume during the sterilization process was constant. Autoclaving proceeded using the same setting (121°C, 15 min) in the same Astell autoclave. After sterilization media were cooled on the bench at ambient temperature and then stored at +4°C, media were discarded if older than six weeks.

Methods detailing sterilization by filtration were recorded in section 2.2.1.

Dispensing of media into required vessels, i.e. conical flasks or plate wells for the bioscreen growth analyser, was performed aseptically in a sterile environment.

3.3.1.1. Variation from described media composition

Section 3.4.6. details a deviation from the standard BPW composition. The concentration of peptone was altered. Peptone was added at concentrations of 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 25 and 30 g l^{-1} and the use of these concentrations was clearly stated where necessary.

3.3.2. Inocula preparation

The bacterial species used in this work, with the exception of those cultures used in section 3.4.8. for heat-shock analysis, were of identical source to enable direct comparison. Bioscreen analysed the OD of up to 200 wells at any time; therefore all test BPW media (inoculated media) in triplicate and triplicate controls (un-inoculated media) were run together after inoculation with the same culture. In order to prevent growth during inoculation the well was incubated on ice and

inoculation of both media (280 µl) and bacterial suspension (20 µl) was performed aseptically in a sterile environment.

Overnight cultures were always incubated in identical conditions: 100 ml of commercially prepared BPW (Merck) in a 500 ml conical flask inoculated by selecting a colony from a NA slope, at 37°C in a reciprocating water bath (Gallenkamp) shaken at 100 strokes min^{-1} for 18 h.

Methods of producing heat shocked inocula of *S.* ser. Typhimurium were recorded in section 2.2.3.2.

3.3.3. Statistical analyses

Comparison of mean generation times and yields obtained during growth in the different peptones and varying conditions was performed using ANOVA for parametric data and the Kruskal-Wallis test for non-parametric data. Minitab™ statistical software version 13.1 was employed to analyse the data.

3.4. Results

Growth analyses of the bacteria *Escherichia coli*, *Salmonella* serovars Typhimurium and Poona and *Staphylococcus aureus* in complex and minimal media were performed in order to give values for comparison with the growth data obtained in various BPW. These species were then cultured in BPW with varying constituent peptone, and subsequent generation times and yields analysed. Investigations into varying methodologies, such as different sterilization techniques and varying medium composition, were performed in order to improve media performance.

3. Growth of bacteria in liquid culture media

The effects of organisms which were sub-lethally injured, on subsequent performance of the media, were performed to assess whether specific peptones may be suited to different applications, i.e. were certain peptones suited to recovery of sub-lethally injured organisms whereas others more suitable for enumeration of healthy bacteria?

3.4.1. Growth of bacteria in commercially available complex media

Exponential phase generation times, and yield of micro-organisms at 24 h, of *E. coli, S.* serovars. Poona and Typhimurium and *S. aureus* were assessed in commercially available, dehydrated NB and TSB (See Appendix 1 for media recipes) using the bioscreen C analyser (Table 3.1.).

Table 3.1. Generation times and yields of some Gram positive and negative bacteria in complex media.

Medium	Organism	Generation Time g (min)	Yield at 24 h (CFU ml^{-1}) × 10^9
Nutrient Broth	*E. coli*	11.4 ± 1.5	3.83 ± 0.76
	S. Poona	23.4 ± 11.7	2.99 ± 0.02
	S. Typhimurium	14.5 ± 0.0	5.86 ± 0.60
	S. aureus	16.1 ± 4.4	3.75 ± 0.03
Tryptone Soya Broth	*E. coli*	26.0 ± 2.0	13.00 ± 0.18
	S. Poona	23.2 ± 0.5	11.60 ± 0.02
	S. Typhimurium	22.5 ± 4.3	13.00 ± 0.34
	S. aureus	25.0 ± 0.0	7.73 ± 0.06

NB produced the lowest values of g for all bacteria compared with TSB. The bacterium displaying the lowest g value was *E. coli* with 11.4 ± 1.5 min, the highest: *S.* ser. Poona with 23.4 ± 11.7. The range of g values between bacterial species for each medium was small: 12 min for NB and 3.5 min for TSB.

The result was reversed with respect to yield: TSB generating higher yields at 24 h relative to NB; high data values were recorded with *E. coli* and *S.* ser. Typhimurium at 1.3 × 10^{10} CFU ml^{-1}. *S. aureus* presented notably lower yields in TSB compared with the Enterobacteria which grew to exceptionally high yields.

3.4.2. Growth of bacteria in a defined medium

Growth of the Enterobacteria in M9 minimal medium (Neidhardt *et al.*, 1974), developed for Enterobacteria and so not appropriate for *S. aureus*, with a supplement of 1% (w/v) glucose produced low yields and values for g (Table 3.2.).

Table 3.2. Generation times and yields of Enterobacteria in the defined medium M9 with an additional 1% (w/v) Glucose.

Medium	Organism	Generation Time g (min)	Yield at 24 h (CFU ml^{-1}) × 10^9
M9 Medium + 1% Glucose	*E. coli*	98.7 ± 7.0	2.19 ± 0.21
	S. Poona	116.8 ± 7.0	1.02 ± 0.11
	S. Typhimurium	87.2 ± 4.4	1.74 ± 0.27

These studies did not show an abnormal metabolism for any of the bacterial strains used in this work, i.e. minimal medium contained ammonium chloride as the sole nitrogen source, therefore strains which required amino acids in the medium would not have been able to replicate, and these strains would not have been suitable for comparison of growth medium.

With regard to values of g: *S.* ser. Typhimurium produced the lowest, followed by *E. coli* and, considerably higher, *S.* ser. Poona. *E. coli* achieved the highest yields; over two fold higher than *S.* ser. Poona.

3.4.3. Generation times of bacteria in selected BPW, with variable peptone constituent, sterilized by autoclaving

Exponential phase generation times (g) of *E. coli*, *S.* ser. Poona and Typhimurium and *S. aureus* in the pre-enrichment medium BPW, of varying peptone content, were analysed using the Bioscreen C analyser (Table 3.3.).

Table 3.3. Generation times of Gram positive and negative bacteria in autoclaved buffered peptone water where the complex ingredient peptone is of differing source. Mean values ± standard deviation are shown where n = 3.

Media		Generation Time g (min) ± Standard Deviation			
		E. coli	*S.* ser. Poona	*S.* ser. Typhimurium	*S. aureus*
Casein	1	5.6 ± 2.0	23.0 ± 1.7	24.9 ± 2.8	27.0 ± 10.1
	2	4.5 ± 1.7	54.5 ± 6.5	15.8 ± 5.2	51.1 ± 22.6
	3	7.3 ± 0.0	24.6 ± 5.5	26.9 ± 0.9	53.2 ± 26.2
	4	4.5 ± 0.7	28.3 ± 5.8	30.7 ± 7.3	25.1 ± 20.8
	5	7.5 ± 1.0	24.4 ± 9.6	14.8 ± 3.1	9.6 ± 3.2
	6	6.3 ± 0.3	26.7 ± 0.0	61.0 ± 6.8	14.6 ± 2.4
	7	6.5 ± 0.3	30.0 ± 3.9	27.4 ± 1.4	10.3 ± 6.0
	8	10.7 ± 0.0	27.7 ± 4.0	35.3 ± 1.4	10.1 ± 0.4
	Mean	6.6 ± 2.0	29.5 ± 9.2	29.6 ± 14.5	25.1 ± 18.0
Gelatin	1	48.3 ± 0.0	41.9 ± 5.4	27.8 ± 9.1	Not Performed
	2	24.5 ± 0.5	75.9 ± 63.6	21.9 ± 1.9	25.3 ± 9.5
	3	56.9 ± 12.2	115.9 ± 41.9	24.4 ± 1.8	63.5 ± 13.0
	4	36.5 ± 0.1	33.4 ± 25.8	29.8 ± 1.4	48.1 ± 10.5
	5	28.3 ± 9.9	50.1 ± 21.7	29.5 ± 2.7	48.6 ± 5.3
	Mean	38.9 ± 13.6	68.8 ± 36.0	26.7 ± 3.4	46.4 ± 15.8
Meat	1	8.1 ± 0.9	8.8 ± 2.1	6.3 ± 3.1	6.8 ± 1.5
	2	9.4 ± 0.0	28.9 ± 6.1	20.7 ± 0.0	17.4 ± 3.5
	3	23.5 ± 3.0	14.2 ± 3.1	20.5 ± 13.7	17.9 ± 1.2
	4	14.2 ± 13.6	18.9 ± 3.2	12.4 ± 3.4	20.0 ± 2.1
	5	16.2 ± 5.5	12.1 ± 2.5	10.3 ± 1.9	11.0 ± 3.0
	6	19.5 ± 5.3	18.1 ± 2.4	48.5 ± 1.8	70.4 ± 0.0
	Mean	15.2 ± 5.9	16.8 ± 7.0	19.8 ± 15.2	23.9 ± 23.3
Soy	1	10.1 ± 2.7	31.3 ± 3.0	26.8 ± 2.4	15.9 ± 2.6
	2	19.5 ± 12.7	23.5 ± 0.9	23.8 ± 1.5	23.4 ± 2.9
	3	10.8 ± 7.9	22.3 ± 14.9	13.2 ± 0.5	6.7 ± 1.1
	4	14.1 ± 1.4	14.8 ± 3.9	25.3 ± 0.7	28.6 ± 14.3
	5	6.2 ± 1.4	20.7 ± 6.1	9.8 ± 0.9	13.4 ± 5.6
	Mean	12.1 ± 5.0	22.5 ± 5.9	19.8 ± 7.7	17.6 ± 8.6
Yeast	1	10.8 ± 1.9	18.9 ± 0.0	24.4 ± 1.4	49.1 ± 5.8
	2	11.5 ± 4.3	24.2 ± 8.4	22.6 ± 1.7	77.5 ± 17.6
	3	25.0 ± 0.1	34.5 ± 2.4	26.7 ± 1.4	56.0 ± 13.5
	4	12.2 ± 1.2	19.9 ± 5.0	25.7 ± 3.1	42.9 ± 2.7
	Mean	14.9 ± 6.8	24.4 ± 7.1	24.8 ± 1.8	56.4 ± 15.1

3.4.3.1. Generation times of *Escherichia coli* in selected BPW sterilized by autoclaving

E. coli showed a significant difference (P = 0.000) in mean g values dependent on peptone class (Table 3.3). Gelatin peptone-based BPW gave the shortest generation time with a mean of 38.9 ± 13.6 min for the category. Three other peptone classes (meat, soy and yeast) were similar with means of 15.2 ± 5.9, 12.1 ± 5.0 and 14.9 ± 6.8 min respectively. Casein peptones as constituents of BPW gave mean generation times at least two fold lower than other peptone classes: 6.6 ± 2.0 min. Comparison of mean g values for peptone classes indicated similarity between groups. There was a significant difference between the mean generation times of casein peptone and gelatin peptone with all other peptone categories. There was not a significant difference between g values during exponential growth in meat peptone and soy peptone (P = 0.388), meat peptone and yeast peptone (P = 1.000) or finally soy peptone and yeast peptone (P = 0.391).

Investigations into differences between generation times of *E. coli* during growth in the same category of peptone, but obtained from a different manufacturer or as different batches, also showed striking differences. Growth curves are shown in Appendix 2.1.

Casein peptone-based BPW: (Appendix 2, Figure 2.1.1.) Individual casein peptone samples, originating from different manufacturers or batches, generated significantly different values of g for *E. coli* (P = 0.035).

Remarkably fast generation times were generated during exponential growth: as short as 4.5 ± 1.7 min in C2 peptone-based BPW. Original OD measurements were

within the linear range of the machine and all data points fell on the linear section of the calibration curve. Optical artefacts, such as dramatic change in cell shape and size or formation of aggregates, which affect light scattering and therefore OD readings may have been responsible (See Chapter 3.4.8). This may be the case for all exceptionally rapid generation times as, even under favourable conditions, cellular processes ultimately limit bacterial growth (Koch, 1997).

Growth curves (Appendix 2, Figure 2.1.1.) demonstrated distinct biphasic profiles with no obvious lag phase. All casein peptones produced similar growth profiles except C7, which was a different batch of nominally identical product from the same manufacturing source to C8, which exhibited a longer lag period before the second phase of exponential growth.

Gelatin peptone-based BPW: (Appendix 2, Figures 2.1.2.1. and 2.1.2.2.) Gelatin was the least effective peptone for growth of *E. coli*; however the source of manufacture and the batch did affect generation time ($P = 0.026$). Cultures on peptone G3 produced the shortest growth of 56.9 ± 12.2 min, compared with 24.5 ± 0.5 min in G2 peptone. Different batches of Merck's gelatin peptone, G1 and G5, gave notably different values of g.

Growth profiles displayed monophasic growth for *E. coli* cultured in all gelatin peptone-based BPW. Growth on peptone G3 exhibited a relatively longer lag phase of 8 h compared with the usual 5 h that other gelatin peptones generate. It was also observed that cultures in G3 peptone show declining cell numbers at 22 h, whereas all other gelatin peptones did not show any signs of this even after 24 h growth. Cultures containing G1 peptone did not enter stationary phase during the 24 h observation period.

Meat peptone-based BPW: (Appendix 2, Figure 2.1.3.)Generation times of *E. coli* cultured in meat peptone were not significantly affected by use of products from different manufacturers or of different batch (P = 0.276) despite the range of 15.4 min. M3 peptone exhibited the lowest value of g with 23.5 ± 3.0 min compared with the highest value of 8.1 ± 0.9 min during culture in M1 meat peptone-based BPW. Again the very fast values for M1 and M2 peptones seem exceptional and may be due to artefacts of the type discussed above. Progress curves of *E. coli* growth in meat peptone distinctly displayed either biphasic (peptone M1) or triphasic (peptones M2, M3, M4, M5and M6) growth curves. Length of time for each phase was similar for each peptone, except M4, which almost attained stationary phase at 8 h compared with ~12 h achieved by the remaining peptones. Again individual batches of Biotechnica's pig meat peptone were markedly different, particularly M4 peptone.

Soy peptone-based BPW: (Appendix 2, Figure 2.1.4.)There was no significant difference between the growth of *E. coli* in the various soy peptones produced by differing manufacturers (P = 0.466). Peptones S1, S3 and S4 produced similar generation times. Culture S5 resulted in a value of 6.2 ± 1.4 min; this was over three-fold lower than cultures in S2 based media at 19.5 ± 12.7 min. Although the initial point on the growth curve presented is at 1 h, OD data accumulated earlier confirm these values. Triphasic growth curves were characteristic of all soy based media with the first and second phases being identical for all soy peptones. The length of the third phase varied with manufacturer with S3 entering stationary phase first, followed by S4 and S1 together, and finally S2 and S5.

Yeast peptone-based BPW: (Appendix 2, Figure 2.1.5.) Yeast peptone based BPW media were not statistically different regarding culture generation times of *E. coli* ($P = 0.154$). Peptone Y3 was noticeably higher at 25.0 ± 0.1 min than other peptones of the same class, 10.8-12.2 min for Y1, Y2 and Y4. Statistically the data were not significantly different: this was due to the large values obtained for standard deviation. These great deviations could be attributed to edge effects within the microtitre plate. Cultures containing Y3 and Y4 peptones, where Y3 cultured *E. coli* grew at half the rate of Y4, is noted as these peptones differ only in batch. Again *E. coli* utilizes certain substrates preferentially to produce biphasic growth curves. Lag phases differ with Y3 and Y4 peptones at ~4 h compared with Y1 and Y2 peptones which did not have a detectable lag phase. The characteristics of the curves are similar from this point onwards; the progress curves for Y3 and Y4 peptone cultured *E. coli* remain delayed due to the initial lag period.

3.4.3.2. Generation times of *Salmonella* ser. Poona in selected BPW sterilized by autoclaving

Peptone class significantly affected mean generation time of *S.* ser. Poona ($P = 0.000$) (Table 3.3). Again gelatin peptone-based BPW resulted in the highest values of g, with a mean value for the class being 68.8 ± 36.0 min. Casein, soy and yeast peptones produced lower values of g, 29.5 ± 9.2, 22.5 ± 5.9 and 24.4 ± 7.1 min respectively. Meat peptones generated the lowest times with 16.8 ± 7.0 min. Meat, soy and yeast peptones did not produce significantly different generation times for *S.* ser. Poona, with a range of 7.6 min; gelatin peptone-based BPW generated significantly different values compared with all other peptone classes. In

contrast, casein peptone media was shown to be statistically different from meat peptone yet remained similar to the other peptone groups in its ability to culture *S.* ser. Poona.

Further analysis of generation times during growth in peptones of the same class, but different manufacturer or batch, showed considerable variation. Growth profiles of *S.* ser. Poona are shown in Appendix 2.2: all growth profiles of this bacterium in all the peptones studied produced monophasic growth curves: only cultures grown in gelatin peptones produced a long lag phase (between 4-5 h).

Casein peptone-based BPW: (Appendix 2, Figure 2.2.1.) Cultures of *S.* ser. Poona in casein-based BPW, whereby manufacturer or batch varied, generated significantly different values of g ($P = 0.012$). Growth in C2 peptone was especially poor at 54.5 ± 6.5 min, whereas all other casein peptones resulted in similar g values. Two different batches of peptones (C7 and C8) of a nominally identical product from one manufacturer resulted in very similar growth.

Gelatin peptone-based BPW: (Appendix 2, Figure 2.2.2.) There was no statistically significant difference between generation times of this organism in the different gelatin peptone-based BPW ($P = 0.306$); however striking distinctions did occur. Cultures using G3 peptone, as was previously observed with *E. coli*, resulted in the shortest growth followed by G2, G5, G1 and, with the lowest value for g, G4. G1 and G5 peptones, two different batches of an identical product, were again comparable. Similar lag phase times of 4-5 h, regardless of peptone manufacturer, were noted for all cultures that used gelatin peptones.

Meat peptone-based BPW: (Appendix 2, Figures 2.2.3.1. and 2.2.3.2.) Peptones of meat origin generated a variety of generation times, within a range of 20.1 min, for *S.* ser. Poona ($P = 0.012$) which altered significantly with manufacturer or batch. M2 peptone, a different batch of a nominally identical product to M4 and M5 peptones, had a value of g distinctly higher than all other meat peptones. Growth in M1 peptone-based BPW was very rapid, whereas all other meat peptones produced growth rates within the values noted for M1 and M2.

Soy peptone-based BPW: (Appendix 2, Figures 2.2.4.1. and 2.2.4.2.) Generation times in soy media were found not to be statistically different ($P = 0.404$) although differences could be measured. Growth in S4 soy peptone was over 2 fold more rapid than in S1 peptone, with a range of 16.5 min for values of g.
A similar lag phase of 5 h was demonstrated in all soy-peptone based media. In this instance, graphical representation of growth, as cell numbers, for *S.* ser. Poona appears to begin at approximately 5 h; the plot of OD against time is therefore also shown in order to demonstrate the lag phase. The lack of data points before 5 h on the graph depicting cell numbers is due to the inaccuracy of the conversion equation used at the lower and higher ends of the linear range of the bioscreen machine.

Yeast peptone-based BPW: (Appendix 2, Figure 2.2.5.) Individual yeast peptones obtained from various manufacturers or different batches did not statistically affect generation time ($P = 0.108$). Media based on Y3 peptone produced slightly slower

growth than the other yeast media, despite the fact that Y3 and Y4 peptones were only different batches of a single manufacturer's product.

3.4.3.3. Generation times of *Salmonella* ser. Typhimurium in selected BPW sterilized by autoclaving

S. ser. Typhimurium was exceptional of all the organisms studied in being the only organism not to display a statistically significant difference ($P = 0.127$) with respect to values of g between categories of peptone: i.e. no significant difference between mean g values in casein, gelatin, meat, soy and yeast peptones as a constituent of BPW medium. This is not surprising as *Salmonella* serovars differ greatly with respect to metabolism and biochemistry. Generation times for the mean of each class were similar with a range of 9.8 min: meat and soy peptones were equally high followed by yeast, gelatin and finally casein peptone-based media as the poorest media to support exponential growth. Growth profiles of *S.* ser. Typhimurium during growth in all casein and gelatin peptone-based media showed monophasic kinetics, whereas in all yeast peptone media the curves plotted were biphasic. Meat and soy peptones differed in that they produced either biphasic or monophasic curves dependent on manufacturer. *S.* ser. Typhimurium did not display a lag phase in any BPW medium tested.

Casein peptone-based BPW: (Appendix 2, Figures 2.3.1.1. and 2.3.1.2.) Growth of this strain of *Salmonella* in varying casein peptones produced significantly different generation times ($P = 0.000$): ranging from 61.0 ± 6.8 min during growth in C6 peptone to 14.8 ± 3.1 min in C5 peptone. Peptones of identical product, but with different batch numbers, generated similar values.

Considerable scattering between data points was observed during growth of this species in casein-based media. Certain casein peptones, in particular C6 and C1, were noted to be of a more turbid, translucent nature in comparison with other peptones which were transparent. These differences in transparency may have affected the light scattering process which indirect techniques such as OD are dependent on.

Gelatin peptone-based BPW: (Appendix 2, Figure 2.3.2.) Relatively good growth of *S.* ser. Typhimurium occurred in gelatin media in comparison with other bacterial strains tested, however no noticeable difference was observed in values of g within the gelatin peptone class ($P = 0.101$). The difference within the range of generation times was slight at 7.9 min; results for peptones G4 and G5 were very similar, being of nominally identical product and differing only in batch.

Meat peptone-based BPW: (Appendix 2, Figures 2.3.3.1. and 2.3.3.2.) Generation times during growth in meat peptones were diverse and were shown to be statistically significant ($P = 0.001$). For *S.* ser. Typhimurium cultured in M6 peptone-based BPW, exponential phase growth was considerably slower then other meat peptones investigated at 48.5 ± 1.8 min; this is in contrast to *S.* ser. Poona and *E. coli* which grew well in M6 peptone. M6 peptone also produced notable diauxic growth during culture of *S.* ser. Typhimurium.

Soy peptone-based BPW: (Appendix 2, Figures 2.3.4.1. and 2.3.4.2.) A statistically significant difference ($P = 0.000$) in generation time was observed during growth in various soy peptones. Peptones clustered into those which produced slower

growth during culture: S1, S2 and S4, and then those which generated faster growth in S3 and S5. These observations were in contrast to the appearance of the curves produced, whereby S1, S3 and S4 peptones resulted in biphasic growth; S2 and S5 peptones gave rise to monophasic curves.

Yeast peptone-based BPW: (Appendix 2, Figures 2.3.5.1. and 2.3.5.2.) Cultures of this strain grown in yeast peptone displayed the most consistent generation times with a low range of g values at 4.1 min ($P = 0.161$).

3.4.3.4. Generation times of *Staphylococcus aureus* in selected BPW sterilized by autoclaving

Cultures of the Gram-positive bacterium *S. aureus* showed a significant difference in mean g values dependent on peptone class ($P = 0.017$). Unusually for this series of experiments yeast peptone as constituent of BPW generated the poorest exponential growth with a mean of 56.4 ± 15.1 min; this was closely followed by gelatin peptones with a mean value of 46.4 ± 15.8 min. Casein, meat and soy peptone-based media gave similar generation times of 25.1 ± 18.0, 23.9 ± 23.3 and 17.6 ± 8.6 min respectively. Growth profiles of *S. aureus* in the differing peptone classes were mostly sigmoid curves, very few exhibited diauxie and were only observed during growth in the meat and gelatin peptone classes.(See Appendix 2.4.). Standard deviation from the mean of most peptone classes was considerable and therefore indicates diversity within the class, peptones which differ only by manufacturer or batch.

Casein peptone-based BPW: (Appendix 2, Figures 2.4.1.1. and 2.4.1.2.) BPW from various sources of casein peptone provided a diverse range of generation times from 9.6 ± 3.2 min in C5 peptone to 53.2 ± 26.2 min in C3, these data were statistically significant (P = 0.004).

Lag phase differed noticeably, with C2, C6 and C5 reaching exponential phase by 1.5 h, C1 entering logarithmic phase at 3.3 h and, finally, C4, C7 and C8 with an extended lag phase of 7.5-8 h.

Gelatin peptone-based BPW: (Appendix 2, Figure 2.4.2.) The range of values within the gelatin group is 38.2 min which was shown to be statistically significant (P = 0.015). G4 and G5 peptones generated similar values and G3 again produced the highest generation time for this bacterium in gelatin BPW: this was also the case for *E.coli* and *S. ser. Poona.* Growth in G3 peptone was biphasic which was in contrast to cultures grown in the other gelatin peptones investigated.

Meat peptone-based BPW: (Appendix 2, Figures 2.4.3.1. and 2.4.3.2.) Growth of *S. aureus* in meat peptone-based media resulted in dissimilar generation times (P = 0.000). As with all bacteria studied in this work M1 peptone generated the lowest value of g: 6.8 ± 1.5 min. M5, M2, M3 and M4 peptones resulted in generation times between 11 and 20 min whilst M6 peptone as a nutrient source gave rise to a particularly poor value of g, 70.4 ± 0.0 min. Growth profiles showed that M2, M4 and M5 peptones gave rise to a diauxic, effect whereas the remaining peptones showed archetypal sigmoid curves. Lag time of *S. aureus* in M4 peptone was 3 h, this was double the lag time during growth supported in other meat peptones.

Soy peptone-based BPW: (Appendix 2, Figures 2.4.4.1. and 2.4.4.2.) Generation times of cultures in soy peptone were also statistically significant (P = 0.002) for the different manufacturers. The lowest exponential generation time was 6.7 ± 1.1 min in S3 peptone and the highest, 28.6 ± 14.3 min; intermediate values were evenly spaced between these two values. Graphical representation of these data showed typical sigmoidal curves with a lag phase of ~3 h for all soy peptones.

Yeast peptone-based BPW: (Appendix 2, Figures 2.4.5.1. and 2.4.5.2.) *S. aureus* was the only bacterial species to present statistically different generation times in the differing yeast peptones (P = 0.029). The most divergent value of g was during growth in Y2 peptone: 77.5 ± 17.6 min. Generation times of this bacterial species were considerably longer in yeast peptone medium compared with the Enterobacteria tested. All growth profiles were sigmoidal with a lag phase of 2.5-3 h.

3.4.4. 24 h Yield of bacteria in selected BPW sterilized by autoclaving

Growth yields of the previously investigated bacterial species after 24 h were determined in the pre-enrichment medium BPW, of varying peptone content, using the Miles and Misra drop count technique. Data are shown in Table 3.4.

For Enterobacteria, the class of peptone which always generated the highest yields, based on the mean of the group, were yeast peptones. This was invariably followed by soy or meat peptone, and then casein peptone-based media. The class of peptone which consistently produced the lowest yields were gelatin peptones. Within the casein class of peptones, C6 based BPW always produced the highest yields; lowest yields generated by a casein peptone varied with bacterial species

cultured. A similar trend occurred during culture in gelatin peptone-based BPW, in that the highest yields were always produced by G2 peptone and the peptone in which lowest yields were displayed varied with bacterial species. Within the meat peptone class M1 peptone resulted in the highest yields for all bacterial species investigated and M4 peptone invariably produced the lowest cell numbers for all species. Yields after 24 h growth in soy peptone based media were similar for all the Enterobacteria: the highest yields were in S2 and the lowest in S3. *S. aureus* was found at highest cell densities in S4 and lowest in S1. Cultures after 24 h in the yeast peptones, for all bacterial species investigated, were always lowest on peptone Y2. Yeast peptone Y1 generated the highest yields after 24 h for all the Enterobacteria, whilst *S. aureus* cultures grew to the highest cell numbers in Y3 peptone.

Table 3. 4. Yield of Gram positive and negative bacteria after 24 h of growth in autoclaved buffered peptone water with constituent peptone originating from various sources and manufacturers. Mean values ± standard deviation are shown where n = 3.

Media		24 h Yield × 10^9 (CFU ml^{-1}) ± Standard Deviation			
		E. coli	*S.* ser. Poona	*S.* ser. Typhimurium	*S. aureus*
Casein	1	2.20 ± 0.19	2.34 ± 0.04	3.81 ± 0.50	1.18 ± 0.90
	2	2.88 ± 0.18	0.49 ± 0.05	2.23 ± 0.08	0.35 ± 0.04
	3	1.80 ± 0.60	1.26 ± 0.01	5.75 ± 1.77	0.08 ± 0.04
	4	1.74 ± 0.50	1.27 ± 0.06	3.54 ± 1.40	1.12 ± 1.80
	5	1.32 ± 0.07	1.35 ± 0.07	2.76 ± 0.18	1.61 ± 0.98
	6	4.26 ± 0.13	4.17 ± 0.09	6.85 ± 1.32	4.18 ± 0.93
	7	1.18 ± 0.10	0.73 ± 0.05	2.42 ± 0.04	0.92 ± 1.23
	8	0.72 ± 0.00	0.58 ± 0.02	1.77 ± 0.04	1.08 ± 0.48
	Mean	2.01 ± 1.12	1.52 ± 1.22	3.64 ± 1.79	1.32 ± 1.26
Gelatin	1	0.62 ± 0.06	0.14 ± 0.04	0.81 ± 0.11	Not Performed
	2	2.39 ± 0.00	0.66 ± 0.04	3.61 ± 0.03	1.25 ± 0.05
	3	0.56 ± 0.06	0.23 ± 0.02	1.22 ± 0.17	0.40 ± 0.17
	4	0.24 ± 0.00	0.13 ± 0.08	1.75 ± 0.22	0.66 ± 0.03
	5	2.12 ± 0.12	0.56 ± 0.10	2.55 ± 0.07	0.36 ± 0.01
	Mean	1.19 ± 0.99	0.34 ± 0.25	1.99 ± 1.12	0.67 ± 0.41
Meat	1	6.53 ± 0.36	5.61 ± 0.03	6.83 ± 0.17	4.40 ± 0.48
	2	2.49 ± 0.18	2.04 ± 0.02	2.75 ± 0.01	2.19 ± 0.04
	3	2.64 ± 0.23	1.89 ± 0.00	2.54 ± 0.03	2.02 ± 0.10
	4	0.50 ± 0.09	0.26 ± 0.00	0.49 ± 0.01	0.19 ± 0.05
	5	5.50 ± 1.13	4.36 ± 0.01	5.19 ± 0.05	3.55 ± 0.10
	6	3.51 ± 0.07	3.25 ± 0.04	3.82 ± 0.04	3.12 ± 0.04
	Mean	3.53 ± 2.19	2.90 ± 1.91	3.60 ± 2.21	2.58 ± 1.46
Soy	1	4.65 ± 0.04	2.82 ± 0.06	5.15 ± 0.03	0.97 ± 0.02
	2	6.02 ± 0.10	4.28 ± 0.03	5.99 ± 0.40	2.93 ± 0.05
	3	2.69 ± 0.17	2.30 ± 0.11	3.33 ± 0.04	1.81 ± 0.02
	4	4.25 ± 0.26	2.61 ± 0.07	4.73 ± 0.03	2.93 ± 0.57
	5	4.42 ± 0.07	2.50 ± 0.04	4.32 ± 0.06	2.10 ± 1.46
	Mean	4.41 ± 1.19	2.90 ± 0.79	4.70 ± 0.98	2.15 ± 0.83
Yeast	1	6.48 ± 0.10	6.55 ± 0.06	7.04 ± 0.02	3.19 ± 0.14
	2	3.47 ± 0.02	3.16 ± 0.05	3.23 ± 0.05	2.39 ± 0.33
	3	6.03 ± 0.08	6.16 ± 0.05	6.48 ± 0.06	4.36 ± 0.45
	4	4.32 ± 0.03	4.60 ± 0.00	5.18 ± 0.07	2.92 ± 0.06
	Mean	5.08 ± 1.54	5.12 ± 1.55	5.48 ± 1.69	3.22 ± 0.88

3.4.4.1. Yield at 24 h of *Escherichia coli* in selected BPW sterilized by autoclaving

E. coli cultures after a 24 h incubation period in buffered peptone water, whereby the peptone constituent was of differing source, exhibited significant differences in yields($P = 0.003$) dependent on the peptone source. Ranking the peptone sources, beginning with those which produced the highest yields, gave the order: yeast, soy, meat, casein and gelatin. The range between the yields produced, based on the mean of each group, was 3.89×10^9 CFU ml^{-1}. Within each peptone class, there was considerable deviation from the mean indicating that there were also differences between peptones of identical source which were produced by differing companies. This was shown to be statistically significant at $P = 0.000$ for each peptone class, demonstrating the diverse effect of batch processes. The most diverse class was the meat peptone category with a range from 5.00×10^8 CFU ml^{-1} in M4 to 6.53×10^9 CFU ml^{-1} in M1 peptone. This was closely followed by the casein peptone class with a range of 3.54×10^9 CFU ml^{-1}. Soy peptones generated yields whereby the highest values were over two fold higher than the lowest yields. Yeast peptones were also divergent with a range of 3.01×10^9 CFU ml^{-1}. Finally gelatin, with the lowest yields, was most consistent with a range of 2.15×10^9 CFU ml^{-1}.

3.4.4.2. Yield at 24 h of *Salmonella* ser. Poona in selected BPW sterilized by autoclaving

S. ser. Poona exhibited statistically different yields, after 24 h incubation, in the various peptone classes investigated ($P = 0.000$). The ranking of peptone class, based on the mean yields, was similar to that of *E. coli*, with the exception of soy

and meat peptones being equal in ability to generate cell numbers after 24 h growth. All peptones within each class also produced significantly different yields at P = 0.000. This demonstrates the great diversity not only between peptone sources, but also within each source whereby batch processes alter the performance of the peptone product. The most diverse class was again meat with a range of 5.35 $\times 10^9$ CFU ml^{-1}, followed by casein, yeast, soy and gelatin with ranges of 3.69$\times$ 10^9 CFU ml^{-1}, 3.39 $\times 10^9$ CFU ml^{-1}, 1.98 $\times 10^9$ CFU ml^{-1} and 0.53 $\times 10^9$ CFU ml^{-1} respectively. This differed from *E.coli* only in the reversing of soy and yeast in the rank order.

10

3.4.4.3. Yield at 24 h of *Salmonella* ser. Typhimurium in selected BPW sterilized by autoclaving

This species was the only species of bacteria which did not display yields which statistically differed with class of peptone (P = 0.057). However, variations were apparent with mean yields of gelatin-based BPW being 1.99 $\times 10^9$ CFU ml^{-1}, whereas yeast-based media produced double the quantity: 5.48 $\times 10^9$ CFU ml^{-1}. Soy-based media were second best, followed by casein- and then meat-based BPW.

Yields of organisms in similar peptone-based media were shown to be significantly

20 different dependent on manufacturer and batch: casein (P = 0.007), gelatin (P = 0.000), meat (P = 0.012), soy (P = 0.009) and yeast (P = 0.000).

The most diverse yields of *S.* ser. Typhimurium generated by one peptone type was meat peptone-based BPW with a range of 6.34 $\times 10^9$ CFU ml^{-1}; this was followed by a range of 5.08 $\times 10^9$ CFU ml^{-1} for casein peptones. Yeast-based media produced a range of 3.81 $\times 10^9$ CFU ml^{-1} whereas gelatin- and soy-based BPW

were similar with 2.80×10^9 CFU ml^{-1} and 2.66×10^9 CFU ml^{-1} respectively. This order was identical to that found for *S.* ser. Poona.

3.4.4.4. Yield at 24 h of *Staphylococcus aureus* in selected BPW sterilized by autoclaving

Mean yields of *S. aureus*, after a 24 h growth period in media of different peptone types, varied significantly ($P = 0.028$); the ranking of highest cell numbers cultured to lowest based on the mean of each group was typical. However the Gram-positive bacteria were distinct from the Enterobacteria previously discussed. Yields after 24 h incubation in BPW were not as high as those of the Gram-negative bacteria investigated, with the highest cell numbers generated in meat-based BPW at 4.40×10^9 CFU ml^{-1}; unlike the Enterobacteria which consistently grew to high values (7.04×10^9 CFU ml^{-1} for *S*T) in yeast-based media.

Yields of *S. aureus* were not significantly different, after culture for 24 h, in the following classes of peptone whereby raw material was identical and manufacturer or batch differed: casein ($P = 0.068$) and soy ($P = 0.054$). In BPW comprising different batches of gelatin ($P = 0.033$), meat ($P = 0.008$) and yeast ($P = 0.015$) there were significant differences.

Meat-peptones produced the greatest range of yields (4.21×10^9 CFU ml^{-1}) followed by casein (4.10×10^9 CFU ml^{-1}), yeast (1.97×10^9 CFU ml^{-1}), soy (1.96×10^9 CFU ml^{-1}) and the consistently poor gelatin (0.89×10^9 CFU ml^{-1}).

3.4.5. Discussion: effect of peptone on growth dynamics of bacteria cultured in selected BPW sterilized by autoclaving

Peptone composition clearly affects the growth of bacteria as a constituent of the medium BPW. Of the bacterial species used in this work, *S.* ser. Typhimurium appeared the most biochemically diverse (i.e. most able to utilize substrates provided for growth of the population), being the least affected by variation in peptone used. This may, especially in this organism, be due to an inherent diversity of protein uptake mechanisms and metabolic pathways. Differences in values of generation times and yields of *S.* ser. Typhimurium are still clearly evident despite these observations not being statistically significant. All other species studied were more sensitive to the peptone constituent of the medium; this sensitivity was as expected due to the biochemical diversity of the peptones used.

The remarkably short generation times achieved, from 4 min, can be attributed to a variety of factors. Light scattering varies dependent on shape, orientation (in the case of bacilli) and size of the organisms in suspension. Aggregates also affect light scattering in comparison with a homogenous suspension. The following chapter details the significant differences observed of the shape and aggregation of certain species which adversely affect light scattering and consequently OD readings. In addition, at the lower limits of detection the bioscreen analyser was less accurate; these low inocula were necessary in order to achieve rapid growth. Therefore the initial log phase produced for some media are deceptive and generate data which are not accurate. The decision to analyse the first exponential phase of growth only for comparison of the different BPW media was flawed. The conclusion was that the Bioscreen analyser was inaccurate at the lower limits of detection, despite these values being in the linear range of the machine. With the

additional morphological variation which affected light scattering the total effect produced exceptionally fast generation times which were not a true representation of actual values.

3.4.5.1. Effect of different peptones on the growth dynamics of bacteria

Pancreatically digested heart tissue of porcine origin would be expected to be complex, in contrast to the refined κ–casein peptone. Outlined below are descriptions of the peptones used in this work, they are as accurate as manufacturer's certificates allow:

Meat peptones originated from a porcine, bovine or poultry origin, the tissue types in most common use were heart, lungs, oesophagus, skin and bones. The characteristic biochemical constituents of these tissue-types differ and consequently affect performance of the medium with respect to their support for microbial growth.

Other peptones originating from animal sources were casein and gelatin. Peptones belonging to the casein group were diverse in their ability to support bacterial growth; this was surprising as caseins (mostly expected to be κ-casein), a phosphoprotein component of bovine milk, was more defined in content compared with other peptones. Thus it was hypothesized that the casein peptones would produce similar growth characteristics, regardless of manufacturer or batch. As this was found not to be the case, manufacturing procedures for casein peptones must differ between companies and between batches, to result in chemically distinct products with different capacities for support of bacterial growth. Certain components of bovine milk (e.g. lactophoricin) have been shown to possess

antibacterial activity (Campagna *et al.*, 2004); casein peptones to be used as the nutritional source within the medium, which were not purified sufficiently to remove such components, may still contain these antibacterial elements which will affect growth of bacteria.

Gelatin is also a more defined substrate compared with other peptones: it is produced by boiling collagen, isolated from animal skin, bones and connective tissues, and subsequent pancreatic digestion. Gelatin peptones are high in glycine, proline and hydroxyproline. Gelatin-based peptones produced consistently poor generation times and yields with all bacteria; analyses showed low tyrosine content relative to other peptones. This and an inadequate carbohydrate source contribute to poor growth of *Salmonella:* as does the tryptophan content which was so low as to be undetectable by the methods employed, Gray *et al.*, 2006.

Soy peptones are prepared by papain-digestion of *Carica papaya*. Growth of all organisms was consistently excellent with soy-based BPW, although differences between manufacturers, even if not as pronounced as for casein peptones, were observed. This again indicated a difference between manufacturers' procedures. Another possibility for variation between the more complex soy peptones was the state of the plant tissue at harvest: if the tissue had been previously damaged, plant defence responses might have included the production of anti-microbials.

Yeast extract produced the best performance of all the peptones tested in this work: autolytically-produced extract should contain all the amino-acids required for growth.

3.4.5.2. Variation in peptone manufacture and media preparation

Source-dependent differences in yields of bacteria tested were evident; however this observation again indicates inconsistent batch processing and different manufacture between companies.

Certain aspects of peptone manufacture which contribute to peptone variability are beyond the manufacturers' control: for all complex peptones (from meat, soy or yeast) biological development processes affect the chemical composition of the organisms. Furthermore, host-response to injury or harsh environmental conditions may induce anti-microbials, immune or chemical responses, all of which have unfavourable consequences when the product is assessed as a constituent of a growth medium. Transport of raw materials may differ between companies: tissues or substances may be frozen immediately, preservatives may be used, and transportation may have been at ambient or freezing temperatures.

However other aspects are controllable: e.g. sample to enzyme and reaction mixture to vessel volume ratios. Other process variables that should be standard include temperature and length of time of the digestion, filtration, centrifugation and drying steps during peptone processing, and vessel cleaning procedures between batches. However these procedures vary between companies and contribute toward the resulting differences in peptone performance; for instance longer digestion periods or higher temperatures may denature certain growth factors which were present in the medium (e.g. B vitamins or other organic compounds).

Procedures in the microbiology laboratory also influence medium performance. These include the overheating of media during autoclaving resulting in production of toxic oxygen species (Mackey and Derrick, 1986b) which differ in concentration with varying peptones (Stephens *et al.*, 2000), inaccurate measurements affecting

medium composition, storage conditions (Mavrommati *et al.*, 1987) and age of medium constituents and prepared media.

All of these factors, from raw material harvest and production processes to laboratory handling, contribute to the differences seen in media performance and consequently experimental outcomes in laboratories across the world. The observations noted for bacteria and media, of identical preparation, in this work are of significant difference.

3.4.6. Growth of *Salmonella* in selected BPW of variable peptone concentration

Determination of the effect of peptone concentration allowed insight into whether the peptone in question was nutritionally poor or whether endogenous inhibitors present in the peptones themselves prevented growth. *Salmonella* ser. Typhimurium grown in various concentrations of S2 and G5 based BPW was analysed and the growth curves are shown in Figure 3.1. and 3.2. respectively.

In S2 based BPW (Figure. 3.1.), there was no significant difference in the generation time with change in peptone concentration ($P = 0.082$); g was 30 min in all cases. The yield however was notably different ($P = 0.018$) with cultures grown in higher concentrations of peptone achieving higher yields. At 1 g l^{-1} the yield recorded was $3.51 \pm 0.00 \times 10^8$ CFU ml^{-1}, in 30 g l^{-1} *Salmonella* cell numbers reached $2.70 \pm 0.00 \times 10^9$.

S2 peptone based BPW produced a higher yield and lower generation time, at all concentrations, compared with G5 peptone.

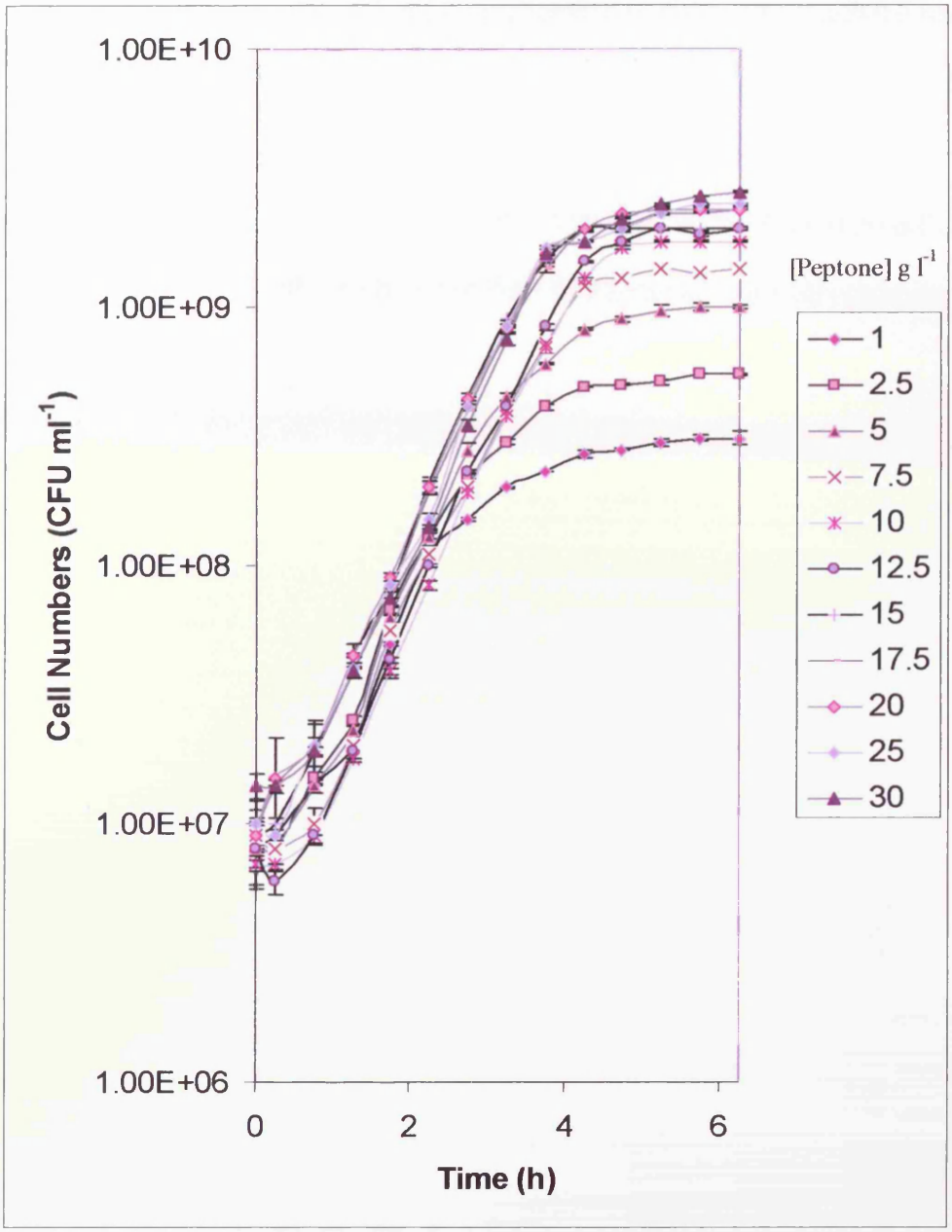

Figure 3.1. Growth curves of *Salmonella* ser. Typhimurium in different concentrations of S2 peptone as a constituent of buffered peptone water pre-enrichment medium. Points represent the mean of duplicate samples from duplicate flasks in the respective media, ± standard deviation where n = 4.

Salmonella grown in different concentrations of G5-based BPW produced distinctly different growth curves (Figure. 3.2.) from those in S2 peptone-based BPW. Generation times were found to be considerably different (P = 0.000), dependent on the concentration of peptone used, the higher the concentration the

lower the generation time. The value of g for *Salmonella* in 2.5 g l^{-1} peptone was 90.8 ± 1.5 min, this decreased to 36.4 ± 0.2 min in 30 g l^{-1} of G5 peptone. Yield was also significantly different dependent on concentration of G5 peptone ($P = 0.023$): 1 g l^{-1} produced a yield of $1.70 \times 10^8 \pm 0.00$ CFU ml^{-1}, whereas 30 g l^{-1} of G5 peptone gave $2.40 \times 10^8 \pm 0.00$.

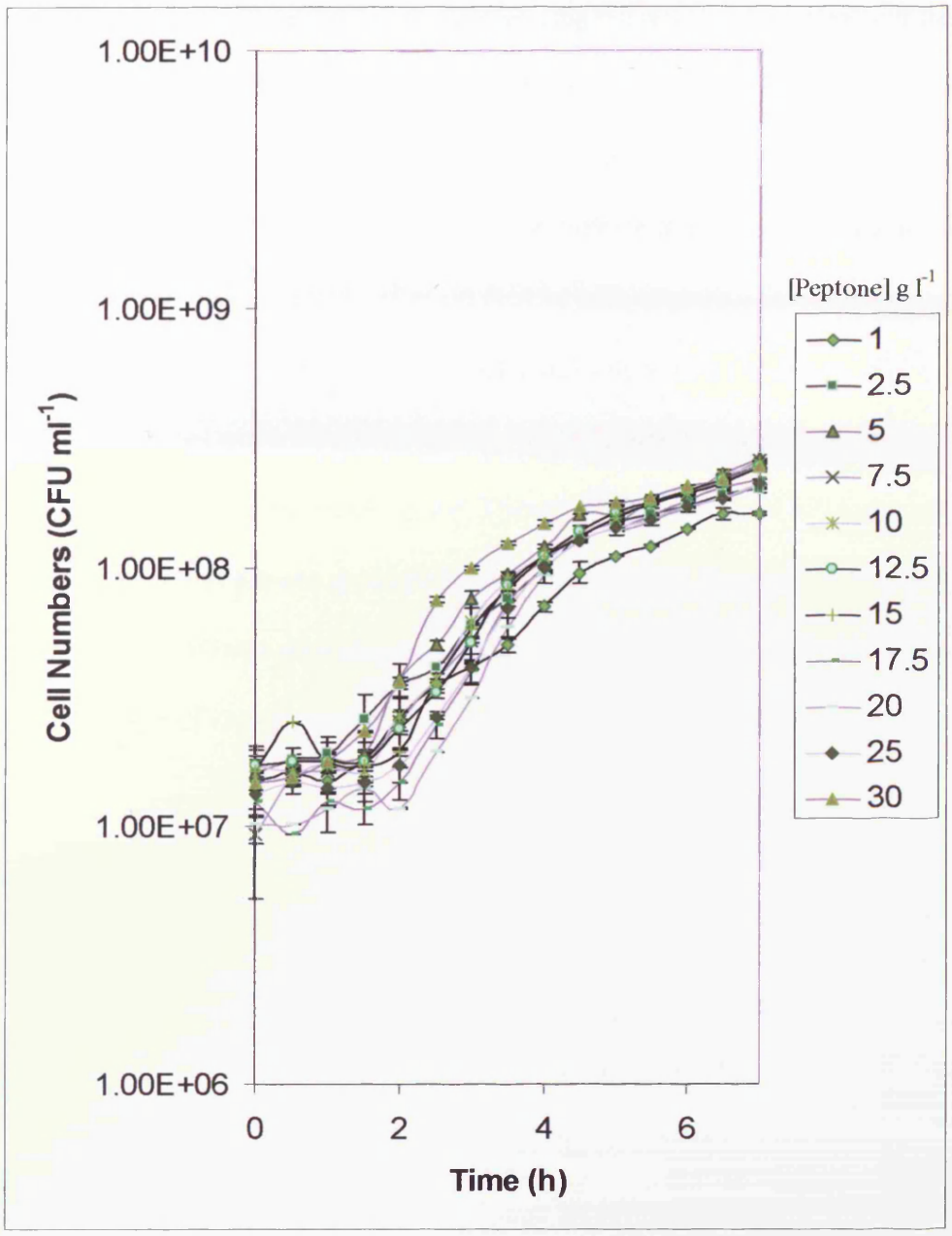

Figure 3.2. Growth curves of *Salmonella* ser. Typhimurium in different concentrations of G5 peptone as a constituent of buffered peptone water pre-enrichment medium. Points represents the mean of duplicate samples from duplicate flasks in the respective media, ± standard deviation where n = 4.

Endogenous inhibitors, such as those described previously (Section 3.4.5), were determined not to be a significant cause for poor growth. If this were the case, then increased peptone concentration would result in a decrease in growth as the concentrations of inhibitors were also increased. In S2 peptone and the

nutritionally poor, G5 peptone-based BPW, this did not occur, suggesting that such inhibitors were not present in sufficient quantities to cause an effect. S2 peptone was observed to provide a nutritionally rich medium, as increasing peptone concentration did not increase the generation time of *S.* ser. Typhimurium. Uptake of nutrients into the organism relies on factors such as secretion of enzymes into the medium for degradation of macromolecules into utilizable forms, diffusion or uptake by permeases or transport systems of these products into the cell and transport across the periplasmic space. These cellular processes limit generation times regardless of growth medium (Koch, 1997). Yield increased with higher concentrations of both peptones suggesting that more nutrients allowed for a denser growth of organisms before other factors became limiting.

3.4.7. Growth of *Salmonella* Typhimurium in selected filter sterilized BPW

Variable factors during media preparation alter the outcome of peptone performance. For instance it has been observed that certain organisms (e.g. the parasitic protozoan, *Giardia intestinalis*) are not as successfully cultured in autoclaved medium (Lloyd, Pers. Com.), and so difference in performance of autoclaved BPW was compared with that of filter sterilized (FS) BPW using *S.* ser. Typhimurium. Generation times (Figure 3.3.) were still affected by the peptone source regardless of the media preparation method used, either autoclaved or FS. Values for g were considerably affected by the method of media preparation ($P = 0.000$). In FS BPW, the g value for growth of *S.* ser. Typhimurium in C6 peptone based-BPW was notably lower than in C6 peptone based-BPW (which was sterilized by autoclaving): 18.12 ± 0.00 min and 61.00 ± 6.80 min respectively.

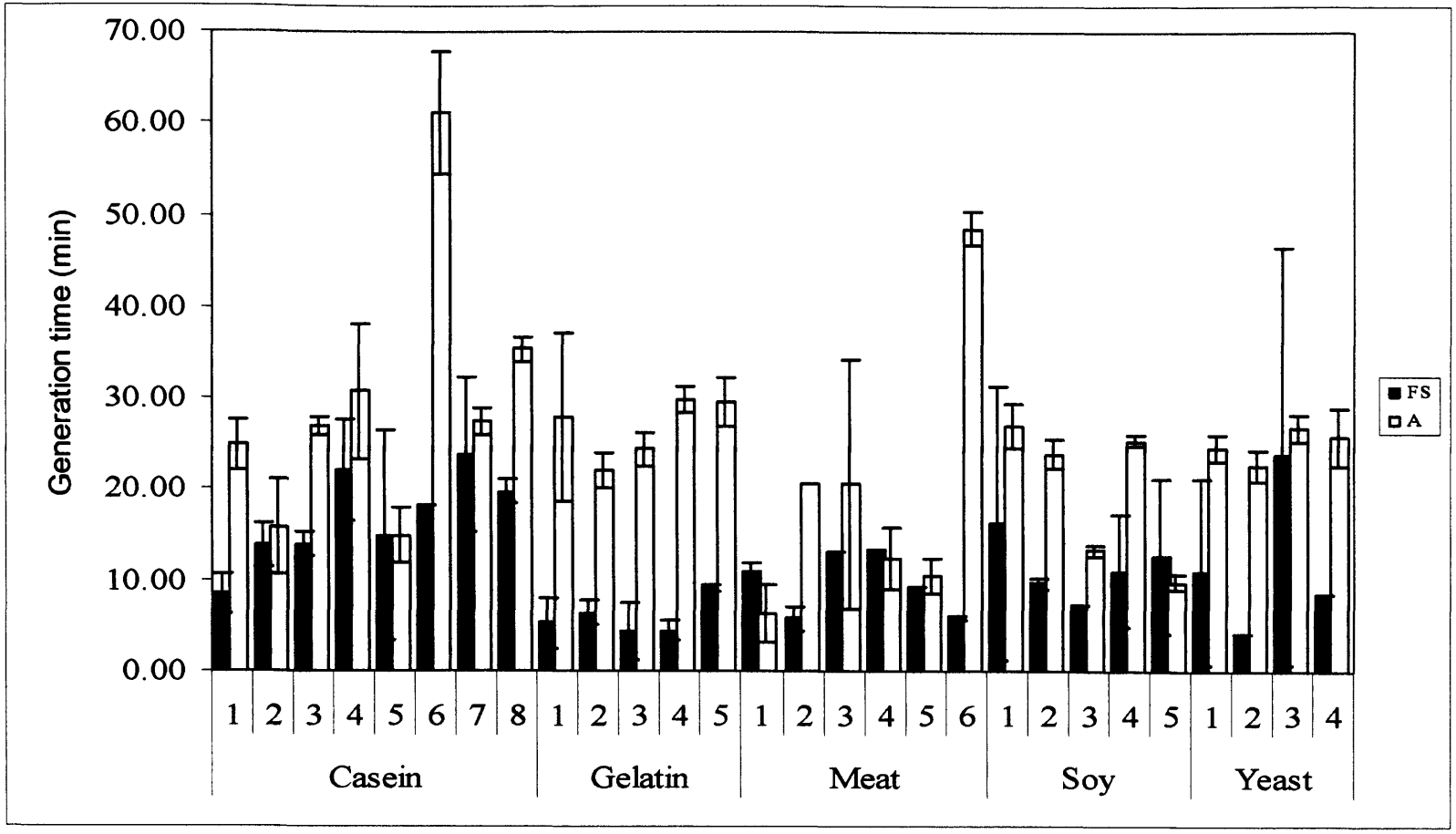

Figure 3.3. Growth characteristics of *Salmonella* ser. Typhimurium: comparisons of the effects of filtration-sterilization (FS) vs autoclaving (A) buffered peptone water on generation times.

3. Growth of bacteria in liquid culture media

Yields of *S.* ser. Typhimurium, after 24 h incubation in BPW constituting different peptone sources, were still affected by peptone source, whether the medium was autoclaved or FS. However, yields were unaffected by the method of media sterilization ($P = 0.998$) and similar cell numbers were achieved in the corresponding FS and autoclaved media (Figure 3.4.).

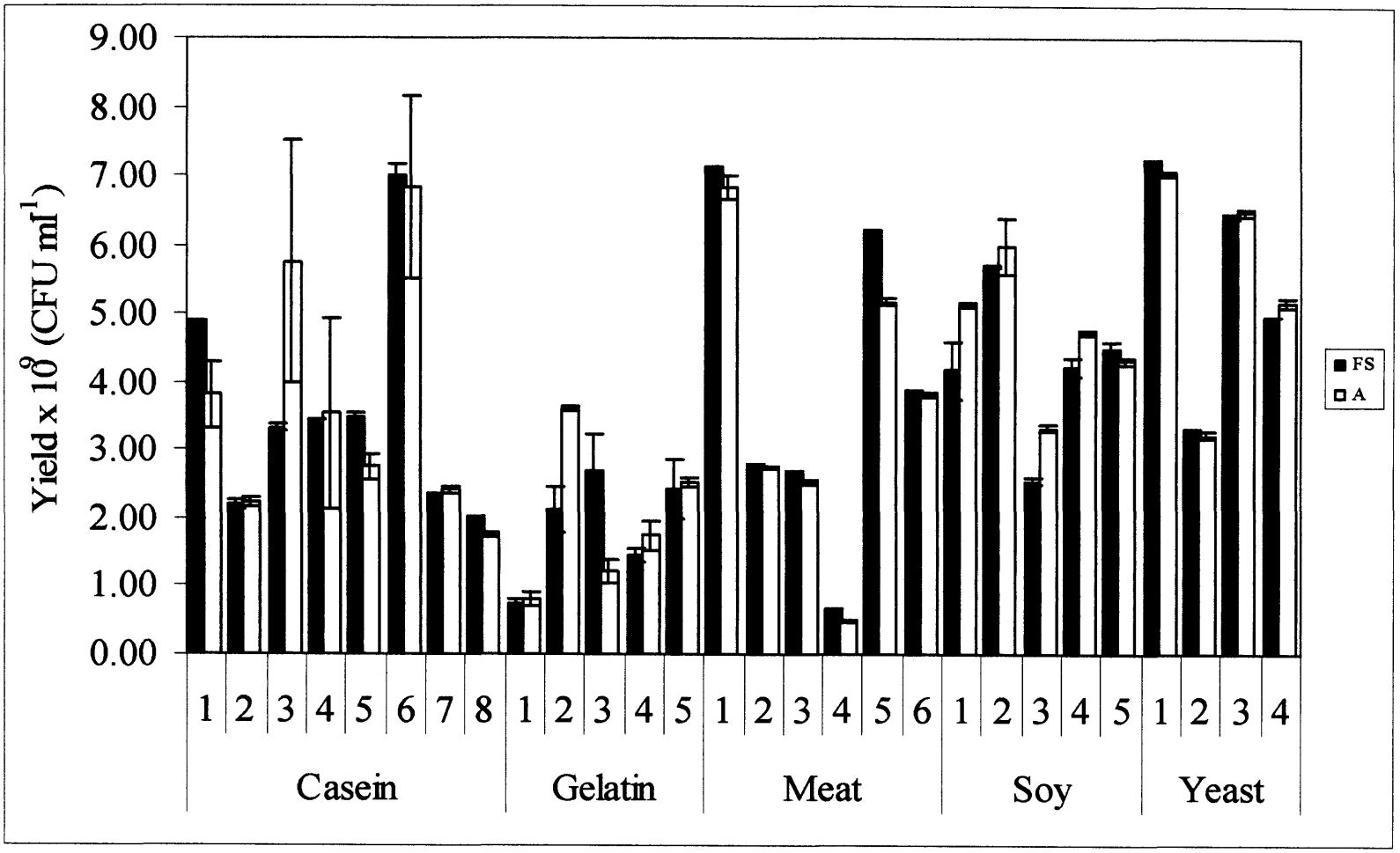

Figure 3.4. Growth characteristics of *Salmonella* ser. Typhimurium: comparisons of the effects of filtration-sterilization (FS) vs autoclaving (A) buffered peptone water on yields.

Effect of sterilization techniques on peptone performance

Sterilization techniques were shown to affect growth of *S.* ser. Typhimurium: generation times in FS BPW were significantly lower in contrast to autoclaved BPW. This may be indicative of the presence of a heat-labile component within the medium, such as an amino-acid (e.g. tryptophan), vitamin (e.g. biotin or thymine) or other growth factors (e.g. purines and pyrimidines). Another possibility is that reconstitution of the peptone with water reactivates enzymes present from the original digestion of raw materials during manufacture; subsequently allowing continued digestion of the peptones in solution. This would result in an increased number of lower molecular weight peptides or utilizable substrates in the FS BPW leading to faster uptake of nutrients and consequently lower values of g. In contrast, enzymes present in the autoclaved BPW medium would be heat-denatured during the autoclaving process, and so higher molecular weight proteins which are more difficult to transport into the cell would remain. The presence of toxic oxygen species in autoclaved media (Mackey and Derrick, 1986 a; Stephens *et al.*, 2000) may also hinder growth of *Salmonella*. However yields were unaffected, indicating that the nutritional value of the medium was not measurably altered during the different sterilization procedures.

3.4.8. Growth of heat-shocked *Salmonella* in selected BPW

Salmonella presented for pre-enrichment may not be of an optimal state, cells are often sub-lethally injured, but recovery of these organisms is essential for subsequent growth and identification on diagnostic media. The ability of these different BPW media to culture *S.* ser. Typhimurium in both optimal and heat-shocked states was assessed. The variation of sub-lethal bacterial growth is of

importance but of indirect interest in this work: the focus being the difference in performance of the various media ingredients. Generation times were statistically different ($P = 0.000$) between healthy and heat-shocked organisms (Figure 3.5.), with heat-shocked organisms having a higher generation time. Previous work demonstrated that after sub-lethal heat treatment less than 90 % of the *Salmonella* population were able to reproduce (Clark and Ordal, 1969) and that aeration was detrimental to recovery (Gomez and Sinskey, 1975); the conditions and number of viable organisms in the inocula may have contributed to the significantly higher generation times. *Salmonella* in G3 peptone had a generation time over 10 fold higher (263.8 ± 26.7 min) after heat shock treatment compared with the standard inocula preparation (24.4 ± 1.8 min). The increase in generation time after heat-shock treatment was expected, but importantly the range of g values during growth in the different peptones increased in heat-shocked salmonellae. Injured *Salmonella* grown in Y2 based BPW showed a g of 41.1 ± 14.2 min, whilst in G3 based BPW g was 263.8 ± 26.7 min. In standard healthy inocula the range for *S.* ser. Typhimurium was from 6.3 ± 3.1 min in M1 based BPW to 61.0 ± 6.8 min in C6. This is of importance as the ability of a medium to allow recovery and subsequent enumeration, of a pathogenic organism which may be sub-lethally injured, is the basis for positive identification of a potential contaminant present on industrial food stuffs. Investigations by Stephens *et al.* (1997) showed that certain pre-enrichment media (BPW, lactose broth, TSB, heart infusion broth and universal pre-enrichment broth) recovered higher numbers of injured *Salmonella* than others. Therefore the inclusion of an unsuitable peptone as a constituent of the pre-enrichment medium may result in failure to recover the organism which, in

subsequently favourable conditions, replicates and results in a bacterial population capable of human infection.

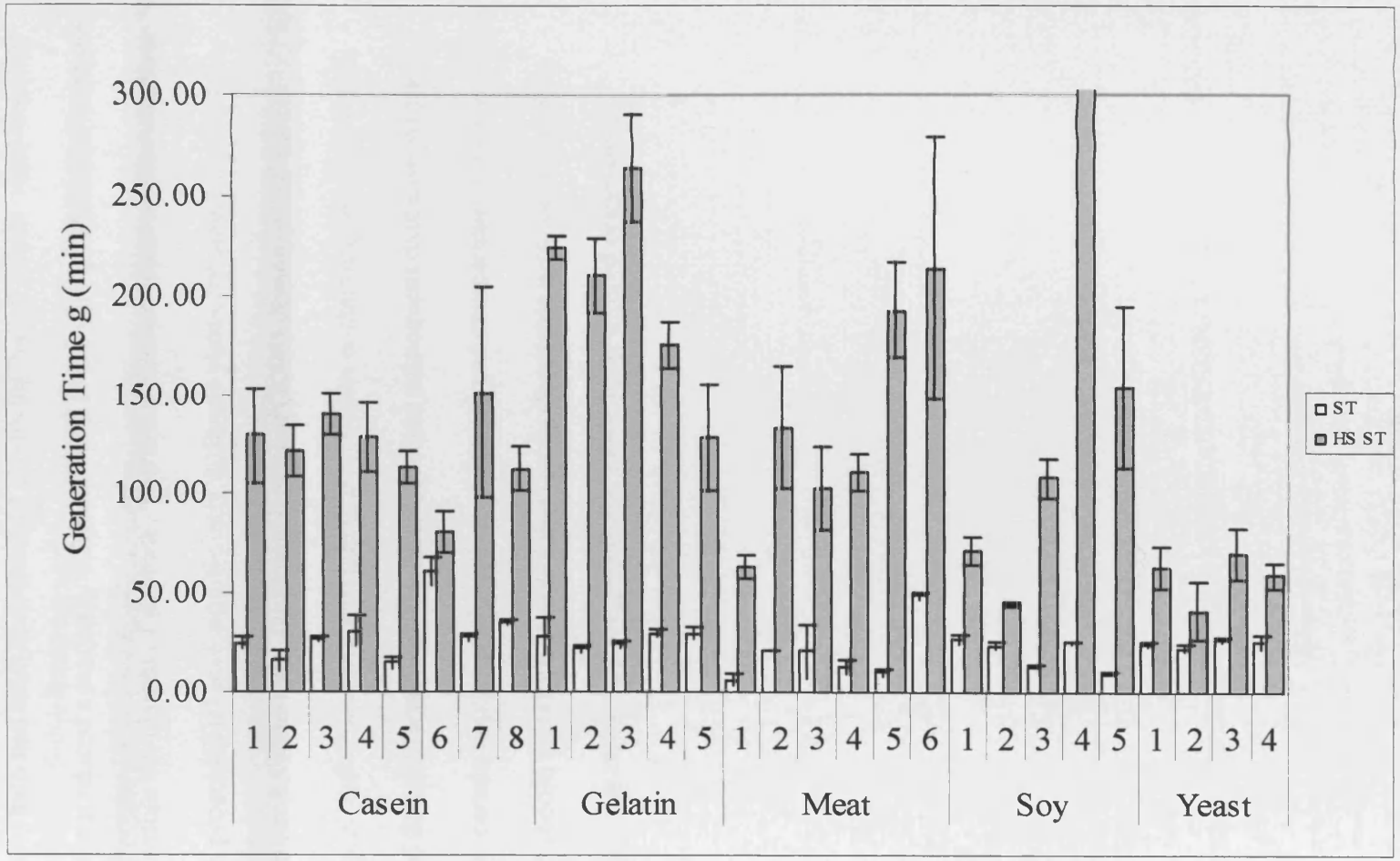

Figure 3.5. Generation times of heat-shocked *Salmonella* ser. Typhimurium (HS ST) compared with standard inocula (ST), during growth in autoclaved buffered peptone water.

Yield after 24 h was also significantly different (P = 0.040) between heat-shocked *Salmonella* and those obtained by the standard preparation (Figure 3.6.), with organisms which had been heat-shocked producing higher yields. Heat-shocked *Salmonella* produced a markedly different yield in the various classes of peptone, and also within the classes of peptone. Therefore class of peptone, manufacturer and batch processes all give significantly different yields for standard and sub-lethally injured *Salmonella*. De Spiegeleer *et al* (2004) demonstrated that harvesting *E. coli* from Luria-Bertani (LB) medium containing tryptone which originated from different manufacturers affected subsequent oxidative stress resistance; certain peptones allowed resuscitation and enumeration whereas others did not. It should therefore be noted that the most suitable peptone for healthy *Salmonella* may result in poor growth for sub-lethally injured organisms.

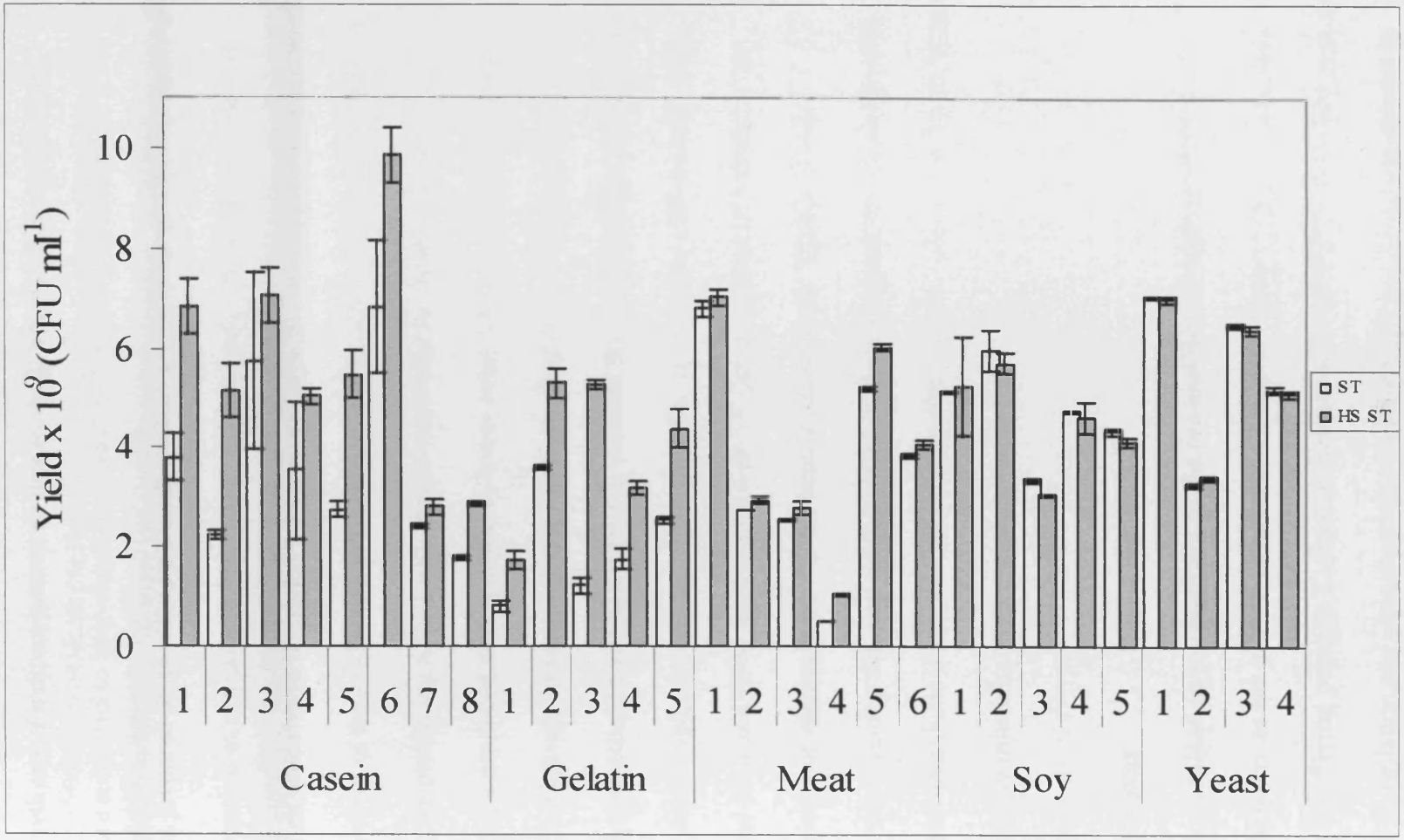

Figure 3.6. Yields after 24 h incubation of heat-shocked *Salmonella* ser. Typhimurium (HS ST) compared with standard inocula (ST), during growth in autoclaved buffered peptone water.

As already discussed the recovery of low numbers of sub-lethally injured pathogens is essential for quantitative diagnostics; however mechanisms of sub-lethal growth are of indirect interest to this work. It was noted that organisms which were subjected to heat-shock generated higher yields in contrast to organisms prepared by the standard method. This observation may be the result of gene expression in response to heat-shock, subsequently allowing expression of genes which give rise to increased tolerance of toxic waste products or oxygenated species present in the media and so producing higher yields. It was also observed that peptones producing a relatively higher value of g for healthy *S.* ser. Typhimurium produced a comparatively lower value for heat-shocked, e.g. C6 produced a relatively lower g value for heat-shocked *S.* ser. Typhimurium, compared with other casein-based media, whereas the same medium produced a relatively higher value in healthy organisms. The reverse was also noticed, with M5, S3 and S5 based BPW performing well for the generation of healthy *S.* ser. Typhimurium but relatively poorly for heat-shocked *S.* ser. Typhimurium.
Thus it is worth noting that certain peptones which do not perform relatively well with healthy organisms may generate comparatively better values with those which are sub-lethally injured.

3.5. Conclusions

Selection of peptone as a constituent of the pre-enrichment medium BPW is of great importance, as the growth kinetics of a bacterial species was shown to vary greatly with differing peptone. Culture techniques are required for purposes such as enumeration, isolation and identification of microorganisms; basic procedures in microbiology forming the foundation for all subsequent work. Organisms that have

signs of vitality and are apparently viable but in practice non-culturable (Lloyd and Hayes, 1995; Bloomfield *et al.*, 1998), little understanding can be gained for these potentially important species. Analysis of bacterial growth under varying conditions, e.g. for identification of potential pathogens within the industrial food environment, may result in false negative results for the presence of these organisms: growth media being of an unsuitable quality leading to lack of growth. Recovery of recombinant bacterial colonies may not occur, as the medium used does not allow growth, rather than the assumed method failure. As the foundation for all experimental work, it is essential that the culture medium chosen allows good growth of the target organisms.

Therefore when the manufacturers perform quality control tests on their media, it would be advisable to investigate performance of the medium with different species and, if the media is recommended as an enrichment or enumeration medium, to test the batch with sub-lethally injured organisms. Perhaps manufacturers could provide quality-assurance statements as to the efficacy of their media for such organisms, and provide increased consideration of the type of peptone specific for these organisms in recovery media (e.g. in BPW, universal pre-enrichment broth, lactose broth or alkaline peptone broth).

This work has also shown the effects of different media preparation techniques; heat-sterilization and the consequent absence of an unknown heat-labile compound(s) often gives rise to higher generation times. Thus the method of sterilization should also be considered during experimental design.

In order to address the issue of variable media effects upon experimental design in the microbiology laboratory I suggest the following procedure:

- Test small samples of different media batches and, after identifying a medium which performs well, buy a batch large enough to complete the work. This will eliminate media variation and ensures that the medium meets the experimental requirements.
- Choose the most suitable sterilization technique and ensure that this remains constant. Different autoclaves will produce variable pressure (and hence temperature) cycles: ideally the same autoclave under identical conditions each time should be employed.
- Vessels used to autoclave media will cause differences: 1 l of liquid media in a 1 l duran bottle will be subject to a slower temperature increase and undergo less oxidation than 250 ml of liquid media in a 1 l duran bottle.
- Rapid cooling of media affects subsequent performance. Media should always be allowed to cool in an identical manner.
- Culture of bacteria in liquid media will vary with media:vessel volume and sample:media volume. Again these variables, well represented within the literature, require consideration and consistency.

Bacterial growth is wholly dependent on culture medium; therefore selection of components is of considerable importance.

3.6. Acknowledgements

I would like to thank the following for their help or support with work described in this chapter:

- University of Wales, Cardiff, School of Pharmacy: for the use of the Bioscreen C growth analyser.

- University of Wales, Cardiff, School of Biosciences: Paul Seaman and Gareth Williams for their kind donations of *Staphylococcus aureus* and *Escherichia coli* respectively.
- Merck Chemicals, Newport: for unlimited use of their laboratory and office facilities.

CHAPTER FOUR

Morphology and motility of bacteria dependent on peptone

4. Morphology and motility of bacteria dependent on peptone

4.1. Summary

Identification of *Salmonella* serotypes are based on flagellar and somatic antigens. The absence of flagella may consequently affect complete identification of the serotype; here it is shown that *Salmonella enterica* serovar Typhimurium exhibits morphological differences dependent on the peptone constituents of the culture medium. Aflagellate *salmonellae* were produced in certain media where the nutritional ingredient was casein, meat or gelatin peptone; in gelatin based peptone aggregates of *Salmonella* were observed. On the other hand, in media containing soy or yeast peptone as the primary nutrient, *Salmonella* displayed a normal flagellated morphology. Transfer of aflagellate *Salmonella* from nutritionally poor media, casein- or gelatin-peptone based, into rich nutrient broth allowed flagella synthesis: indicating that the aflagellate form is still able to produce flagella. Amino acid sequencing of the peptones producing aflagellate organisms showed a relatively low tyrosine concentration: only 0.03 ± 0.01 g l^{-1} for gelatin-based buffered peptone water compared to 0.21 ± 0.01 g l^{-1} for soy-based buffered peptone water. Tyrosine is essential for flagellin which is the subunit of the *Salmonella* flagellar filament. Addition of 200 μM tyrosine to casein peptone-based media produced flagellate *salmonellae;* 2 mM glucose was needed in addition to tyrosine to achieve a similar morphology in gelatin-based media.

Quantitative swim migration assays, assessing the motility of *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Poona and *Escherichia coli* during cultivation in the various peptones, found that peptone variation considerably affected motility. The organisms differed in motility on identical media, however non-motile forms were observed only on casein-, gelatin- or meat-based BPW. Soy and yeast

peptones consistently produced motile organisms: *E. coli* cultivated on S4 peptone based BPA achieved a swim rate of 7.77 ± 0.33 mm h^{-1}. Thus motility is markedly affected by the peptone constituent of the medium.

4.2. Introduction

Media composition affecting degree of flagellation or motility has been previously observed with certain basic constituents of bacterial culture media, including different agars or peptones. It was found that a broad spectrum of adverse conditions produced *E. coli* and *Salmonella* ser. Typhimurium which were lacking in flagellin, the subunit of the flagellar filament, and consequently motility. These included high temperatures, high concentrations of inorganic salts, high concentrations of carbohydrates, low molecular weight alcohols and the presence of gyrase inhibitors, which were all shown to decrease or prevent motility in these organisms (Li *et al.*, 1993; Shi *et al.* 1993). *Proteus* species have been observed to alter the wavelength of flagella (i.e. distance from one wave-crest to the next) in response to different concentrations of casitone and yeast extract as medium constituents (Leifson *et al.*, 1955). *Salmonella* ser. Pullorum, identified by the Kauffmann-White scheme as a non-flagellate and non-motile serovar affecting avian hosts, was noted to produce flagella and consequently become motile under certain conditions. Medium induced motility was dependent on agar concentration, and type and concentration of carbohydrate (Holt and Chaubal, 1997). Guard-Petter (1997) similarly achieved flagellate cultures of *Salmonella* ser. Pullorum on Hektoen-enteric agar (HEA) with a supplement of 100 mM glucose. *Salmonella* ser. Enteritidis during growth in identical conditions (HEA supplemented with 100 mM glucose) became hyper-flagellate; in this form the organism swarmed and penetrated the agar (Guard-Petter, 1997). Previous work

identifies organisms which were noted as aflagellate only in certain conditions: aflagellate cultures of *Salmonella typhosa* strain O-901 were observed during cultivation on a minimal medium, yet were of normal serology and morphology after a period of growth on a more complex media (Tulley and Gaines, 1961).

Harshey and Matsuyama (1994) demonstrated that the swarming ability of *E. coli* and *Salmonella* ser. Typhimurium was critically dependent on the manufacturer of the agar used, with certain agars supporting swarming to a lesser degree, despite there being no difference in the chemical composition of the agar. Concentration of agar critically affected motility and morphology of many bacteria, for example *Pseudomonas aeruginosa, Listeria monocytogenes, Salmonella typhimurium* and *Escherichia coli*, with higher agar concentrations inhibiting motility (Mitchell and Wimpenny, 1997).

Swim migration assays performed with *Photorhabdus temperata* exhibited variable motility which was dependent on the different peptones used as the nutritional ingredient of the medium. The motility of this organism deviated from the values obtained during growth on the original medium recipe after the addition of 0.5 % or 1 % yeast extract (Hodgson *et al.*, 2003).

Here I will show how the use of peptones from various origins and sources, as the nutritional ingredient of the culture medium, affects *Salmonella* and *Escherichia* morphology and motility.

4.3. Materials and methods

Deviations from the standard methods which were detailed in Chapter 2, and were used in this chapter, are described below:

4.3.1. TEM of *Salmonella* incubated in a nutrient rich medium whereby the inoculum was aflagellate

Conical flasks (100ml) containing the nutrient rich medium NB (20 ml) were inoculated with aflagellate *Salmonella* suspensions (20 µl). These inocula were taken from C2, C5, G1 and G3 peptone based BPW which had been incubated in the respective media for 22 h at 37°C, shaken at 100 cycles min^{-1}. The resulting NB cultures were then incubated for 4 h at 37°C, shaken at 100 cycles min^{-1}. Suspensions of *Salmonella* cultured in BPW, used to inoculate the NB, and the resulting NB cultures were then examined using TEM as described in chapter 2.2.5.

4.3.2. Calculation of protein content for peptones

Amino acid analysis was performed on each peptone (Chapter 2.3.2.) and from these data the protein content, as the summation of all amino acids, was calculated as a % w/w of the original peptone sample.

This method was employed instead of the Bradford method as the latter produced inaccurate data; this was due to the low quantities of aromatic amino acids in some peptones, and the requirement for two peptide bonds (a tri-peptide) in order for the colorimetric shift to be determined. Due to digestion methods most peptones did not fit this requirement and so results obtained were incorrect. Gelatin was named as a protein for which the Bradford method was unsuitable (Bio-Rad protein assay

instruction manual, 1984).Thus summation of amino acid content, giving the total percentage composition of protein based material, was used.

4.3.3. Supplemented buffered peptone water

BPW was made up according to the methods detailed in chapter 2.1 and appendix 1. Where indicated supplements were added to the standard BPW recipe as follows:

- Tyrosine (Sigma): A range of concentrations were assessed from 0.03-0.1 μM
- Tryptophan (Sigma): 100 μM
- Glucose (Fisher): 1.84 mM

4.3.4. Statistical analyses

Statistical tests employed to determine significant differences between protein concentration and carbohydrate concentration of the various peptones, and swim migration assays in BPW medium comprising different peptones, were dependent on the data being normal and the variances homogenous. ANOVA was used to analyse parametric data, the Kruskal-Wallis test was applied to non-parametric data. Analyses were performed by the statistical software Minitab™ version 13.1.

4.4. Results

Electron microscopy (transmission EM) was employed to determine significant differences in morphology of *Salmonella* in the differing peptone based BPW media. This was essential as variable shape and size of organisms in the respective media would affect optical density measurements taken to assess growth dynamics, therefore leading to inaccurate comparison of bacterial growth in the different peptones.

4. Morphology and motility of bacteria dependent on peptone

Differences in morphology were found and nutrient deficiencies of certain peptones were shown to cause these diverse phenotypes.

Initial morphological differences were noted as observations using transmission electron microscopy and so quantitative analysis of *Salmonella* and *Escherichia* motility was then performed using the swim migration assay.

4.4.1. Transmission electron microscopy (TEM) of *Salmonella* ser. Typhimurium

TEM of *Salmonella* showed distinct morphologies which were dependent on the peptone constituent of the medium. The typical phenotype of *Salmonella enterica* is that of peritrichously flagellated rods, possessing 6-8 flagellar filaments per organism (Manson *et al.*, 1998), which are present singularly or in small clusters. These typical phenotypes were observed in most yeast and soy peptone based media. Interestingly, the cultivation of organisms in most gelatin peptone BPW generated aflagellate aggregates: flagella were not observed throughout the sample either attached to an organism or broken, and so present in the medium. Casein and meat peptones as constituents of BPW produced both flagellate and aflagellate morphologies, and varying degrees between the two. Manufacturer and batch processes within the source peptone groups affected the degree of flagellation of the resulting cells, and so each peptone within the group is described in detail.

Casein peptone-based BPW: Degree of flagellation was noted after cultivation of *Salmonella* ser. Typhimurium in BPW which contained casein peptone as the sole nutritional source (Figure 4.1.). Examination of organisms which had been incubated in peptones C6, C7 and C8 revealed the typical flagellate, non-aggregate phenotype;

whilst those observed from C2, C3 and C5 peptone based media were considerably diminished with respect to quantity of flagella identified throughout the sample. *Salmonella* grown in C1 formed aggregates but displayed flagella whereas those cultured in C4 peptone based media were present in tight clusters, the surrounding media containing insignificant numbers of detached flagella. Dark halos surrounding cells were artefacts of staining, and occurs in all samples, as organisms were not washed before examination by microscopy.

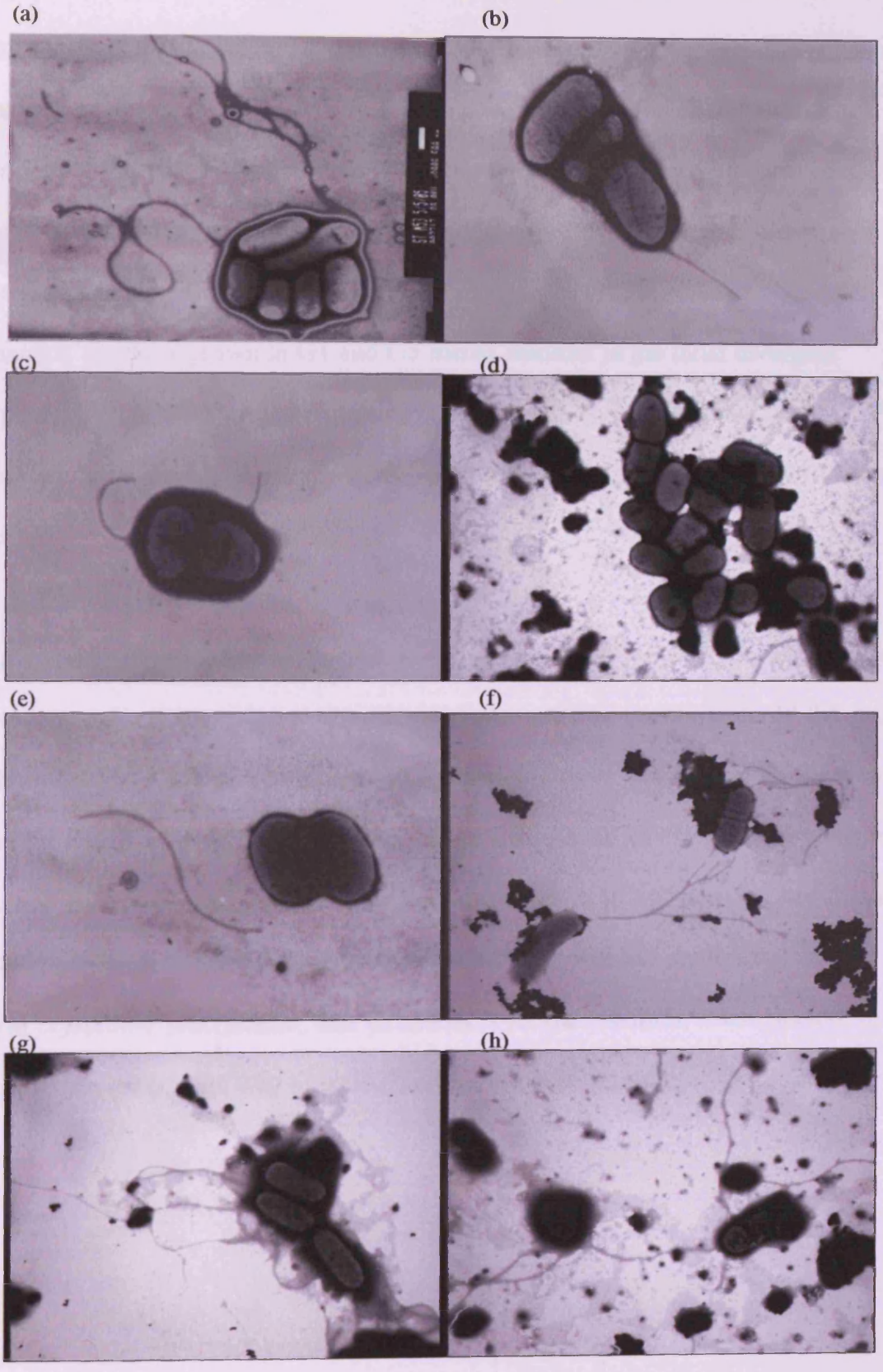

Figure 4.1. Transmission electron micrographs of *Salmonella* ser. Typhimurium cultured for 24 h in BPW containing peptones of different casein origin: (a) C1, (b) C2, (c) C3, (d) C4, (e) C5, (f) C6, (g) C7 and (h) C8.

10

4. Morphology and motility of bacteria dependent on peptone

Gelatin peptone-based BPW: Media containing G2 and G4 peptones as the nutritional ingredient exhibited *Salmonella* which were observed to be flagellate, although quantities of flagellar filaments in G2 cultures were insignificant (Figure 4.2.). Only G2 and G5 peptones generated cells which were noted to be present in either small clusters or in a singular state, all other gelatin peptones produced large aggregates of organisms. Cultures grown in G1 and G3 media resulted in the most divergent morphology from the typical description: i.e. as tight aggregates which were completely aflagellate and cells which appeared unable to separate efficiently after division

Meat peptone-based BPW: Figure 4.3. shows the different degrees of flagellation dependent on variable source of meat peptones used as constituents of the growth medium BPW. The morphologies of *Salmonella* after cultivation in these meat peptones varied from small, aflagellate clusters displayed in M1 and M6 cultures, to very few flagellate organisms being noted after growth in M4 peptone and finally to well flagellated cells following cultivation in M2, M3 and M5 peptones.
Dense crystalline precipitates, due to artefacts of the medium, were present in M6 samples, less precipitate was observed in other peptone samples.

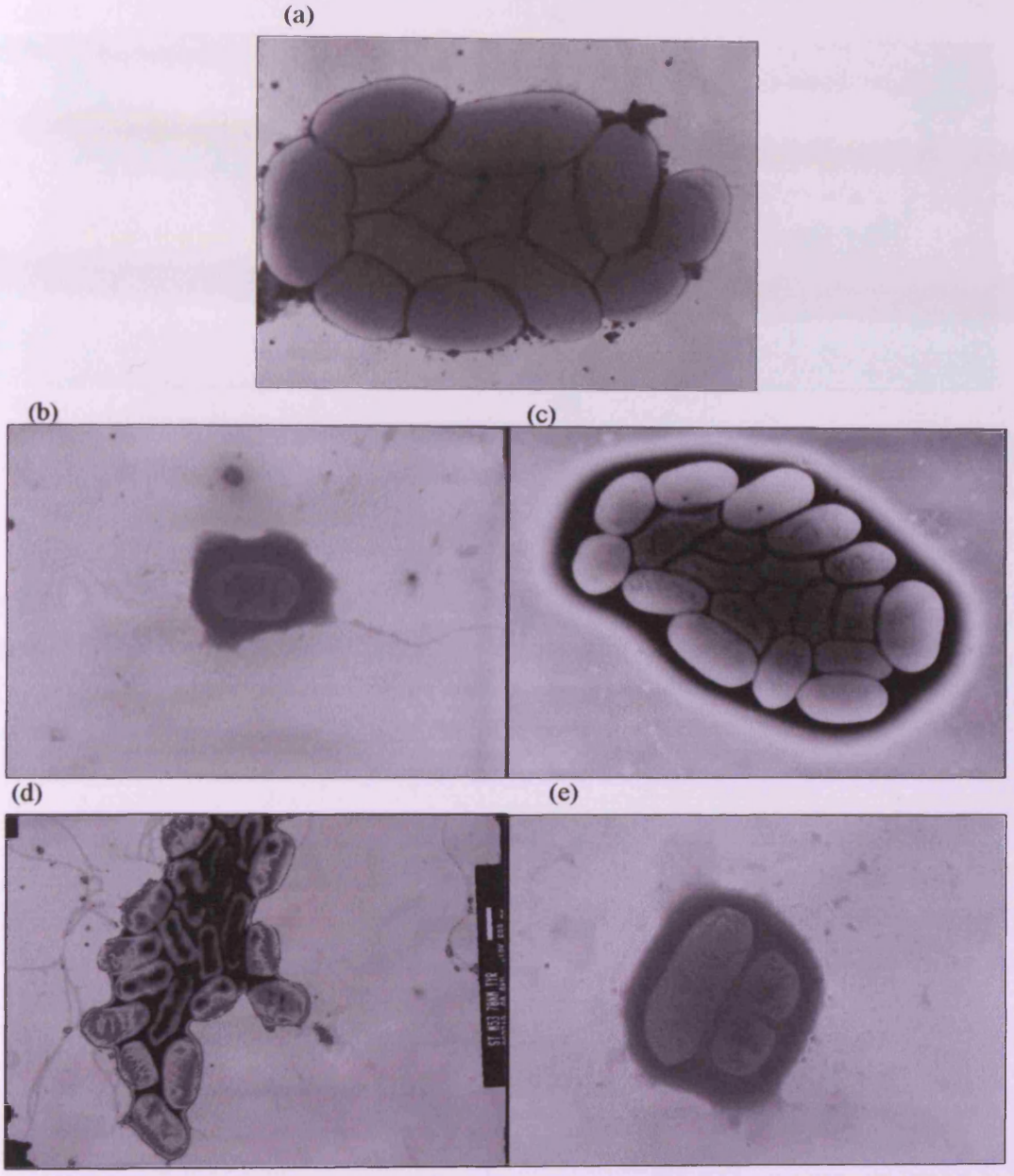

Figure 4.2. Transmission electron micrographs of *Salmonella* ser. Typhimurium cultured for 24 h in BPW containing peptones of different gelatin origin: (a) G1, (b) G2, (c) G3, (d) G4 and (e) G5.

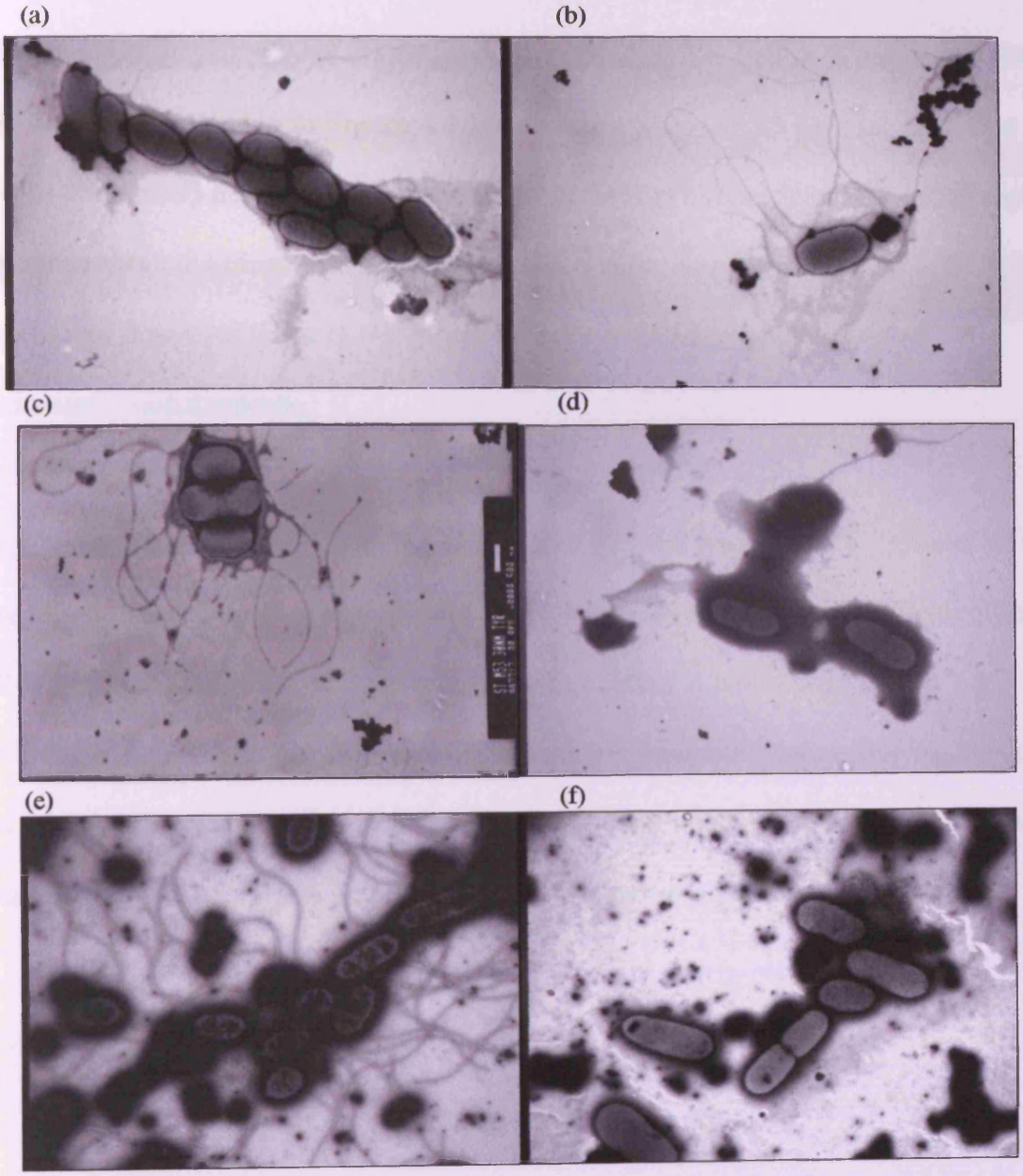

Figure 4.3. Transmission electron micrographs of *Salmonella* ser. Typhimurium cultured for 24 h in BPW containing peptones of different meat origin: (a) M1, (b) M2, (c) M3, (d) M4, (e) M5 and (f) M6.

4. Morphology and motility of bacteria dependent on peptone

Soy peptone-based BPW: All organisms observed after incubation in soy based BPW were flagellate, as shown in Figure 4.4. *Salmonella* grown in S5 peptone appeared to contain diminished numbers of flagella per organism in comparison with other soy peptone generated cultures. Organisms produced aggregates when grown in S1 and S4 peptones; however those in S1 appeared more irregularly packed and of inconsistent conformation.

Yeast peptone-based BPW: *Salmonella* cultured in yeast peptones Y1, Y2 and Y4 were highly flagellate, as seen in Figure 4.5. Deviations from the typical description of this organism were the presence of aggregates found in cultures grown in Y4 peptone medium, and cultures grown in peptone Y3 were noticeably less flagellate than other yeast peptone cultivated organisms; in comparison to other peptone sources a high degree of flagellation was still observed in cultures grown in Y3 peptone.

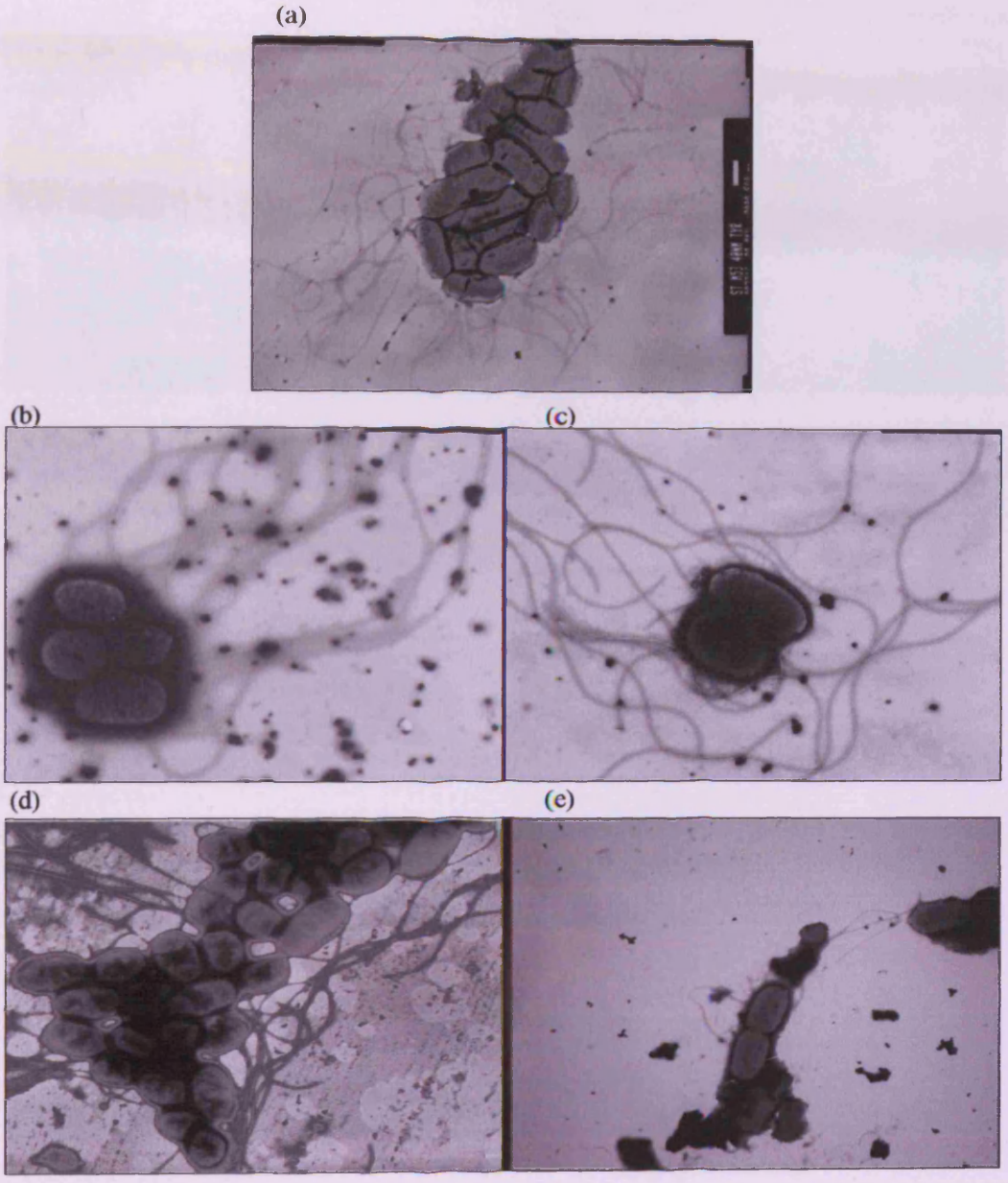

Figure 4.4. Transmission electron micrographs of *Salmonella* ser. Typhimurium cultured for 24 h in BPW containing peptones of different soy origin: (a) S1, (b) S2, (c) S3, (d) S4 and (e) S5.

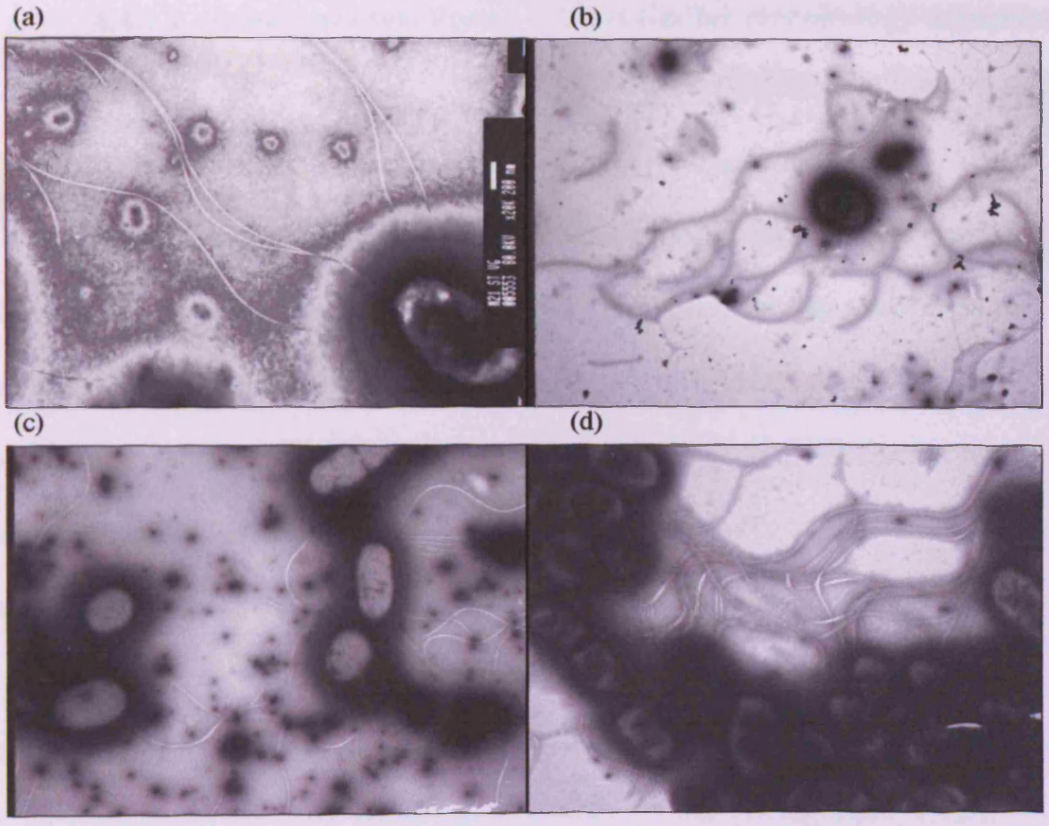

Figure 4.5. Transmission electron micrographs of *Salmonella* ser. Typhimurium cultured for 24 h in BPW containing peptones of different yeast origin: (a) Y1, (b) Y2, (c) Y3 and (d) Y4.

4. Morphology and motility of bacteria dependent on peptone

4.4.1.1. *Salmonella* ser. Poona exhibit similar morphology dependent on peptone

Cultures of *Salmonella* ser. Poona (*S*P) were also investigated using TEM, after incubation in BPW containing peptones which produced atypical phenotypes of *Salmonella* ser. Typhimurium (*S*T), shown in Figure 4.6. This was performed to determine whether the effect of peptone was limited to the serovar of *S*T used. Although *S*P exhibited different morphologies to *S*T (in general the former was more flagellate) the effect was still striking. Medium containing C2 peptone gave rise to cultures which were observed to be of typical morphology for *S*P; this was in contrast to *S*T which, after growth in C2 peptone was of aflagellate morphology. BPW medium containing C5 peptone as a nutritional source generated similar morphologies for both *Salmonella* serovars. BPW incorporating G1 and G5 peptones, which produced aflagellate cultures of *S*T, displayed normal flagellate forms of *S*P. BPW which was comprised of G3 peptone gave rise to aflagellate *S*P, which was in accordance with the aflagellate *S*T cultures generated by this medium. However, appearance of aggregates occurred during growth of *S*T and this was not noted in *S*P cultures of the same medium. The effect of tyrosine deficiency was not therefore limited to a specific serovar.

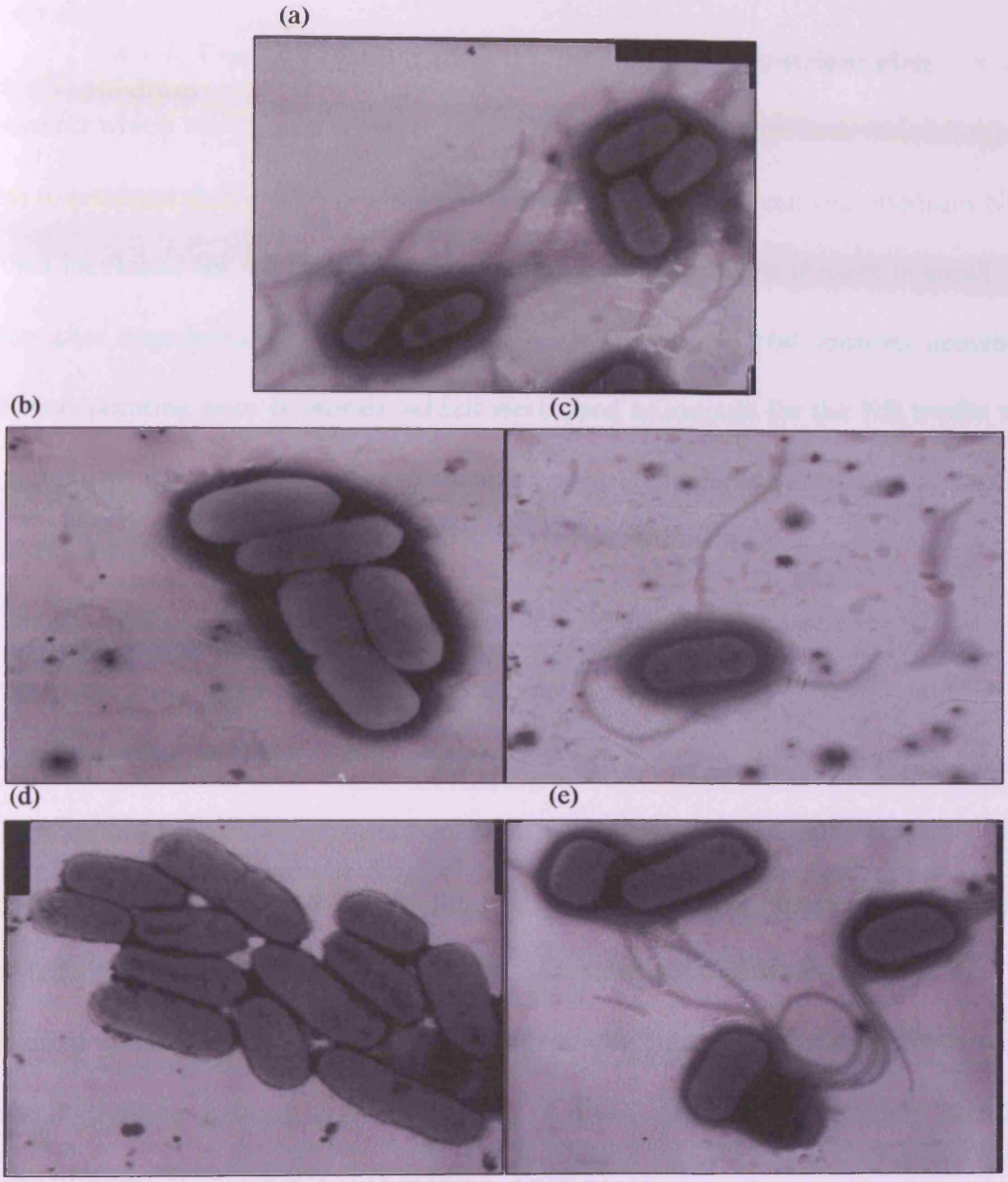

Figure 4.6. Transmission electron micrographs of *Salmonella* ser. Poona cultured for 24 h in BPW containing peptones of different sources and origins: (a) C2, (b) C5, (c) G1, (d) G3 and (e) G5.

4.4.1.2. Transfer of aflagellate *Salmonella* into a nutrient rich medium

Salmonella which were transferred, from BPW comprised of peptones which were shown to produce abnormal morphology into the complex nutrient rich medium NB, and then incubated for 4 h were observed as flagellate organisms present in small clusters after examination by a TEM (Figure 4.7). The *Salmonella* cultures, grown in BPW incorporating poor peptones, which were used as inocula for the NB media were also examined; this confirmed that the inocula were aflagellate and of similar form as previously described.

This demonstrated that the aflagellate, aggregate forms of *Salmonella* ser. Typhimurium and Poona observed in certain BPW media were reversible, and indicated a deficiency (chemical or otherwise) within the peptones themselves. Previous studies on a different strain of *Salmonella*, whereby the inocula were aflagellate, have been carried out and also demonstrated regeneration of the organelle. *Salmonella typhimurium* strain SW 1061 (which had been deflagellated by mechanical methods) showed regeneration of flagellar filaments during growth in a chemically defined medium (a buffered salts medium containing a complete amino acid mixture and 0.2% Glucose) (Kerridge, 1959 a).

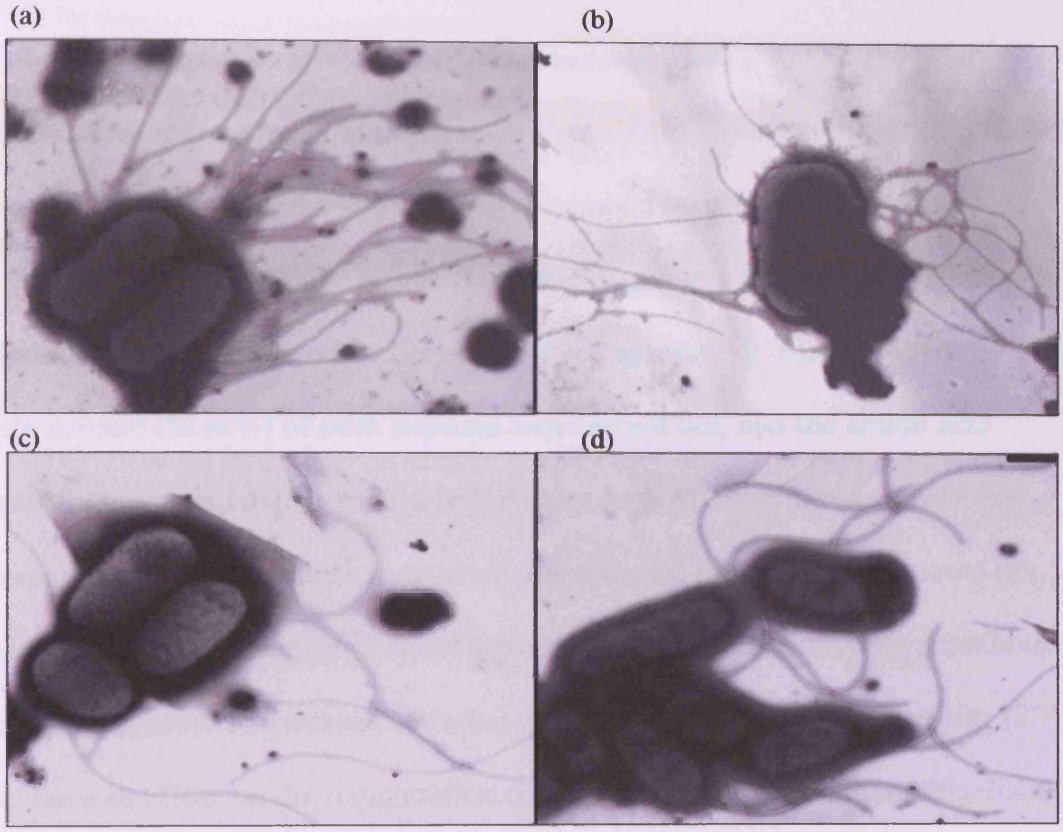

Figure 4.7. Transmission electron micrographs of *Salmonella* after culturing for 4 h in nutrient broth, whereby the inocula were aflagellate, having been harvested from poor buffered peptone water medium: *Salmonella* ser. Typhimurium in NB after culture from (a) C5, (b) G1, (c) G3 and *Salmonella* ser. Poona in NB after culture from (d) G3.

4.4.2. Chemical analysis of peptones investigated

Determination of the suspected deficiency, contributing to the atypical morphologies observed, was initially performed by basic chemical tests to establish differences between peptones which gave rise to flagellate organisms and peptones which produced aflagellate organisms. Therefore investigation into the carbohydrate and protein content (% w/w) of each peptone was carried out, and the amino acid composition was also analysed. Growth factors such as vitamins (e.g. thiamine, biotin), purines or pyrimidines may also have affected morphology but were not investigated in this study. Addition of a purine and pyrimidine mixture (xanthine, hypoxanthine, adenine, guanine, thymine and uracil) was shown by Kerridge (1959 b) not to have an effect on the regeneration of flagella by mechanically deflagellated organisms.

4.4.2.1. Carbohydrate content

Carbohydrates can be utilized by organisms as an energy source during respiration or fermentation, or may be stored as an energy reserve in the form of granules (e.g. glucose forms polymers of starch or glycogen). Glucose specifically is an important constituent of the cell wall polymer peptidoglycan, forming the polysaccharide backbone. Therefore the presence of carbohydrates in the culture medium is of great advantage. Organisms grown in culture media which are deficient in carbohydrates are able to synthesize this important hexose sugar by gluconeogenesis; however this requires consumption of an energy source. Figure 4.8. shows the percentage of carbohydrate present in each peptone investigated; the mean average values for each peptone group were determined to be statistically different ($P = 0.000$) with soy peptones containing the highest percentage of carbohydrates (21.92 % w/w), followed

by yeast (14.68 % w/w), meat (7.04 % w/w), gelatin (2.30 % w/w) and finally casein peptones with no carbohydrates detectable by the method employed (0.00 % w/w). The lack of carbohydrates in casein peptones, without exception, was not surprising as casein is a relatively more defined material in comparison with the other peptones studied. The peptone groups comprising gelatin (P = 0.029), meat (P = 0.000), soy (P = 0.000) and yeast (P = 0.046) sources were all significantly different within their respective groups; i.e. carbohydrate as a composition of the peptone varied with manufacturer and batch.

Most gelatin peptones contained considerably low levels of carbohydrates, the lowest value obtained was G4 with 0.27 % w/w; the exception was G2 peptone which was noticeably higher in comparison (5.70 % w/w). Peptone G3 generated an average mean value which was 0% however the standard deviation was high, as shown in Figure 4.8.

Meat peptones displayed diverse values for carbohydrate content, for example M4 peptone constituted a 2.03 % w/w concentration of carbohydrate compared with M3 peptone which was five fold higher: 10.98 % w/w.

Soy peptones contained the highest proportion of carbohydrates and were the most diverse group, the highest being 38.12 % w/w in S5 peptone, and the lowest peptone S1 with a carbohydrate content of 11.71 % w/w.

Yeast peptones, like soy peptones, all contained noticeable amounts of carbohydrate from 10.98 % w/w in Y2 to 17.72 % w/w in Y4 peptone. Yeast peptones were relatively consistent with regards to macromolecular composition.

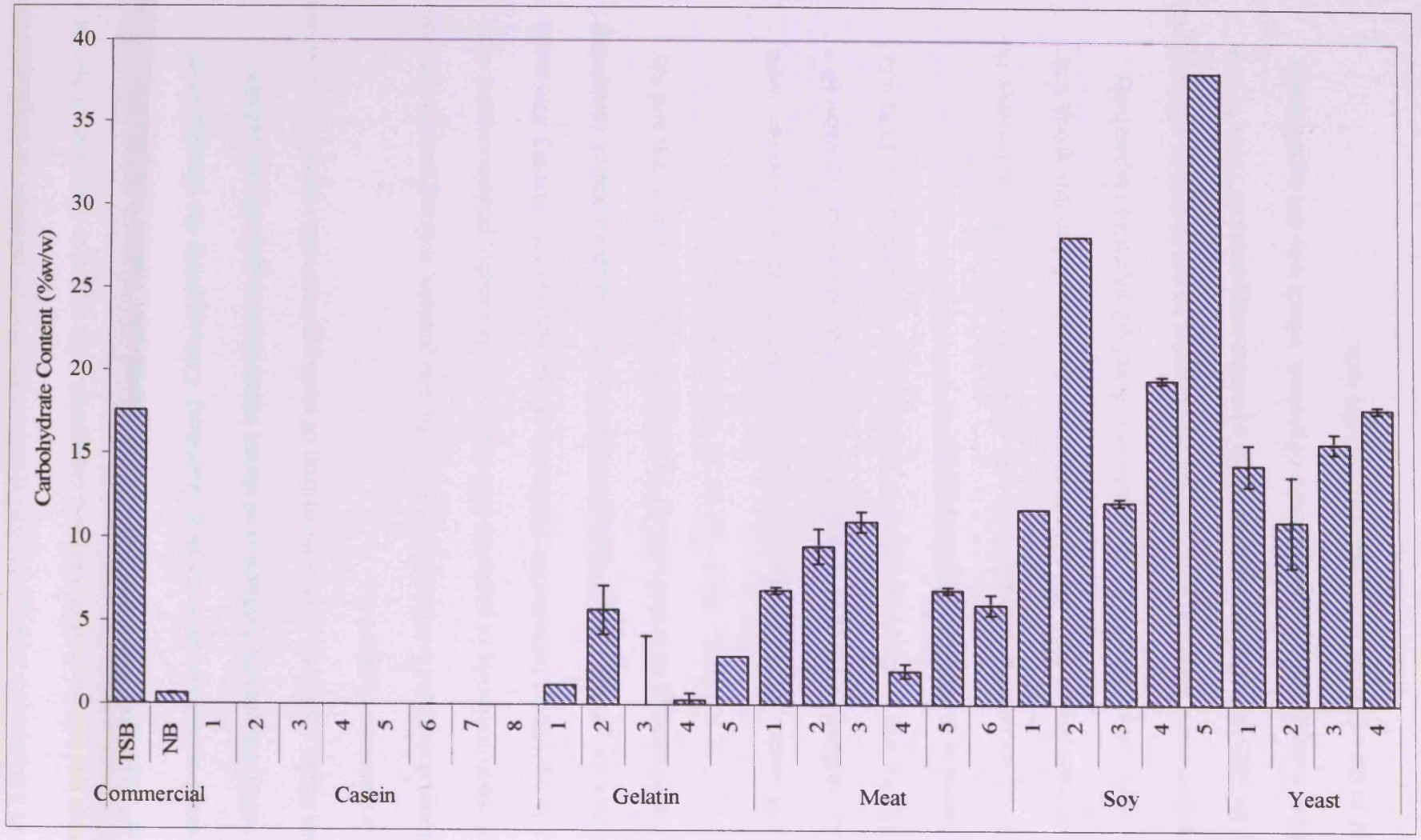

Figure 4.8. Comparison of carbohydrate content (%w/w) for each peptone. Error bars represent mean averages ± standard deviation where n=3.

4.4.2.2. Protein determination of peptone

Protein as a constituent of peptone, and therefore the growth medium, is of great importance and can be divided into two categories with regard to function within the organism: those utilized for cell structure (e.g. membrane, wall or cytoplasmic components) or catalytic proteins (e.g. enzymes). Consequently the abundance of protein within the growth medium is of direct importance to growth and cellular processes of the organism. The composition of each peptone with respect to its protein content is shown in Figure 4.9.

All peptones comprised a minimum of 40.16 % w/w protein, making protein the most abundant macromolecule in peptones investigated in this study; protein content was statistically significant between the different peptone sources ($P = 0.028$). The mean average for each peptone group highlighted gelatin as the peptone source containing the highest percentage of protein (83.37 % w/w), then casein, meat, yeast and soy with values of 72.70, 72.02, 59.85 and 55.24 % w/w respectively.

Peptones of casein origin, obtained from differing manufacturers or batches, were statistically different from each other with respect to concentration of protein ($P = 0.043$). The highest protein concentration was found in C5 peptone (92.88 % w/w) and the lowest in C8 peptone (51.90 % w/w).

Protein composition of gelatin peptones was not significantly different between the various samples ($P = 0.221$). The lowest protein value recorded in this group was 71.53 % w/w, compared with 85.38 % w/w in G4 and G2 peptones respectively.

Meat peptones were shown to be consistent with respect to concentration of protein ($P = 0.073$) for each peptone investigated. With a protein component of 79.01 % w/w M5 peptone contained the highest amount of protein, which was not significantly dissimilar to the value for M3 peptone of 68.14% w/w.

4. Morphology and motility of bacteria dependent on peptone

Peptones originating from a soya source were statistically different (P = 0.000) as S3 peptone contained only 40.16 % w/w protein as a constituent of the peptone, whereas S5 soy peptone comprised 61.61 % w/w.

Yeast peptones, despite appearing consistent with respect to protein concentration, were shown to be statistically different (P = 0.024). This may be due to small values for standard deviation from the mean average which increases the power of statistical analysis. Thus the difference between protein in Y2 (63.96 % w/w) and Y3 (57.11 % w/w) peptones is significant.

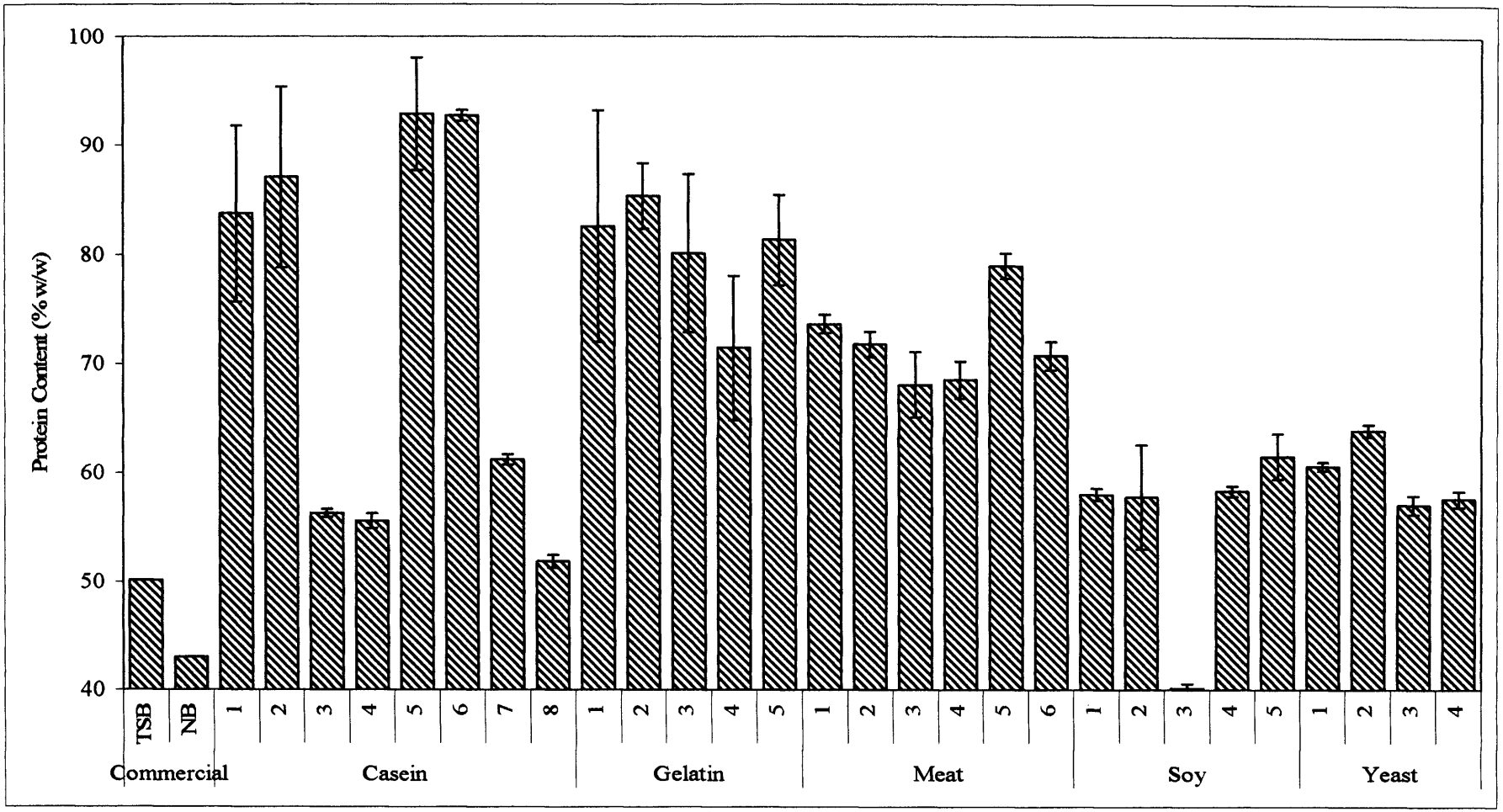

Figure 4.9. Comparison of protein content (%w/w) for each peptone. Error bars represent mean averages ± standard deviation where n=3

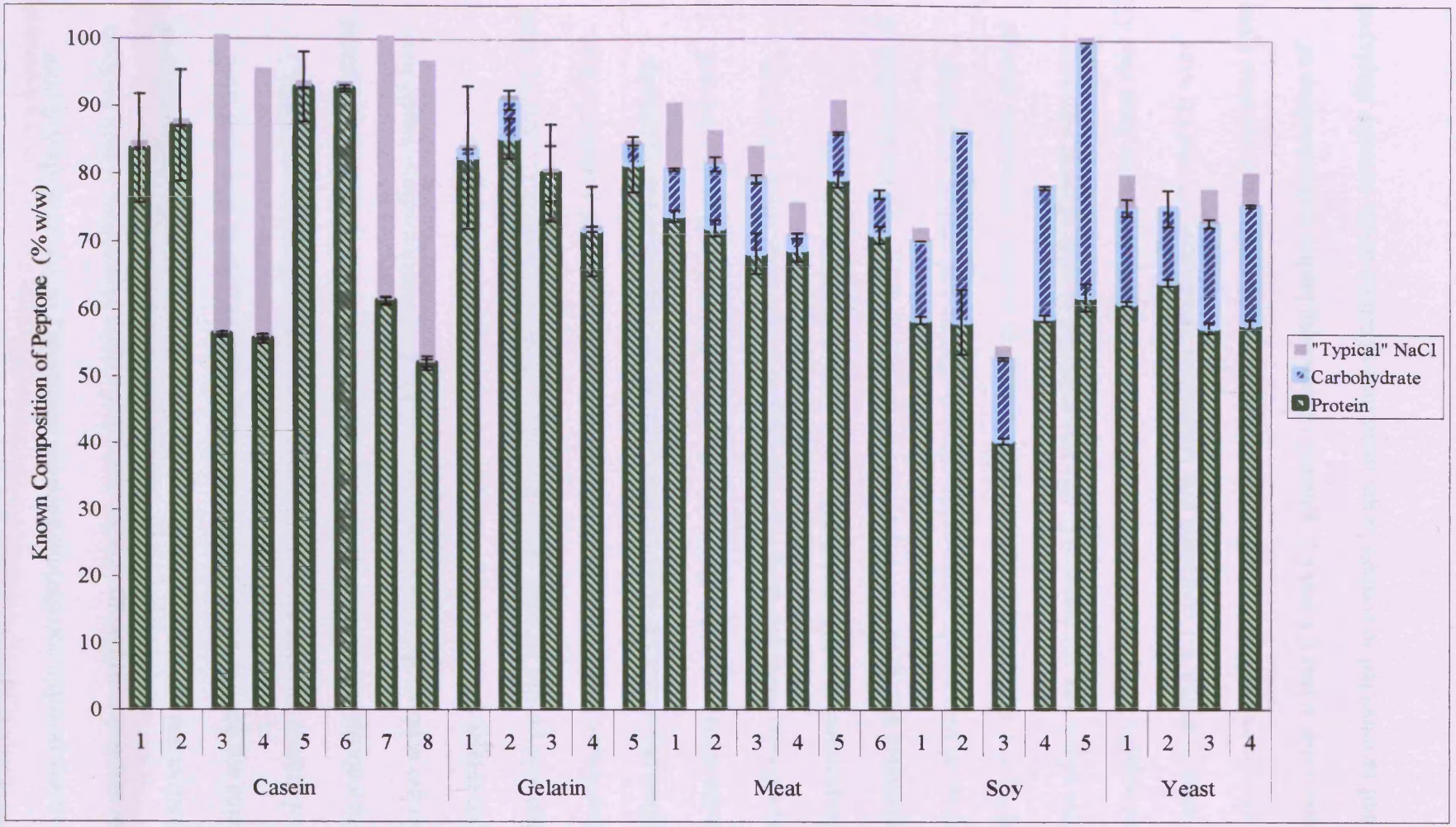

Figure 4.10. Summary table of peptone composition. M6 NaCl concentration is unknown. NaCl values are typical and are taken from the respective manufacturers' manuals.

4.4.2.3. Amino acid analysis of peptone

The amino acid composition of each peptone was determined chromatographically in order to assess possible correlations between availability of amino acids and poor growth or atypical morphology. As the monomeric units of proteins, which perform essential structural and metabolic roles within the organism, specific deficiencies of amino acids are detrimental to growth. All Enterobacteria used in this work were capable of growth by utilization of ammonium chloride as sole nitrogen source: indicating a normal amino acid metabolism. However, uptake and direct deployment of free amino acids or short peptides present within the growth medium would have been more energy efficient.

The amino acid profile for each casein peptone is shown in Figure 4.11., and for each gelatin peptone in Figure 4.12. Both peptone groups demonstrated a relatively high level of diversity within the specified class; in contrast to soy and yeast peptones (which were consistent within their groups) and are depicted in Figures 4.14. and 4.15. respectively. Meat peptones were intermediate, exhibiting slight variations within the peptone group (Figure 4.13).

Casein peptones displayed diversity for the all the amino acids with the exception of the lower molecular weight amino acids, glycine, alanine and cystine. In general peptones C3, C4, C7 and C8 contained lower quantities of every amino acid (except tryptophan which was not measured), this was expected as each of these peptones contained notably less protein (Figure 4.10). There were few aberrations from this: C2 was deficient in serine, C1 contained low amounts of methionine, C1 and C6 were lacking in arginine. Of interest is the aromatic amino acid tyrosine; this deviated from the general trend in that C1 and C5, generally containing higher concentrations of protein and so expected to contain larger quantities of amino acids, actually displayed

relatively low tyrosine. In contrast, peptones C3 and C7, despite relatively low protein concentrations, showed comparatively high levels of tyrosine.

Gelatin peptones were less diverse, in comparison with casein, with noticeable differences in fewer amino acids which included: serine and glutamic acid (both polar), proline and isoleucine (both non-polar), histidine and arginine (both basic). Peptone G4 was found to contain 71 % protein material, with all other gelatin peptones containing between 80-85 % (the ranking from highest protein to lowest protein concentration was: G2, G1, G5, G3 and finally G4). This resulted in decreased quantities of amino acids in G4. Deviations in amino acid composition, within the gelatin group which were not due to total protein content, were established in G3 (low quantities of serine and arginine) and G2 (comparatively lower concentrations of alanine and isoleucine).

Meat peptones demonstrated slight variation in glutamic acid, proline, glycine and methionine (non-polar) and arginine. More specifically, M1 meat peptone contained comparatively high levels of glutamic acid and low concentrations of glycine. Peptone M6 was deficient for proline and lacking in arginine, whereas M4 lacked both methionine and tyrosine.

Peptones based from soy material were mostly consistent, the exception being glycine for which peptones S1 and S5 were relatively high. Peptone S3 contained considerably less protein material and therefore amino acid concentrations were lower when compared with other soy peptones.

Yeast extracts were consistent for all amino acids.

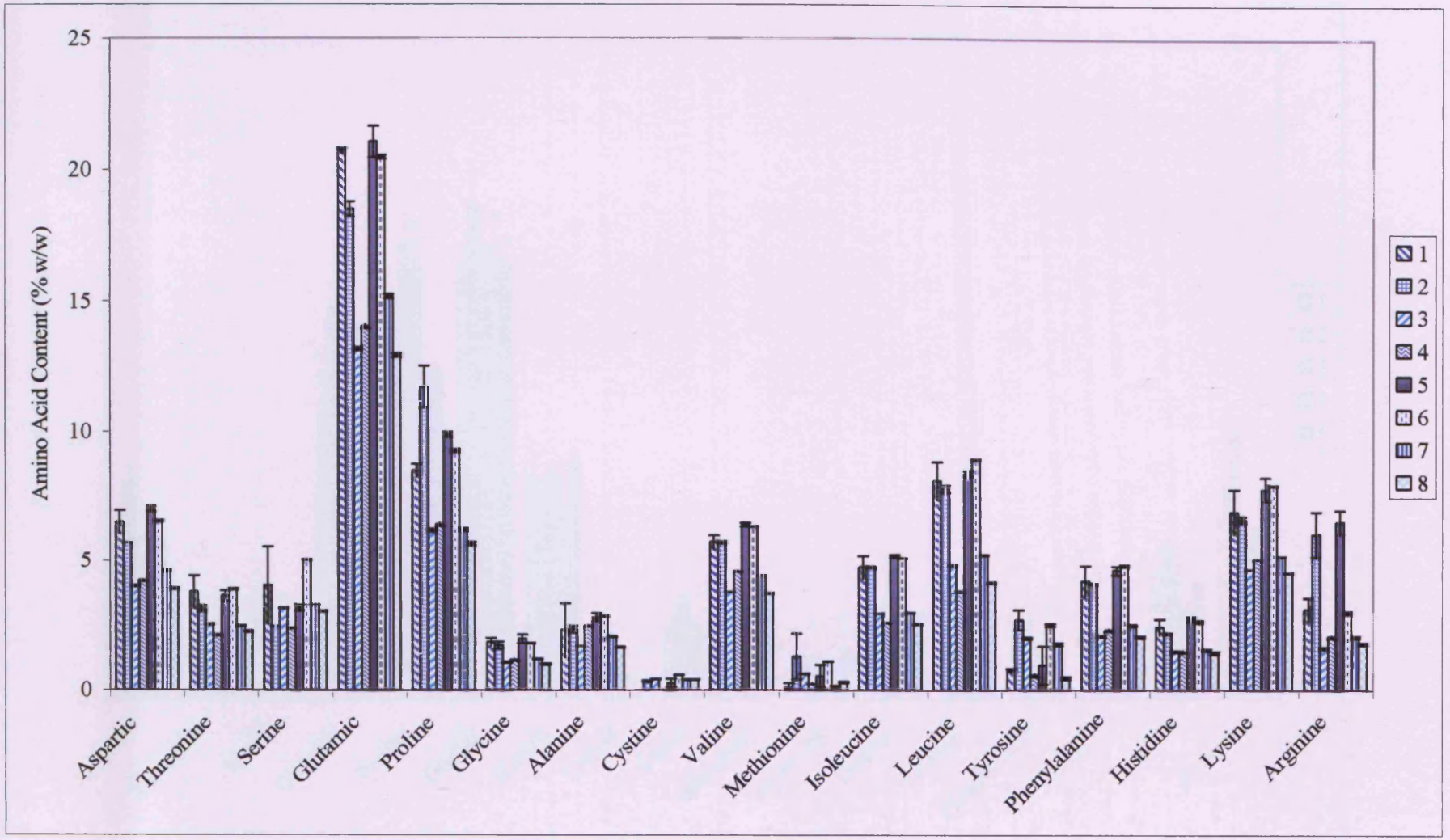

Figure 4.11. Comparison of amino acid content (%w/w) for each casein peptone. Error bars represent mean averages ± standard deviation where n=3.

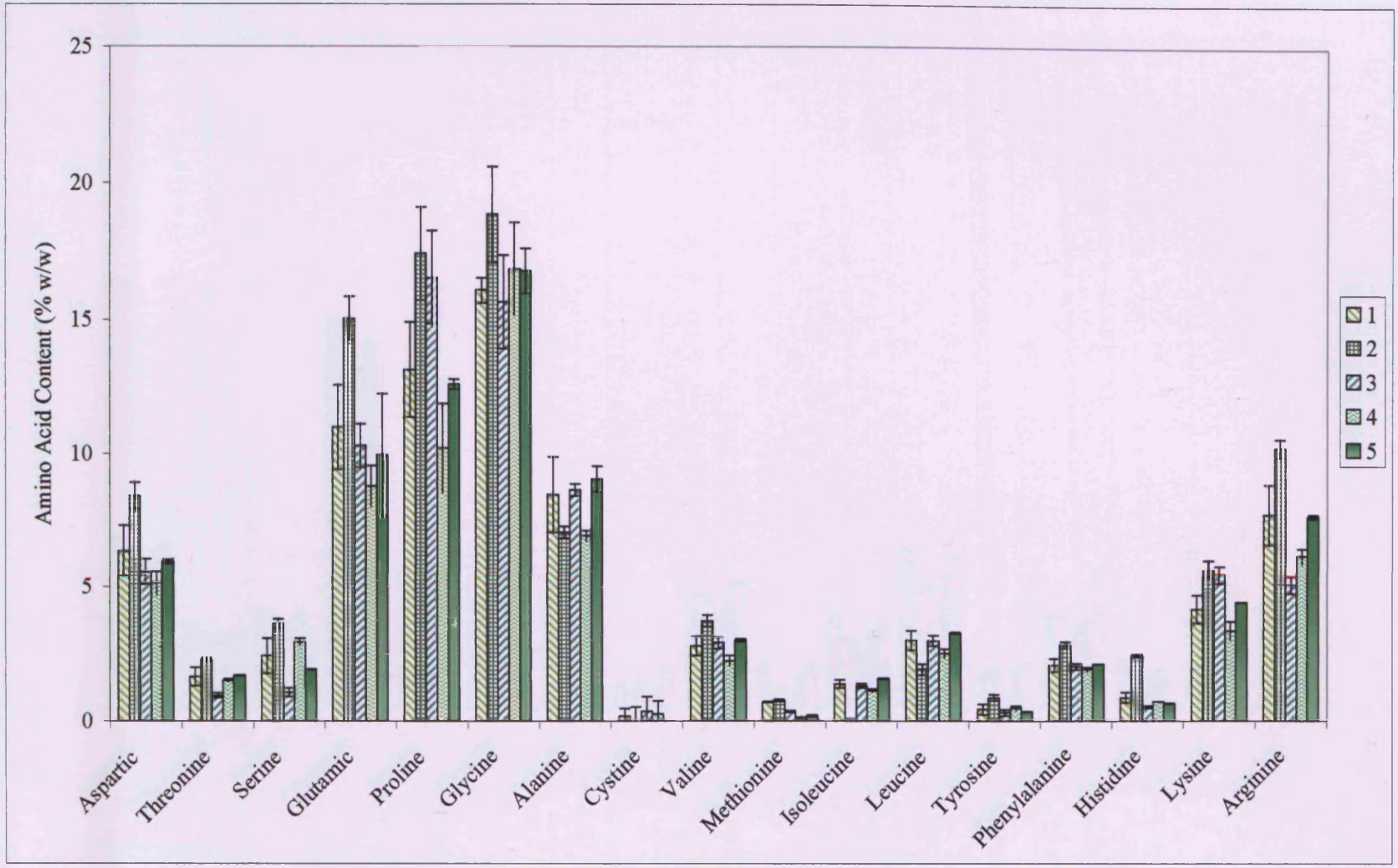

Figure 4.12. Comparison of amino acid content (%w/w) for each gelatin peptone. Error bars represent mean averages ± standard deviation where n=3.

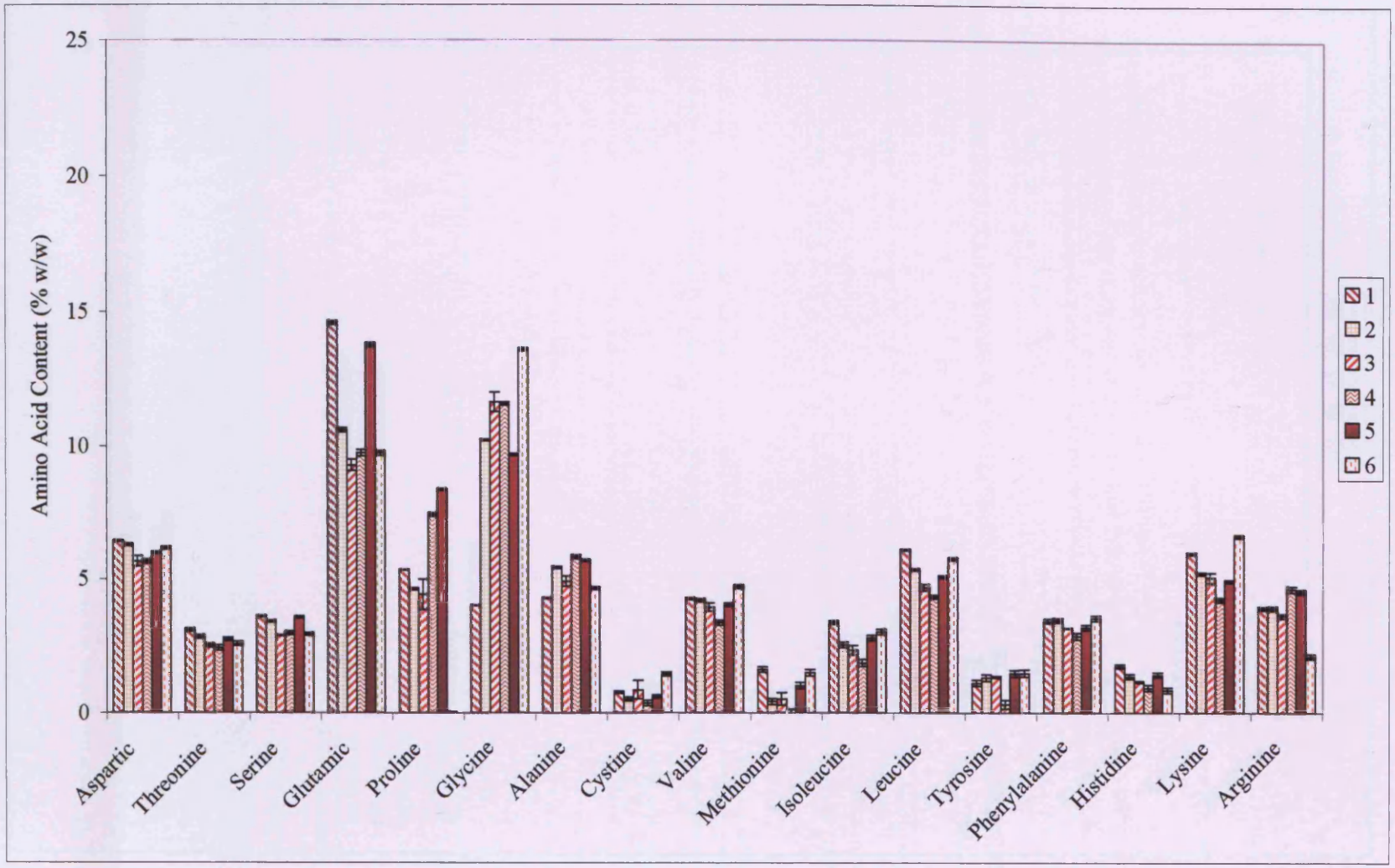

Figure 4.13. Comparison of amino acid content (%w/w) for each meat peptone. Error bars represent mean averages ± standard deviation where n=3.

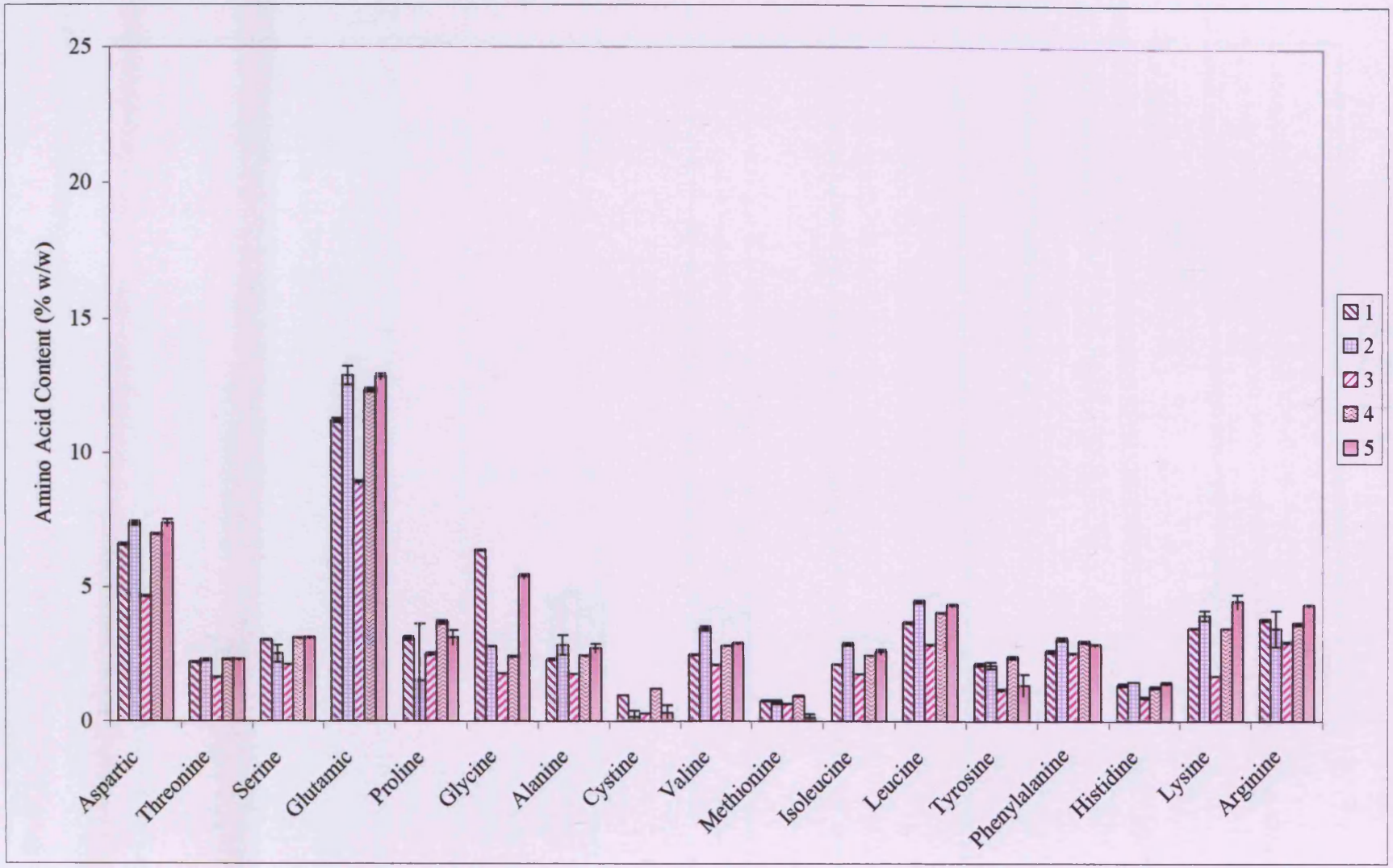

Figure 4.14. Comparison of amino acid content (%w/w) for each soy peptone. Error bars represent mean averages ± standard deviation where n=3.

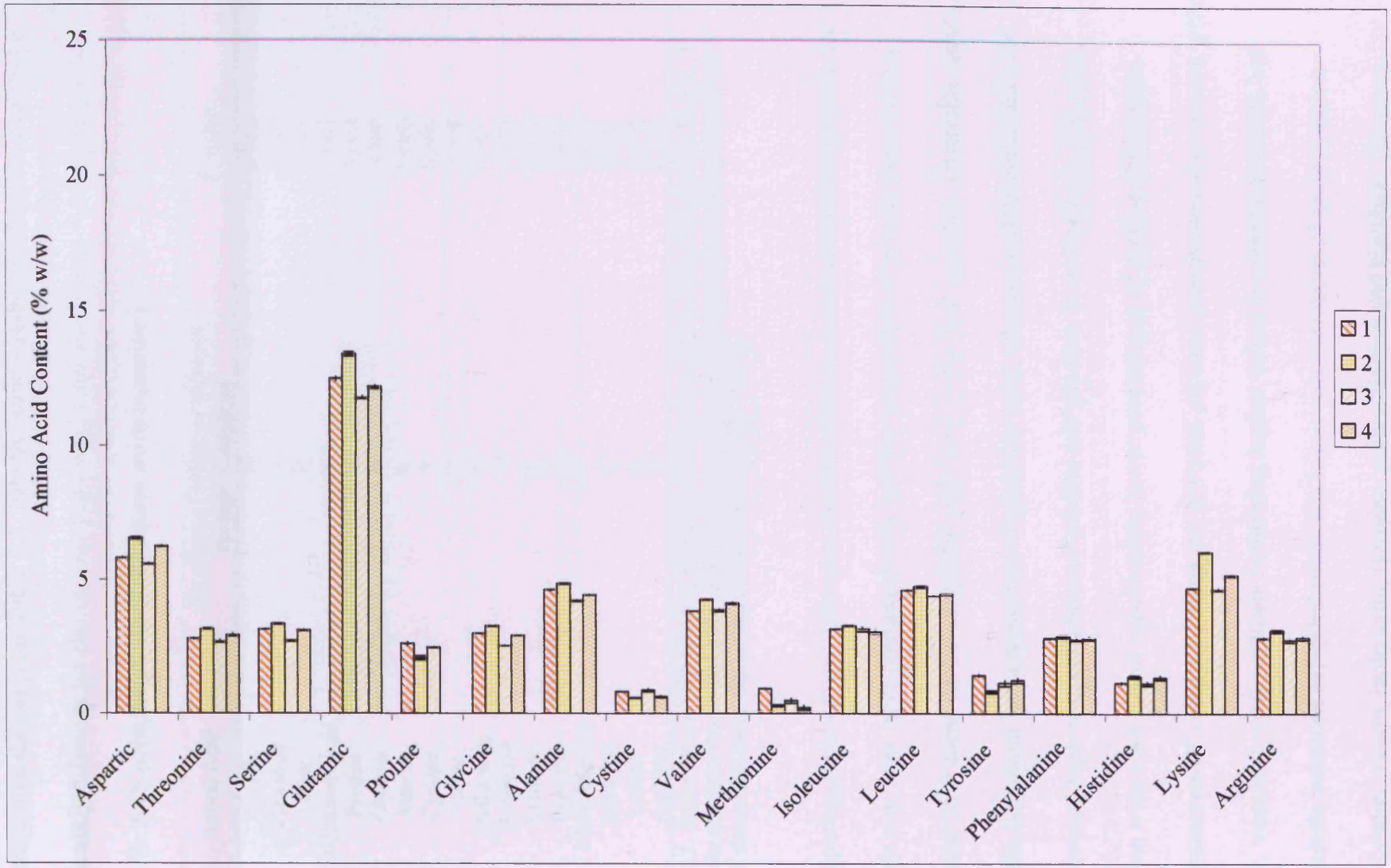

Figure 4.15. Comparison of amino acid content (%w/w) for each yeast peptone. Error bars represent mean averages ± standard deviation where n=3.

4. Morphology and motility of bacteria dependent on peptone

Of greater significance in this investigation was that of the difference between the peptone groups themselves. Table 4.1. lists the amino acids which were noted to be significantly different for the various peptone groups and Figure 4.16. displays the mean for each peptone group of all amino acids examined.

Amino Acid	Significant Difference Between Peptone Categories	P Value
Aspartic	N	0.242
Threonine	Y	0.003
Serine	N	0.145
Glutamic acid	Y	0.001
Proline	Y	0.000
Glycine	Y	0.000
Alanine	Y	0.000
Cystine	Y	0.006
Valine	Y	0.000
Methionine	N	0.464
Isoleucine	Y	0.001
Leucine	Y	0.001
Tyrosine	Y	0.005
Phenylalanine	Y	0.040
Histidine	Y	0.006
Lysine	Y	0.002
Arginine	Y	0.008

Table 4.1. Statistical analyses showing relationship of various amino acids with peptone source. Y represents that there was a statistical difference between the peptone groups for that amino acid ($P < 0.05$), N shows that there was no significant difference ($P > 0.05$).

In comparing these amino acids care must be taken to note that certain peptones, for example soy, may have contained only half the proportion of protein material (e.g. compared with some casein or gelatin peptones) in the first instance. Generally casein peptones contained large quantities of glutamic acid, whereas gelatin peptones were comprised of high concentrations of proline and glycine. In contrast soy and yeast peptones were made up of significantly lower percentages of proline and glycine. Concentrations of alanine between the different peptone groups were also diverse with gelatin, meat and yeast peptones containing higher values in contrast to casein and soy. Gelatin peptones exhibited relatively lower concentrations of several amino acids: cystine, valine, isoleucine, tyrosine, phenylalanine and histidine. However

arginine concentrations were noticeably higher for gelatin peptones compared with other sources.

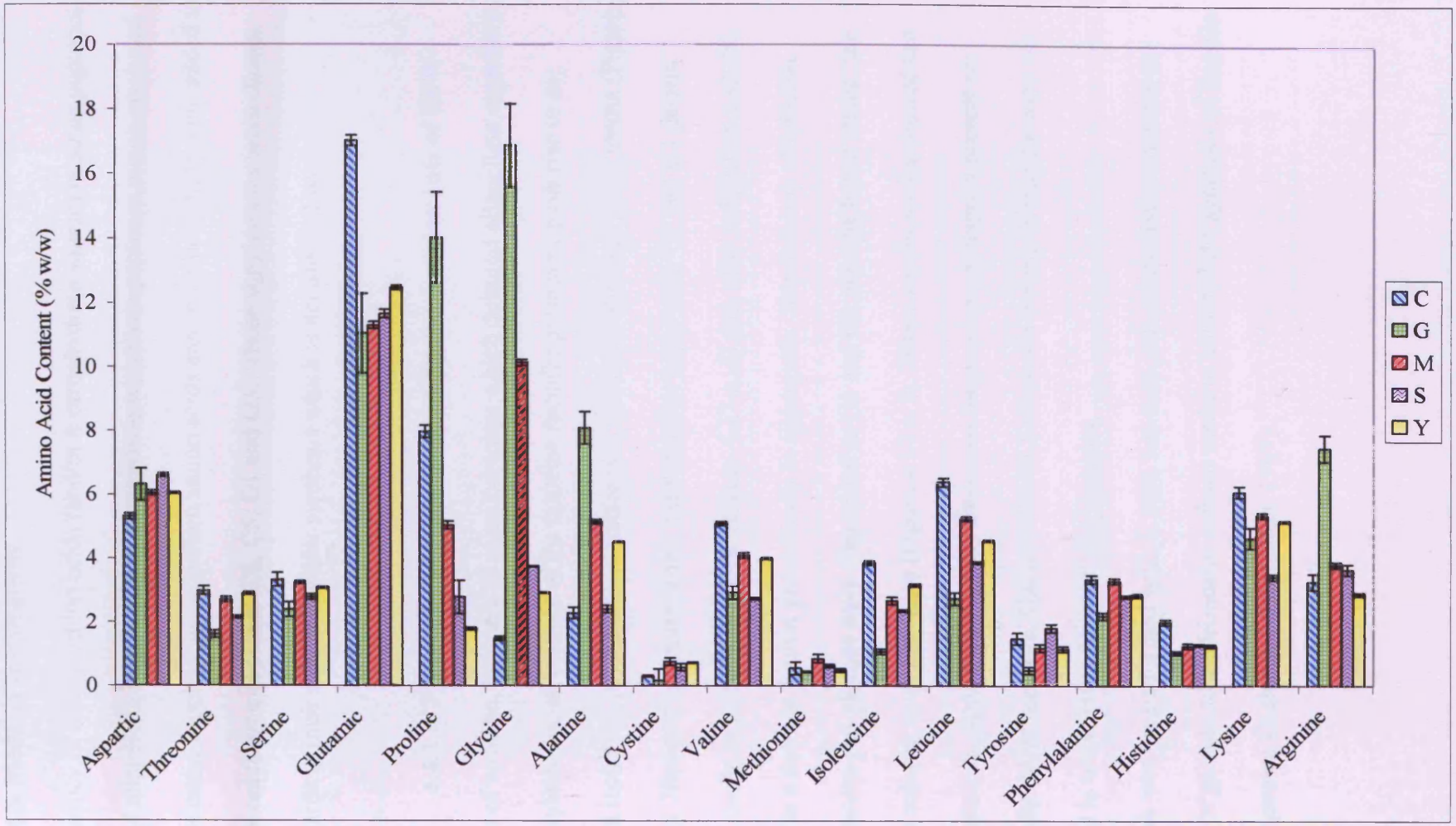

Figure 4.16. Comparison of amino acid content (%w/w) for the group average of each source peptone. Error bars represent mean averages ± standard deviation where n=3.

4.4.3. Medium supplements

As deficiency of certain amino acids (and/or a carbohydrate source) in some peptones produced aflagellate organisms (by comparison with those peptones which produced flagellate ones) supplements of certain amino acids and of carbohydrate were added to the BPW containing peptones C2, C5, G1 and G3.. These supplements were chosen with regards to their role in flagellar synthesis which is detailed below.

4.4.3.1. Tyrosine, phenylalanine and glucose supplements of BPW

Addition of tyrosine, deficient in some peptones which cultured aflagellate organisms, and phenylalanine, an inducer of the tyrosine specific permease, gave rise to an increased incidence of flagellate organisms in the casein peptone based media (Figure 4.17 a, b). However *Salmonella* ser. Typhimurium cultured in G1 and G3 gelatin peptone media remained aflagellate (Figure 4.17 c, d) and, after cultivation with G1 peptone as a nutrient source, present only in aggregates. Supplements of tyrosine, phenylalanine and glucose were then added to G1 and G3 peptone based media; the resultant cultures were flagellate (Figure 4.17 e, f). Where casein media showed few flagella glucose supplements greatly improved the numbers of flagella present on *Salmonella*. Meat peptones which produced *Salmonella* lacking in flagella were not subjected to work with additional supplements.

This work was not performed with *E. coli*; however, it is of interest to note that the addition of glucose to a chemically defined medium inhibited the synthesis of flagella in this organism (Adler and Templeton, 1967).

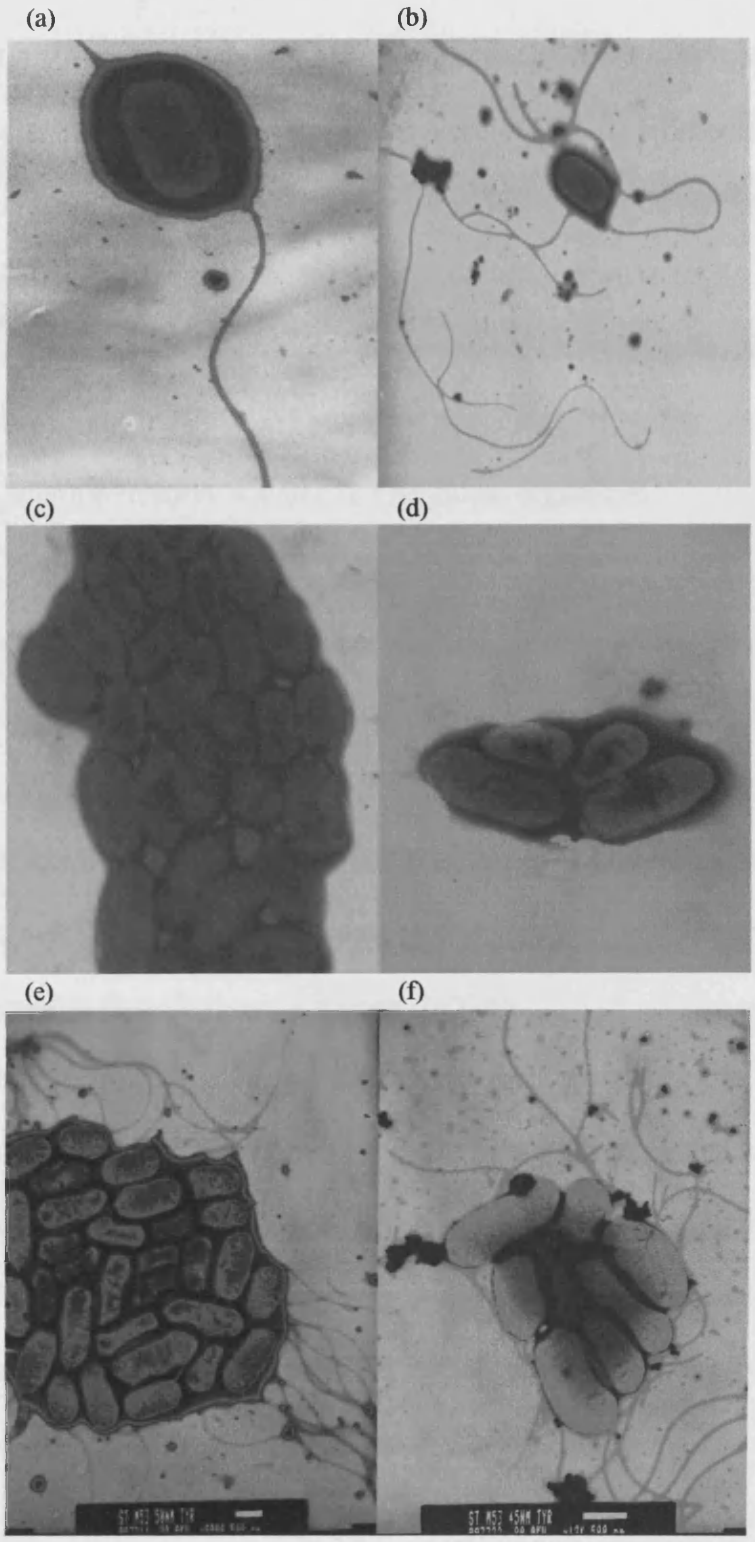

Figure 4.17. Transmission electron micrographs of *Salmonella* ser. Typhimurium after culture in (a) C2, (b) C5, (c) G1 and (d) G3 BPW with additional supplements of tyrosine (200 μM) and tryptophan (100 μM); (e) G1 and (f) G3 BPW with additional supplements of tyrosine (200 μM), tryptophan (100 μM) and glucose (2 mM).

4.4.3.2. Determination of absolute concentrations of tyrosine

Additions of tyrosine below 0.01 μM failed to produce flagellate organisms in all media which, without supplements, consistently generated organisms which were observed to be aflagellate. Supplements above 0.01 μM and up to 0.05 μM of tyrosine resulted in flagellate organisms with considerable numbers of flagella. Additions of tyrosine from 0.055 μM to 0.060 μM produced fewer flagella; values above 0.065 μM tyrosine as an addition to BPW resulted in aflagellate organisms.

4.4.3.3. Possible effects of peptone composition on flagellation

Tyrosine, leucine and glutamic acid are essential amino acids present in the medium which are required for the synthesis of flagellin of *Salmonella*; the flagellar filament subunit for this species lacks tryptophan, cysteine and hydroxyproline. Mutants which required for growth the amino acids leucine, glutamic acid or tyrosine could not regenerate flagella in their absence (Kerridge, 1959b).

An extreme deficiency of tyrosine may therefore prevent flagellar synthesis, e.g. in the case of *Salmonella* cultured in gelatin and in some casein and meat based BPW. Tyrosine transport into both *Salmonella* (Ames, 1964) and *E. coli* (Whipp and Pittard, 1977) is by two permeases: uptake is by way of the aromatic permease which transports all aromatic amino acids, or by using the tyrosine-specific permease which shows absolute specificity for tyrosine. Aromatic amino acids present at relatively higher concentrations inhibit uptake of those present at lower concentrations (Ames, 1964; Whipp and Pittard, 1977). Tyrosine was present in similar quantities to histidine, and at significantly lower concentrations compared with phenylalanine, in most peptones used in this study. Tyrosine uptake via its specific permease is driven by a proton motive force (PMF) (Antonucci and Oxender, 1986) or ATP for its

generation. This thesis hypothesizes that organisms cultured with most gelatin-based BPW do not produce flagellated *Salmonella* for two reasons: 1. Higher concentrations of phenylalanine inhibit tyrosine uptake by the aromatic permease, and 2. Lack of an energy source suitable for the production of a PMF that would power the tyrosine specific permease. Thus, in supplemented gelatin medium containing tyrosine and glucose, flagellated organisms were produced. Peptones G2 and G4 exhibited noticeable quantities of carbohydrate and, G2 in particular, higher levels of protein material in comparison with other gelatin peptones. Thus these peptones were able to produce cultures containing some flagellate organisms. Casein based C4 and C5 peptones contained relatively low concentrations of tyrosine, and C5 especially comprised higher quantities of phenylalanine and histidine in comparison with the level of tyrosine. In this case *Salmonella* cultured in C4 or C5 peptones may not have resulted in flagellated organisms as high concentrations of the competitive amino acids (phenylalanine and histidine) prevented uptake of tyrosine by the aromatic permease. Lack of a suitable substrate for and generation of a proton motive force or synthesis of ATP rendered the tyrosine specific permease inactive also. Thus tyrosine was not easily taken up by the organism and flagellin was not synthesised. Supplements of additional tyrosine, to overcome competition for uptake by the aromatic permease, resulted in flagella being produced. Peptone C6 displayed similar amino acid and carbohydrate composition to C2; however, cultures grown in C6 peptone were relatively more flagellate. Other growth factors (such as vitamins), which were not investigated in this work, may have been responsible. Furthermore, typical values of NaCl (obtained from the respective company manuals) were exceptionally high for casein peptones digested by acid hydrolysis (Figure 4.10). It is known that high concentrations of inorganic salts can be inhibitory to synthesis of

flagellar (Li *et al.*, 1993; Shi *et al.*, 1993). Furthermore the results from TEM are qualitative rather than quantitative.

Meat peptone M4, which was lacking in the amino acids tyrosine and methionine and contained relatively low quantities of carbohydrate, could be expected to produce more highly flagellate cultures of *Salmonella* after additional supplements consisting of tyrosine and glucose. Methionine is not required by *Salmonella* for flagellar synthesis, despite this amino acid being present in flagellin in small quantities (Kerridge, 1959 b). Peptones M1 and M6 were not deficient in tyrosine or carbohydrate: an absence of proline in M6, which is present in small quantities in the flagellin of *Salmonella*, may have been responsible for the aflagellate morphology observed. Peptone M1, a meat extract, has a typical NaCl concentration of 10 (% w/w); this is comparatively high compared to the other meat peptones and may have an inhibitory effect on synthesis of flagellin. Again other factors not investigated may have been responsible, and quantitative analysis of motility (Chapter 4.4.4) demonstrated that cultures grown in M1 medium were motile.

Yeast and soy based peptone media consistently produced flagellate cultures of *Salmonella* ser. Typhimurium; peptones examined from these categories contained all amino acids and a substantial carbohydrate source. Therefore these peptones as a nutrient supply provided all essential elements for growth and synthesis of flagella by this bacterium.

In summary, a deficiency in the growth medium of tyrosine or a suitable substrate for ATP synthesis, or a lack of required growth factors not investigated in this study, may result in aflagellate cultures of *Salmonella*. The presence of various inhibitors, for example high concentrations of salts or alcohols (e.g. methanol, ethanol or isopropanol), is also known to affect flagellar synthesis.

4.4.4. Swim migration assay

Quantitative analysis of motility dependent on the peptone constituent of the medium was assessed using "sloppy" buffered peptone agar (BPA). This was made up using the same ingredients as BPW with an additional 0.3% agar and allowed migration of the bacteria across the surface of the medium. Many media containing peptones which had previously produced cultures observed to be aflagellate, during examination using electron microscopy, proved to be non-motile. Apparent values for motility were achieved for organisms which were effectively non-motile as, during the incubation period, a colony developed at the site of inoculation; subsequent migration did not occur (Figure 4.18a). As expected, the observations during EM were not always definitive and some *Salmonella* which appeared aflagellate were in fact motile on BPA. Where flagellation, and consequently motility, was successful the bacteria spread across the surface of the medium forming concentric rings (Figure 4.18b). Motility of the different bacterial species, during growth on media containing variable peptones, were noticeably different but were not statistically different ($P = 0.194$). All species displayed increased diversity with respect to motility on casein, gelatin or meat peptone based media; and therefore more consistent values for swim migration were seen during growth of the Enterobacteria on soy and yeast media.

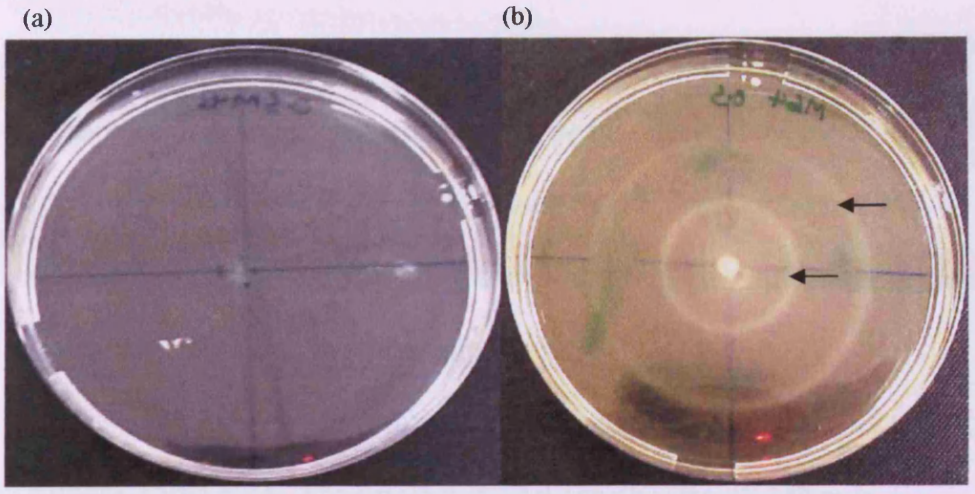

Figure 4.18. Swim rates of *Salmonella* ser. Typhimurium in various Buffered Peptone Agar media (BPA) were obtained by measuring the diameter of swim circles after 5 h growth at 37°C. Shown are photographs of *Salmonella* migration in (a) G1 and (b) S1 peptone based-BPA. Arrows highlight concentric circles which were present in all replicates of *Salmonella* and *Escherichia* which successfully migrated.

4.4.4.1. Swim migration assay of *Salmonella* ser. Typhimurium

Motility of *Salmonella* was shown to be significantly different dependent on the source peptone as a constituent of the medium ($P = 0.018$). Swim rates in mm h^{-1} of *Salmonella* during a 5 h incubation period on differing peptones are shown in Figure 4.19. Group averages determined that yeast peptones generated the fastest swim rates: 4.96 ± 1.07 mm h^{-1}. Soy, casein and meat peptone-based media produced similar rates with 4.79 ± 0.65, 4.69 ± 1.33 and 4.45 ± 1.90 mm h^{-1} respectively. Gelatin peptone-based BPA gave rise to the slowest swim rate at 2.15 ± 0.91 mm h^{-1}. Commercially available, nutrient rich media (e.g. NB or TSB), produced the fastest mean average swim rate of 6.54 ± 2.16 mm h^{-1}; this was unsurprising due to the more complex nature of the media providing all necessary components for growth, flagellation and consequently motility. However NA produced notably faster swim rates: 8.07 ± 0.10 mm h^{-1} compared with TSA which generated a mean average swim rate of 5.01 ± 0.71 mm h^{-1}.

4. Morphology and motility of bacteria dependent on peptone

Within the groups of source peptones there was, as seen during analysis of growth dynamics, diversity within some of the groups which could be attributed to manufacturing and storage as discussed previously (Chapter 3). Outlined below is a detailed discussion of each source peptone in relation to the swim rate of *Salmonella* ser. Typhimurium.

Casein peptone-based BPA: The casein peptones which were of different manufacturer or batch were statistically different in their ability to culture motile *Salmonella* ($P = 0.025$). Peptone C4 was a particularly poor peptone regarding motility, with a slow rate of 2.42 ± 0.15 mm h^{-1}; this matched observations from EM; whereas C3 and C8 peptones generated exceptionally fast swim rates with 6.15 ± 0.20 and 6.55 ± 0.53 mm h^{-1} respectively. Peptones C3 and C5 were noted to culture *Salmonella* ser. Typhimurium with few flagella, however this quantitative method indicates otherwise.

Gelatin peptone-based BPA: Gelatin peptone-based BPA allowed swim rates which were found to be statistically significant ($P = 0.000$) with a range of 2.36 mm h^{-1}. The slowest rate was G3 with a value of 0.80 ± 0.39 mm h^{-1}; however this was not due to migration: the data can be attributed to growth of inocula at the site of inoculation (Figure. 4.17a) and not the subsequent migration of bacteria due to their motile ability. The fastest swim rate obtained on gelatin based media was 3.16 ± 0.14 mm h^{-1} during cultivation on G5 peptone; this was still substantially slower than most peptones of different biological source. It is worth noting that where cell numbers were low due to the medium being unsuitable for the support of dense growth, motility was still apparent (where

applicable); translucent concentric rings of the migrating bacteria were easily seen despite the lack of dense growth. These data generally confirmed the micrographs obtained using TEM, the exception being G5 cultures which demonstrated motility despite appearing aflagellate.

Meat peptone-based BPA: In contrast to growth dynamics data, meat peptones as a medium for motility were less diverse. They were statistically different ($P = 0.000$) as peptone M6 failed to generate motility, growth occurring only at the point of inoculation and so giving a value of 0.78 ± 0.53 mm h^{-1}. This therefore gives a large range of 5.14 mm h^{-1} for the group when, ignoring M6, the range is small at 1.66 mm h^{-1}. The fastest rate was obtained during cultivation on M2 peptone with 5.92 ± 1.02 mm h^{-1}.

Again these data support the qualitative images achieved using TEM, the anomaly being that cultures grown on M1 media were motile; exceptional inconsistencies of this type arise from the qualitative nature of TEM.

Soy peptone-based BPA: Soy peptones as constituents of BPA were the only peptones which produced consistent swim rates during cultivation of *Salmonella* ser. Typhimurium ($P = 0.067$). Peptone S3 generated *Salmonella* which produced a marginally slower swim rate at 4.00 ± 0.84 mm h^{-1} compared with S4 peptone which gave rise to a value of 5.60 ± 0.48 mm h^{-1}.

Yeast peptone-based BPA: The range of swim rates obtained during growth on BPA containing variable yeast peptone was 3.07 mm h^{-1}, with Y3 peptone producing the slowest rate and Y4 the fastest: 3.12 ± 0.30 mm h^{-1} and $6.19 \pm$

1.19 mm h^{-1} respectively. The difference in swim rates obtained was statistically significant at P = 0.006. Cultures of *Salmonella* ser. Typhimurium were noted to be less flagellate during examination by TEM in Y3 peptone media, by comparison with organisms cultivated in the other yeast peptones investigated.

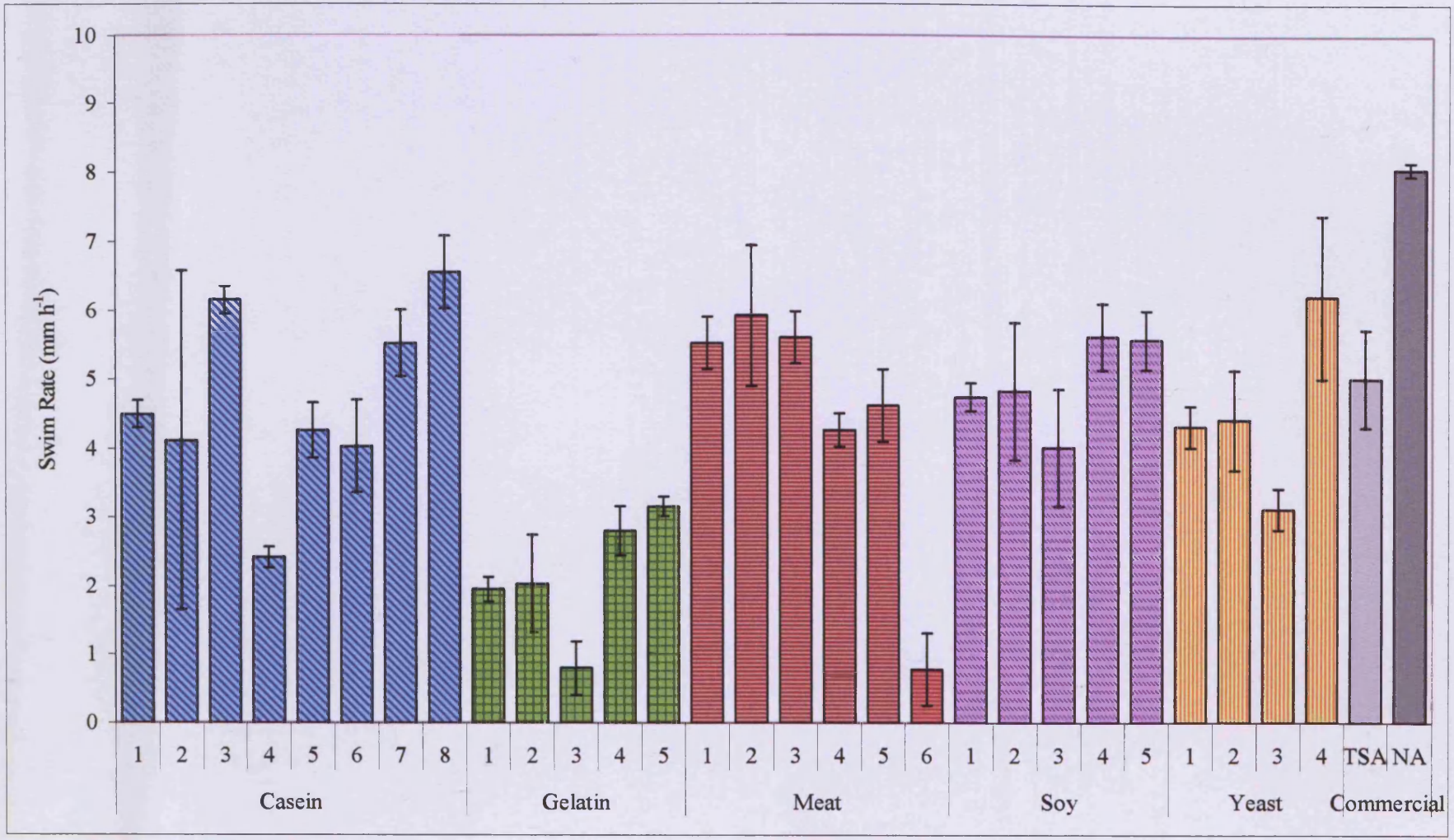

Figure 4.19. Swim rates of *Salmonella* ser. Typhimurium in BPA made with 0.3% agar. Radii of swim circles were measured after 5 h growth at 37°C and the swim rate calculated for experimental BPA, nutrient rich "0.3% agar" nutrient agar (NA) and tryptone soya agar (TSA). Error bars indicate ±standard deviation (n=3).

4.4.4.2. Swim migration assay of *Salmonella* ser. Poona

Swim rates of *Salmonella* ser. Poona, shown in Figure 4.20. were significantly different depending on the source of peptone used as an ingredient of their medium (P = 0.000). Gelatin peptones generated notably slower swim rates with a mean group average of 2.68 ± 1.31 mm h^{-1}, whilst soy and yeast peptones displayed predictably faster swim rates: 4.82 ± 0.48 and 5.89 ± 0.77 mm h^{-1} respectively. Rates with casein (3.06 ± 0.89 mm h^{-1}) and meat (4.71 ± 1.52 mm h^{-1}) were intermediate.

Casein peptone-based BPA: Casein peptones originating from various manufacturers generated statistically different swim rates (P = 0.000). The range was 2.31 mm h^{-1} with the slowest values being produced in C3 and C6 peptones (1.73 mm h^{-1}) and the highest in C1 peptone (4.04 ± 1.52 mm h^{-1}). These values deviated from expected values based on TEM: media based on peptone C2 appeared to produce highly flagellate organisms, which was in contrast to the aflagellate appearance of *Salmonella* ser. Poona cultivated in C5 peptone (Figure 4.6). However, motility was similar during growth on both peptones as constituents of BPW.

Gelatin peptone-based BPA: *Salmonella* ser. Poona generated swim rates in certain gelatin peptone based media which were faster than those measured in casein based BPA. Motility was observed on all gelatin media, with the slowest value being 1.73 mm h^{-1} (G1, G3 and G4 peptones) and the fastest at 4.26 ± 1.13 mm h^{-1} in G2 peptone. Media comprising G5 peptone also gave rise to a comparatively fast swim rate of 3.97 ± 0.33 mm h^{-1}. These data were shown to be statistically different (P = 0.000). An inconsistency between the electron micrographs of *Salmonella* ser. Poona and swim migration occurred with respect to G1 peptone: TEM displayed flagellate

organisms which lacked in motility. All other electron micrographs were confirmed by the migration assays.

Meat peptone-based BPA: Peptones originating from a meat source, as the sole variable ingredient in the culture medium, showed the greatest range (3.11 mm h^{-1}) of swim rates for *Salmonella* ser. Poona; this was shown to be statistically significant at P = 0.017. This serovar of *Salmonella* exhibited motility on M6 meat peptone based BPA, where ser. Typhimurium did not. Swim rates were spread evenly between the fastest and slowest values: 6.36 ± 1.44 mm h^{-1} recorded after cultivation in M3 peptone based medium and 3.25 ± 0.22 mm h^{-1} in M5.

Soy peptone-based BPA: Soy peptone as a constituent of BPA gives a more consistent set of swim rates (Figure 4.20.), however they are still statistically significant at P = 0.000. There was a small range of 1.15 mm h-1 between the data values, the slowest being 4.19 ± 0.13 mm h^{-1} in S5 peptone and the fastest 5.34 ± 0.12 mm h^{-1} in S2.

Yeast peptone-based BPA: Significantly different swim rates were noted during growth of *Salmonella* ser. Poona on yeast peptones (P = 0.038). Media comprising Y3 peptone generated a considerably slower rate (4.84 ± 0.45 mm h^{-1}) relative to Y1 (6.50 ± 0.22 mm h^{-1}).

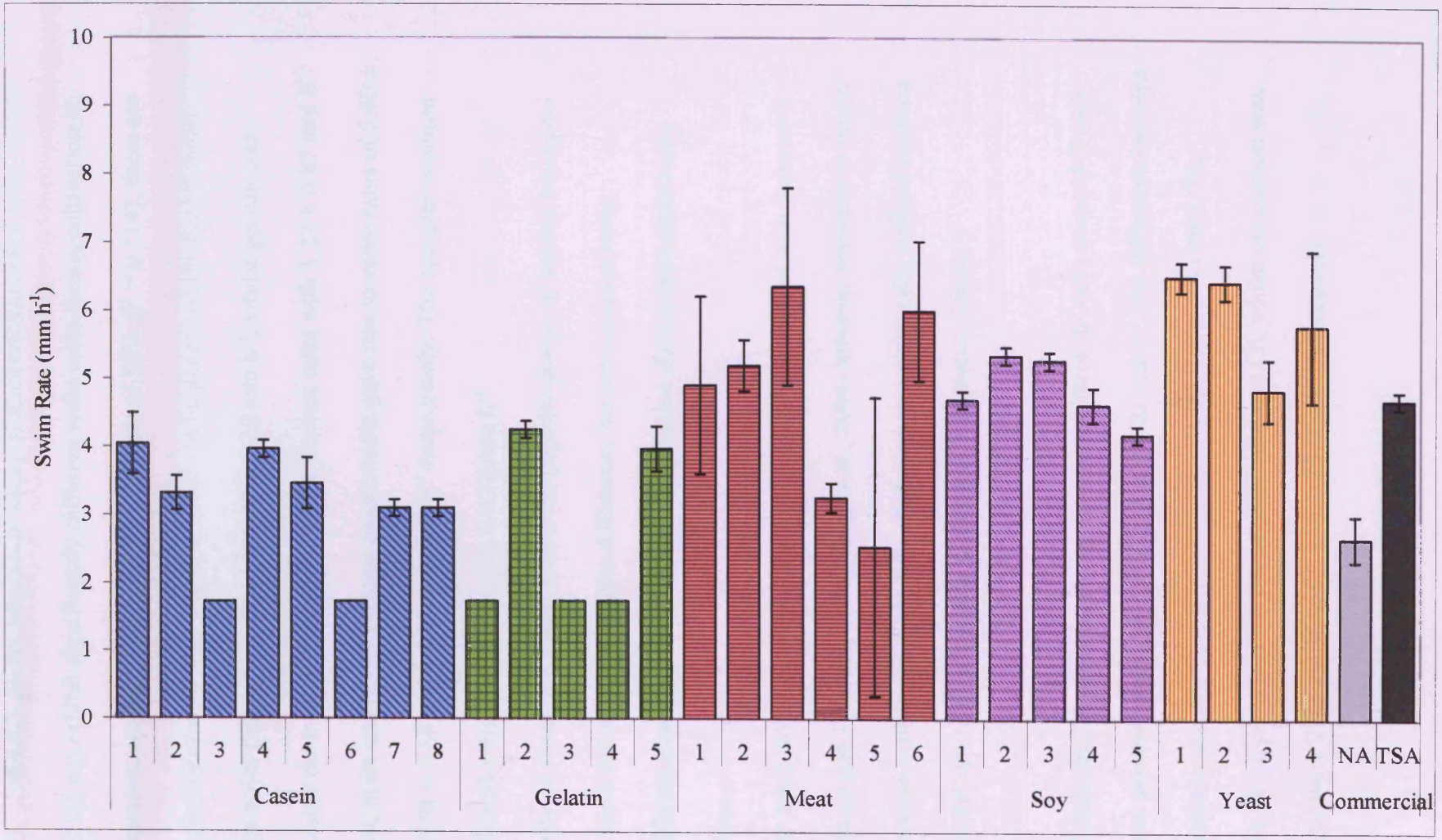

Figure 4.20. Swim rates of *Salmonella* ser. Poona in BPA made with 0.3% agar. Radii of swim circles were measured after 5 h growth at 37°C and the swim rate calculated for experimental BPA, nutrient rich "0.3% agar" nutrient agar (NA) and tryptone soya agar (TSA). Error bars indicate ±standard deviation (n=3).

4.4.4.3. Swim migration assay of *Escherichia coli*

E. coli did not exhibit significantly different swim rates during cultivation on the various sources of peptones as constituents of BPA ($P = 0.114$); these are shown in Figure 4.21. Soy peptone-based media generated the fastest mean average swim rate, with a value of 5.82 ± 1.29 mm h^{-1}, whilst gelatin was predictably the nutrient source giving the slowest rates with 3.22 ± 0.87 mm h^{-1}. Yeast, meat and casein peptone based media gave rise to swim rates of 5.40 ± 0.29, 5.08 ± 1.68 and 4.45 ± 1.51 mm h^{-1} respectively. The complex medium NA allowed a fast swim rate: 7.92 ± 0.33 mm h^{-1}.

Diversity of swim rate values within the peptone groups of similar biological origin were still apparent despite differences between sources being insignificant; yeast peptones were the only source shown to be statistically consistent.

Similar mechanisms of amino acid uptake exist for *E. coli* as with *Salmonella* (Antonucci and Oxender, 1986; Yang *et al.*, 2004) however response to media supplements is dissimilar; addition of glucose to a chemically defined medium inhibits the synthesis of flagella (Adler and Templeton, 1967).

Casein peptone-based BPA: The range of swim rates for cultivation of *E. coli* on casein peptone media was notably high: 5.02 mm h^{-1}; the difference between swim rates in variable casein peptones was significant at $P = 0.002$. The peptone which generated the fastest swim rate was C6, whilst the slowest was C7; these were 7.92 ± 1.66 and 2.90 ± 0.33 mm h^{-1} respectively.

4. Morphology and motility of bacteria dependent on peptone

Gelatin peptone-based BPA: *E. coli* swim rates within this group were diverse ($P = 0.001$) with a range of 2.12 mm h^{-1}. The peptone producing the slowest swim rate was G3; however migration of *E. coli* was noted to give a rate of 2.43 ± 0.11 mm h^{-1}. The fastest swim rate was obtained with G1 peptone: 4.55 ± 0.00 mm h^{-1}.

Meat peptone-based BPA: Diversity of swim rates during cultivation of *E.coli* on meat peptone was considerable ($P = 0.000$). Significantly, M6 peptone, which yielded aflagellate, non-motile *Salmonella* ser. Typhimurium, generated the fastest swim rate: 6.95 ± 0.07 mm h^{-1}. Media containing M1 and M2 peptones also gave rise to fast rates, with values of 6.83 ± 0.55 mm h^{-1} and 5.96 ± 0.22m h^{-1} respectively. Poor motility was achieved on M3, M4 and M5 peptone based media with the slowest swim rate being 3.29 ± 0.00 mm h^{-1} during cultivation on M4 medium.

Soy peptone-based BPA: Inconsistent swim rates were displayed by *E. coli* cultured in soy peptone manufactured by different companies ($P = 0.002$); from a fast 7.77 ± 0.33 mm h^{-1} in S4 peptone media, to relatively slow 4.39 ± 0.00 mm h^{-1} in S1.

Yeast peptone-based BPA: Yeast peptones, regardless of batch or manufacturer, were highly consistent ($P = 0.360$), with swim rate values having a range of 0.71 mm h^{-1} for the group. The fastest was 5.73 ± 0.11 mm h^{-1}, the slowest 5.02 ± 0.44 in peptones Y2 and Y4 respectively.

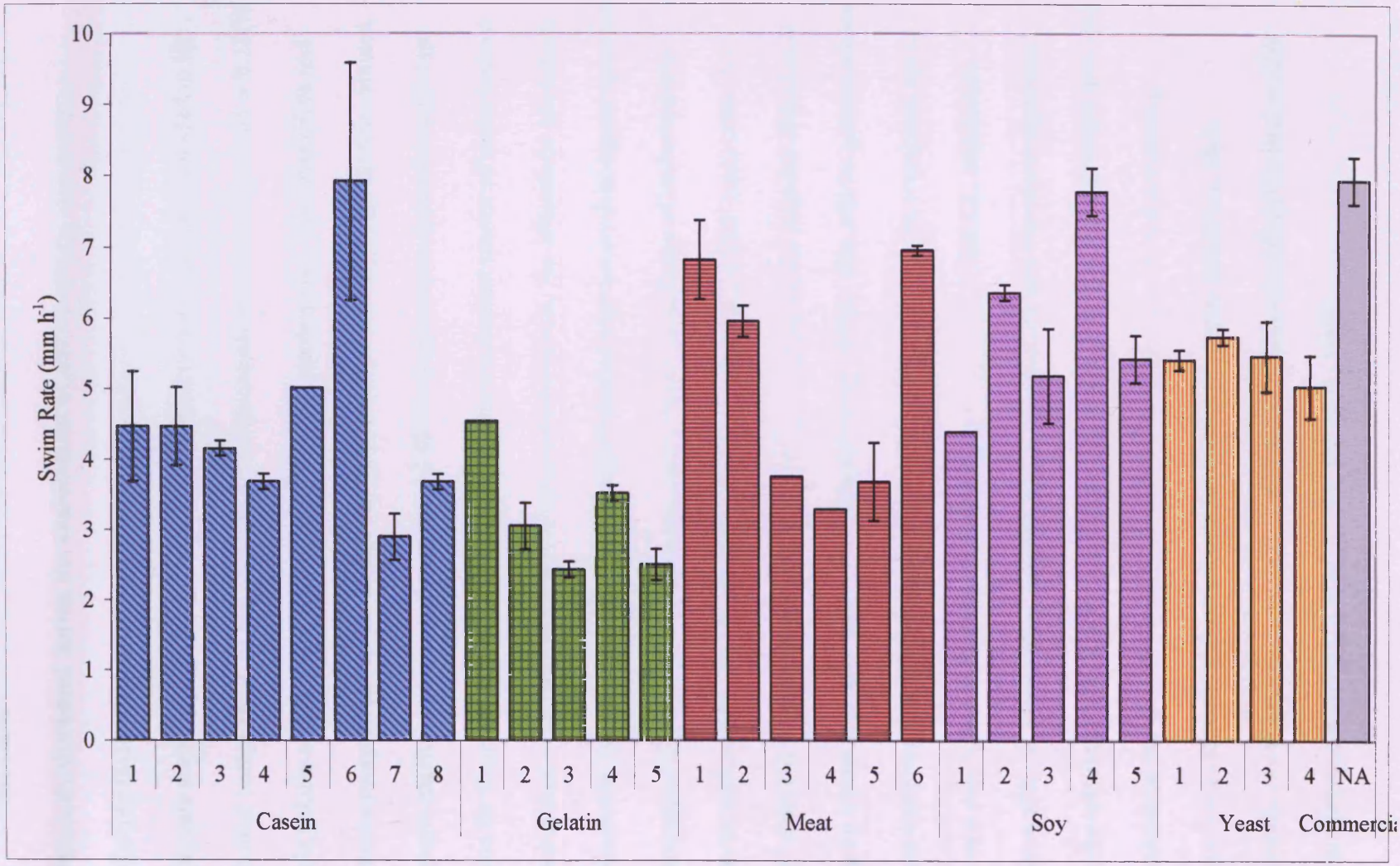

Figure 4.21. Swim rates of *Escherichia coli* in BPA made with 0.3% agar. Radii of swim circles were measured after 5 h growth at 37°C and the swim rate calculated for experimental BPA and nutrient rich "0.3% agar" nutrient agar (NA). Error bars indicate ±standard deviation (n=3).

4. Morphology and motility of bacteria dependent on peptone

4.4.4.4. Variation in motility dependent on peptone composition of the medium

As previously discussed during the examination of *Salmonella* by microscopy, contributing factors towards bacteria becoming non-motile on specific medium constituents are numerous. Organisms which are non-motile can be attributed to the amino acid composition of the peptone: low concentrations of tyrosine (0.31 ± 0.11 % w/w) in gelatin and in some casein-based peptones correspond with aflagellate and non-motile morphology of the Enterobacteria thereby cultured. High protein content, as in some casein-, gelatin- and meat-based BPW, does not correlate with sufficient tyrosine for flagellin synthesis. Often where there is a limited source of carbohydrate in combination with relatively low tyrosine concentrations, the aflagellate phenotype is exacerbated. Soy- and yeast-based peptones contain over seven-fold higher tyrosine concentrations (2.37 ± 0.05 % w/w) and also a higher constituent of carbohydrate compared with gelatin, and consistently produce highly flagellated organisms. An energy source is of great importance for motility: rotation of the flagellar filament is driven by a proton motive force (PMF) (Bardy *et al.*, 2003); this will be greater where glucose was provided as a substrate for glycolysis. High NaCl concentrations, up to 45 % w/w was found to be typical of casein peptones C3, C7 and C8, might also inhibit motility as synthesis of flagellin might be inhibited and therefore organisms would be less flagellate. In addition certain growth factors (such as vitamins) may not be available in the growth medium, therefore hindering growth and preventing motility; the exact composition of certain peptones remains unknown; high concentrations of certain compounds would act as inhibitors of flagellin and would therefore prevent motility (Li *et al.*, 1993; Shi *et al.*, 1993).

4. Morphology and motility of bacteria dependent on peptone

Bacteria observed to be flagellate (by TEM) but non-motile (using the swim migration assay) in the same media (e.g. *Salmonella* ser. Poona cultured in G1 peptone based BPW) could represent organisms with flagella that are too short to support motility. This has previously been observed in *Salmonella* by Kerridge, (1959 b). An additional explanation for diversity of the swim rates obtained is that the bacteria were flagellate and motile, but not migrating outwards by positive chemotaxis. *Escherichia coli* and *Salmonella* spp. respond to spatial gradients of attractants; these include serine, aspartate, maltose, ribose, galactose and glucose. *E. coli* is also attracted to dipeptides, whereas *Salmonella* exhibits positive chemotaxis towards citrate. Therefore, if there were no chemical attractants (for example in media lacking carbohydrates and containing only very low concentrations of the attracting amino acids) the bacteria would move in a random 3-dimensional walk, which would result in a slower outward migration. In contrast, growth in media containing relatively higher concentrations of the chemo-attractants would suppress tumbles, giving rise to longer run periods towards the attractant (Manson *et al.*, 1998). This would result in a seemingly faster migration rate.

It was noticeable that the various bacterial species, despite not being significantly different, showed slight differences in motility on the BPA containing peptones of varying source. *E. coli* was comparatively more motile, achieving faster swim rates and exhibiting motility on media which gave rise to non-motile cultures of *Salmonella*. *E. coli* is chemotactically attracted towards dipeptides, whereas *Salmonella* are not; this may explain the faster swim rates generated by this species, as protein material was present in all peptones. Also *E.coli* seemed to be more inhibited by the high concentrations of NaCl, which appeared not to affect motility of

Salmonella, in C3, C4, C7 and C8 casein based media. The lowest swim rate obtained for *Salmonella* ser. Poona was 1.73 mm h^{-1}, whereas *Salmonella* ser. Typhimurium generated a value of 0.78 mm h^{-1}; it would seem that *Salmonella* ser. Poona was increasingly motile particularly on M6 meat peptone whereas *Salmonella* ser. Typhimurium failed to migrate outward on this medium.

The patterns of concentric rings noted as organisms migrated away from the central inoculation point were a result of the bacterial response to spatial gradients of chemo-attractants generated by uptake and catabolism (Budrene and Berg, 1991). For *E. coli* and *Salmonella* ser. Typhimurium these complex patterns have been observed during exposure to intermediates of the tricarboxylic acid cycle. This resulted in secretion of aspartate by the bacteria, which is a potent chemo-attractant, and caused organisms to form high density aggregates (Tyson *et al.*, 1999). Periodic swarming of *Proteus mirabilis* provided an explanation for similar patterns when this species was grown on solid agar, whereby *Proteus* alternated between hyper-flagellate and vegetative states. The hyper-flagellate, elongated swarmer cells migrated away from the colony rapidly; then stopped. By a series of cell fissions they then reverted to the vegetative state, whereby cells grew normally for a short period. Reverting to a vegetative state from a swarmer state is termed consolidation. Formation of swarmer cells are then re-initiated at the outermost zone and rapidly migrate away; this pattern repeats itself. Concentric zonations result as the process continues in periodic cycles (Czirók *et al.*, 2001).

4.5. Conclusion

Identification of the Enterobacteria can often involve microscopic examination and an evaluation of motility: the work reported here indicates that these characteristics are dependent on the culture medium. For work whereby motility or morphology is evaluated, it is recommended that a consistent batch of medium be used in order to prevent possible interference by commonly occurring components; where work is ongoing, for example in the clinical microbiology setting, it would be necessary to compare old and new media batches in order to achieve consistent results.

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CHAPTER FIVE

Public health analyses

5. Public health analyses

5.1. Summary

The identification of *Salmonella* serovars Poona and Typhimurium by serotyping was assessed and the quantities of H antigen, expressed on the flagellar filament, were observed to alter with the peptone used as the sole nutrient source in the media buffered peptone water (BPW). The serological results obtained were often either incomplete or inconclusive, especially with gelatin peptones, and so gave incorrect or incomplete identification of the serovar. Peptones originating from a soy or yeast source proved more consistent and gave conclusive results, with organisms cultured in those media producing strong agglutination reactions. Where weak reactions were observed after addition of the H antisera, these correlated well with known motility of the organisms on those media; *Salmonella* which were non-motile and aflagellate were not able to express the H antigen present on the flagellar filament, therefore these cultures did not produce a serological reaction.

Detection of the O antigen for *Salmonella* by serological methods was not affected by peptones of different sources or manufacturers as a constituent of the medium BPW.

The complex medium tryptone soya broth (TSB), containing both casein and soy peptones resulted in similar agglutination reactions of *Salmonella* suspensions despite the different sources or manufacturers of the soy and casein peptone constituents.

The antibiotic resistance for several antimicrobial compounds was quantitatively recorded for *Escherichia coli*, *Salmonella* ser. Typhimurium, *Salmonella* ser. Poona and *Staphylococcus aureus* on buffered peptone agar (BPA) containing peptones of different source. It was found that after growth on certain peptone-based media, particularly the

peptones gelatin and casein, antagonized activity of streptomycin against *Salmonella* ser. Typhimurium and *Escherichia coli*. This resulted in *Salmonella* ser. Typhimurium, which is sensitive to all aminoglycosides after growth on a commercially available Iso-sensitest agar (ISA) and yeast or soy based BPA, being completely resistant to the antimicrobials. The resistance of *Salmonella* ser. Typhimurium to gentamicin was also dependent on the source or manufacturer of the peptone. *Salmonella* ser. Poona and *Staphylococcus aureus* were not observed to vary notably (after growth on the different peptone-based BPA) in their susceptibility to the antibiotics examined.

In six different laboratory-made ISA containing different peptones it was found that, in the more complex media the effect of peptone on the activity of the antibiotic was not as pronounced. The inhibition zones were shown to be significantly different dependent on peptones used as constituents of the medium but the organisms did not become resistant to any of the antimicrobials investigated.

This work will highlight the need for a consistent culture medium for clinically important diagnostics, which has yet to be achieved.

5.2. Introduction

Cultures of *Salmonella* serovars Typhimurium and Poona produced notably different serological reactions to H antigen antisera dependent on the peptone constituent of the growth medium BPW. Extensive work on the serology of bacteria has been performed by Craigie (1931); the chemistry and genetics of flagella are reviewed by Iino (1969). Comprehensive works on the effect of differing growth media recipes on clinically

important diagnostics, including bacterial identification by serology and antibiotic susceptibility to various antimicrobial compounds, have been published.

With respect to different media constituents which have been acknowledged to affect serological identification, a member of the D1 group *Salmonella enterica* ser. Pullorum deviated markedly from the expected H-antigen immunoreactivity when supplemented with 100 mM maltose. This serovar, classified by the Kauffman-White identification scheme as non-motile and aflagellate, produced flagella after addition of maltose to HEA medium; thus expressing the H antigen present in flagellin and consequently an altered serology, which was inducible by addition of the maltose (Guard-Petter, 1997). Medium-induced motility of *Salmonella* ser. Pullorum, described by Harshey and Matsuyama (1994), presented cultures which exhibited strong agglutination reactions with antisera to group G flagellar antigens (Holt and Chaubal, 1997), demonstrating a need to review the original identification scheme. Tulley and Gaines (1961) produced cultures of *Salmonella typhosa* which were found to have a significant decrease or loss of H antigen and flagella after growth on minimal medium. Also, the sulphate reducing bacteria cultured in different commercially-available media expressed different membrane proteins which were demonstrated by fluorescent antibody and SDS-PAGE techniques (Cloete and de Bruyn, 2001).

However, these previous literature articles focus on the effect of variable media recipes on the serotype obtained; this work specifically examines the undefined nutritional constituent of BPW and TSB: peptone.

Investigation into the effect of peptone, as a constituent of the simple medium BPA, illustrated that the activities of various antibiotics on the organisms *Salmonella*,

5. Public health analyses

Escherichia and *Staphylococcus* were remarkably dependent on the growth medium. This effect was not noticeable with laboratory-prepared versions of the complex ISA medium. Again, clinical diagnostics (e.g. antibiotic susceptibility testing) and subsequent therapeutics are based on methods which are fatally flawed in that they are dependent on the growth medium and this is not consistent. This fact, as with serological identification, is noted repeatedly in the literature.

Peptone "antagonism" of antibiotics hindered the determination of bacterial sensitivity to antimicrobials; specifically, sensitivity of Gram negative bacteria to nitrofurantoin and sulfamides in work performed by Marica *et al.* (1989). The solution suggested by these authors was the use of a medium which was chemically defined (and so free of antagonists) and that this medium should be established for this purpose. The effect of the bacterio-static boron-containing compounds was shown to be altered upon addition of 1% casein hydrolysate to Gram negative organisms grown in a minimal medium; in some cases, addition of casein to boron-containing cultures allowed a more rapid growth rate than in control cultures, whereby the antimicrobial compound was absent (Bailey *et al.*, 1980). Agar concentration and variation in growth medium recipes influenced the sensitivity of bacteria to different antibiotics; the growth media examined included BHI agar which proved to be best for nafcillin and methacycline; oleandomycin was best in synthetic amino acid medium, Mueller-Hinton agar for vancomycin and chloramphenicol; and G & R medium for novobiocin. Huys *et al.* (2002) demonstrated that the antibiotic resistance profiles of lactobacilli and enterococci were altered after growth of these organisms on different media: Iso-sensitest medium, de Man, Rogosa, Sharpe or MRS agar. The organisms were more sensitive to ampicillin and tetracycline

on MRS agar compared to Iso-sensitest; this was in contrast to the antimicrobials gentamicin, bacitracin and erythromycin which produced smaller inhibition zones on MRS agar. Man medium produced incorrect resistance profiles to bacitracin and gentamicin. Gentamicin sulphate exhibited medium-dependent activity on Enterococci: the organisms were sensitive to this antimicrobial on Mueller-Hinton agar (5 % sheep blood), but were found to be resistant to the same compound when grown in BHI and trypticase soy broth or agar (Raymond and Traub, 1971). Similarly, the antibiotic susceptibility of *Haemophilus influenzae* to ampicillin varied dependent on the growth medium used, with identical isolates ranging from susceptible to resistant after growth on the experimental media. The media investigated were: *Haemophilus* test medium (commercial and laboratory prepared), supplemented BHI medium, enriched chocolate agar medium and Mueller- Hinton chocolate agar medium (Mendelman *et al.*, 1990). *Moellerella wisconsensis* exhibited marked medium-dependent differences in susceptibility to the antibiotics tetracycline, clindamycin, fosfomycin and the macrolides; noticeable differences were also observed with the aminoglycosides, quinolones and lincosamides. The media used were IsoSensitest broth and cation-adjusted Mueller Hinton broth (Stock *et al.*, 2003).

Chapter 4 of this thesis explored the variation in morphology which was shown to be inconsistent after culture in different peptones. Published works have established that morphological variations can also affect antibiotic susceptibility; for example, resistance of *Salmonella typhimurium* to antimicrobial peptides was different for starved or log phase organisms (McLeod and Spector, 1996). Swarm-cells, rather than vegetative cells, of *Salmonella* ser. Typhimurium resulted in elevated resistance to many antibiotics; thus

it was demonstrated that the morphology of the organism played an important role in its resistance to antimicrobials (Kim *et al.*, 2003). The mechanism for elevated resistance was thought to be a result of swarmer cells having a more positively charged LPS core (Kim and Surette, 2003).

This work aims to demonstrate that peptone variation in diagnostic media, which can not be controlled through purchase of commercially dehydrated culture media (as they do not stipulate peptone type) significantly affects clinical identification, antibiotic profiles and subsequent choice of chemotherapy within the public health and research sectors.

10

5.3. Materials and methods

Two methods indispensable to public health are identification of the organism and its level of resistance to possible chemotherapies; for *Salmonella*, serotyping determines identity and for antibiotic resistance: the disk diffusion method is used.

5.3.1. Serology

Serological identification of the *Salmonella* serovars is outlined in Chapter 2.2.8. These were performed with *Salmonella* ser. Poona and Typhimurium in laboratory prepared BPW and TSB, containing peptones of different sources and origins. The recipe for TSB

20 medium is detailed in Appendix 1.2.1. Commercially prepared TSB and NB (both Oxoid) were also used for comparison.

5.3.2. Antibiotic profiles

The antibiotic profiles of *Salmonella* ser. Typhimurium and, as a reference strain, *Escherichia coli* were determined in laboratory made BPA and ISA containing variable peptones which originated from different sources and manufacturers. M9 defined medium (with the addition of 1.5 % agar (BD)) was also used to assess antibiotic susceptibility; these media recipes are detailed in Appendix 1.

5.3.2.1. Antibiotic profiles on laboratory prepared ISA

The commercially available dehydrated ISA used was product CM471 from Oxoid. Laboratory prepared ISA, containing different selected tryptone (casein peptones) and peptone (peptones other than casein peptones) was made up to determine whether altering peptone source affected the susceptibility of the bacteria to various antibiotics. Table 5.1. outlines the different peptone combinations used and Table 5.2. gives the manufacturer of each component.

Table 5.1. Full outline of ISA medium recipe is detailed in Appendix 1.2.3. The tryptones and peptones listed above were used in the make up of laboratory prepared ISA, the commercially available dehydrated ISA was obtained from Oxoid.

Iso sensitest	Undefined components	
Medium	Tryptone	Peptone
1	C1	M2
2	C4	M4
3	C6	S5
4	C7	M1
5	C7	G2
6	C6	G3
Commercial	ISA (Oxoid)	

Table 5.2. ISA recipe.

Ingredient	Concentration (g l^{-1})
Tryptone (Various)	11
Peptone (Various)	3
Glucose (Fisher)	2
NaCl (BDH)	3
Starch (Soluble) (BDH)	1
Na_2HPO_4 (BDH)	2
Sodium Acetate (BDH)	1
Mg Glycero PO_4	0.2
Ca Gluconate (Sigma)	0.1
Cobaltous SO_4 (BDH)	0.001
Cupric SO_4 (BDH)	0.001
Zinc SO_4 (BDH)	0.001
Ferrous SO_4 (BDH)	0.001
Manganous Cl	0.002
Menadione (Sigma)	0.001
Cyanocobalamin (Sigma)	0.001
L-Cysteine HCl (Sigma)	0.02
L-Tryptophan (BDH)	0.02
Pyridoxine (Sigma)	0.003
Pantothenate(Sigma)	0.003
Nicotinamide (BDH)	0.003
Biotin (Sigma)	0.0003
Thiamine (Sigma)	0.00004
Adenine (Sigma)	0.01
Guanine (Sigma)	0.01
Xanthine (Sigma)	0.01
Uracil (Sigma)	0.01
Agar (Sigma)	8

5.3.3. Statistical analyses

Serological identifications were not subject to statistical analysis as the results were semi-quantitative, with the strength of agglutination reactions based on observations only.

One way ANOVA was employed to analyse zones of inhibition, produced by the disk diffusion method, for determination of antibiotic susceptibility where data were parametric. Kruskal-Wallis was used to analyse non-parametric data.

5.4. Serological Results

Agglutination reactions, based on the work published by Craigie, (1931), were employed to determine if serological identification of the *Salmonella* serovars were altered when

different peptones, as constituents of the growth media, were used. The strength of the agglutination reactions for both somatic (O) and flagellar (H) antigens were semi-quantitatively recorded based on observations. The *Salmonella* serovar was known (Poona or Typhimurium) and the aim was to show that different peptones, as constituents of both BPW and TSB, may give inaccurate or incomplete serological results.

5.4.1. Serotyping of the *Salmonella* somatic (O) antigen in various BPW

All somatic (O) antigens were easily detectable after cultivation of both *Salmonella* ser. Typhimurium and ser. Poona in BPW which contained varying peptones of any source and any manufacturer or batch. Therefore peptone variation had no effect on the O antigen of these *Salmonella* serovars. Similar results, i.e. consistent O antigen production regardless of culture medium, were obtained with *Helicobacter pylori* in various commercial media: BHI, Brucella broth, Mueller-Hinton broth and tryptone soya broth (Walsh and Moran, 1997).

5.4.2. Serotyping of the *Salmonella* flagellar (H) antigen in various BPW

The two *Salmonella* serovars underwent full serological identification of H antigens (results shown in Table 5.3.) and it was noticeable that certain H antigens (usually phase two antigens) were present in higher concentrations than others. For example, *Salmonella* ser. Poona expressed a stronger reaction to the H6 antisera (in contrast to the H1 and Hz antisera) whereas *Salmonella* ser. Typhimurium exhibited a stronger agglutination reaction to H2 antisera (in contrast to the H1 and Hi antisera); it is commonly known that

Salmonella often express one H antigen more strongly than others (Silverman and Simon, 1977).

Generally, *Salmonella* ser. Poona was shown to produce stronger agglutination reactions in comparison with *Salmonella* ser. Typhimurium; the only antigen in common was the phase two antigen H1, for which *Salmonella* ser. Poona exhibited relatively stronger agglutination reactions in response to the antisera.

With respect to the different peptones as constituents of the growth medium BPW, soy and yeast peptones produced the strongest serological reactions in comparison with casein, gelatin and meat.

Both *Salmonella* serovars cultured in peptones C8 and G3 showed no obvious signs of agglutination to any of the antisera; noticeable signs of reaction to the H antisera were not produced by cultures of *Salmonella* ser. Typhimurium after growth in the peptones C2, C4, G1, G5 and M2. Peptone Y4 based BPW gave notably strong agglutination of all H antigens for *Salmonella* ser. Poona.

In comparison with the swim migration assay data, most peptones which cultivated *Salmonella* ser. Poona with low motility, for example C6, G1, G3, G4, M4 and M5, all generated organisms which did not exhibit strong reactions to the H antisera. This confirms that lower numbers of flagella are present on organisms grown on these media; they are less motile and produce less H antigen which is expressed on the flagellar filament. Furthermore, those organisms which demonstrated increased motility were cultured on BPA containing the peptones M3, M6, and all soy and yeast source peptones. This again correlated well with the agglutination reactions shown in Table 5.3., which were stronger for *Salmonella* ser. Poona cultivated in those media.

Salmonella ser. Typhimurium also demonstrated a correlation between the swim migration assays and the serological reactions to H antisera. Peptones which exhibited particularly poor swim rates, for example C4 and G3, also produced agglutination which was barely visible. However, most casein and meat peptones generated *Salmonella* ser. Typhimurium which were equally as motile as those cultured on soy or yeast peptones. Serological analysis showed a greater difference between these groups, clearly indicating that soy and yeast peptones did produce higher quantities of the H antigen in comparison with casein and meat.

Again, it should be noted that the serological analysis was semi-quantitative, based on observation only. Apparent anomalies such as *S*T being non-motile during growth on M6 peptone-based BPA, but motile on all other meat peptones, yet showing similarly weak serological reactions to all antisera can therefore sometimes be expected.

Table 5.3. The reaction strengths of serotyping *Salmonella* sers. Poona and Typhimurium after culture for 18 h in BPW media containing various peptones as sole nutrient source. Strengths are indicated with × being inconclusive, ×× reaction barely visible, ××× weak reaction, ×××× easily visible reaction, ××××× strong reaction. TSB and NB reactions are shown for comparison.

Media		Detectability of Flagellar (H) Antigen					
		Salmonella ser. Poona			*Salmonella* ser. Typhimurium		
		Z	1	6	i	1	2
Casein	1	××	××	××××	××	××	×××
	2	×	××	××	×	×	××
	3	×××	×××	×××	××	××	×××
	4	×	××	××	×	×	××
	5	××	××	××	×	××	××
	6	××	××	××	××	×	××
	7	××	××	×××	×	××	×××
	8	×	×	×	×	×	××
Gelatin	1	××	××	××	×	×	××
	2	××	××	××	××	××	×
	3	×	×	×	×	×	××
	4	××	××	××	××	××	××
	5	××	××	××	×	×	××
Meat	1	××	××	××	××	×	××
	2	××	××	×××	×	×	××
	3	××	××	××××	××	××	×××
	4	××	××	××	×	××	××
	5	××	××	××	××	××	××
	6	××	×	××××	××	×	×××
Soy	1	××	××	×××××	××	××	×××××
	2	×××	××	××××	×××	×	×××
	3	××	××	×××××	×	××	××××
	4	××	××	××××	××	××	××××
	5	××	××	××××	××	××	××××
Yeast	1	×	××	×××××	×	××	×××××
	2	×	×	×××××	××	×	×××××
	3	×	×	×××××	×	××	××××
	4	×××	×××	×××××	××	×××	××××
TSB		×	×	××	××	××	×××
NB		××	××	××××	××	××	××××

5.4.3. Serotyping of the flagellar (H) antigen in laboratory-made TSB

The serological analysis of *Salmonella* in laboratory-made TSB generated stronger reactions to the H antisera in comparison to those produced after growth in BPW (Table 5.4). Soy peptone has been previously shown to improve agglutination (Chapter 5.4.2); this was attributed to increased numbers of flagella thus presenting higher quantities of H antigen and so a stronger serological reaction in response to the addition of the antisera. Again the two serovars produced different strengths of serological reaction with *Salmonella* ser. Typhimurium observed to present less H antigen in comparison with *Salmonella* ser. Poona.

Casein peptone is included in the TSB recipe at a concentration of 15 g l^{-1}, whereas soy is only 5 g l^{-1}; therefore the soy peptone was not present at a high enough concentration to disguise the effect of the casein peptone. This was observed as *Salmonella* ser. Poona generated low quantities of H antigen in BPW, particularly in BPW medium, where the peptone was C8. Similarly, where C8 was the casein constituent of TSB, *Salmonella* ser. Poona produced relatively weak agglutination reactions, compared with other casein peptones, after growth with BPW and TSB (whereby the constituent casein peptones were C1 and C3). Different soy peptones as constituents of TSB did not have an obvious effect on serological analysis.

Salmonella ser. Typhimurium exhibited similar reactions irrespective of peptone manufacturer. There were few exceptions: firstly peptone C2, which proved to be a poor nutrient as a constituent of TSB in addition to BPW; organisms cultured in peptone C8-based TSB generated relatively strong agglutination reactions in response to the H antisera, this was in contrast to the organisms grown in C8-based BPW, which was noticeably poorer.

Table 5.4. The reaction strengths of serotyping *Salmonella* sers. Poona and Typhimurium after culture for 18 h in laboratory-made TSB media containing various casein and soya peptones as nutrient sources. Strengths are indicated with × being inconclusive, ×× reaction barely visible, ××× weak reaction, ×××× easily visible reaction, ××××× strong reaction.

Peptone Components of TSB Recipe		Detectability of Flagellar (H) Antigen					
		Salmonella ser. Poona			*Salmonella* ser. Typhimurium		
Casein	Soy	Z	1	6	i	1	2
C1	S1	××	××	××××	×	×	×××
	S2	×××	××	×××××	×	××	×××
	S3	××	××	×××××	×××	×××	××××
	S4	××	××	××××	×	×	×××
	S5	××	××	××××	×	×	××××
C2	S1	××	××	×××	×	×	××××
	S2	××	××	×××	×	×	××××
	S3	×	××	×××	×	×	×××
	S4	×	××	×××	×	×	×××
	S5	×	××	×××	×	×	××××
C3	S1	××	×××	××××	×	×	××××
	S2	×××	××	××××	××	×	×××
	S3	×××	××	××××	×××	×	××××
	S4	××	××	××××	××	×	×××
	S5	××	×××	×××	××	×	×××
C4	S1	×	××	×××	×××	×	××××
	S2	××	××	×××	×××	×	××××
	S3	×	××	×××	×××	×	××××
	S4	××	××	×××	×	×	×××
	S5	×	××	×××	×	×	×××
C5	S1	××	××	×××	×××	×	××××
	S2	×××	××	×××	×××	×	××××
	S3	××	×	×××	×××	×	××××
	S4	××	××	×××	×	×	×××
	S5	××	××	×××	×××	×	××××
C6	S1	××	××	×××	×××	×	××××
	S2	×××	××	×××	×××	×××	××××
	S3	××	××	××	×××	×	××××
	S4	××	××	×××	×	×	×××
	S5	××	××	××	×	×	××××
C7	S1	××	××	×××	×××	××	××××
	S2	××	××	×××	××	×	×××
	S3	××	××	××××	×××	×	××××
	S4	×××	××	×××	××××	×	××××
	S5	××	××	×××	××××	×	××××
C8	S1	××	×	××	××	×	×××
	S2	××	×	××	××	×	××××
	S3	××	×	×××	××××	×	××××
	S4	××	××	××	×××	×	××××
	S5	××	×	××	××××	××	×××××

5.4.4. Effect of peptone on the serological analysis of *Salmonella* serovars

It was clear from the serological data, that TSB was more reliable for serological analysis and subsequent identification of *Salmonella* serovars. It was also observed that *Salmonella* ser. Typhimurium generated lower quantities of respective H antigen on the media assessed in comparison with *Salmonella* ser. Poona; of particular note the quantity of shared phase-two H1 antigen, produced by both serovars, after growth on the various TSB media. Considerable differences in strength of the agglutination reactions were noticed, in many cases the tests were inconclusive (i.e. no agglutination was seen) in some casein and gelatin media, yet in soy or yeast media the antisera generated a strong reaction. These effects were not as apparent in the medium TSB, however, differences were still observed.

Within the public health setting it is of great importance to accurately identify the *Salmonella* serovar responsible for an outbreak. Therefore it is likely that use of inadequate media leading to incomplete or incorrect serological identification will hinder investigators. In certain TSB media (e.g. those containing C2 casein and S3 and S4 soy peptones) *Salmonella* ser. Typhimurium may only have positive identification of the phase 2 H2 antigen. As the O antigen was proven to be unaffected by the different peptone sources, this will give an incomplete serological identification; however if all H antigens were absent, this would lead the investigator to the inaccurate conclusion that the organism was aflagellate.

The observations of serological differences dependent on peptone as a constituent of the growth medium can be explained by the results obtained in Chapter 4. The H antigen is presented on the flagellar filament, therefore if flagella are not synthesized, the H antigen will not be expressed; this consequently leads to added antisera not producing

agglutination as there is no H antigen present. Briefly, causes of aflagellate *Salmonella* which were identified in Chapter 4 include low tyrosine concentrations (the condition being exacerbated by high concentrations of competitors for uptake into the organism such as phenylalanine and histidine), low concentrations of carbohydrate or high concentrations of NaCl.

5.5. Antibiotic susceptibility

Antibiotic susceptibility was shown to be greatly affected by the variable composition of peptones obtained from different sources and manufacturers, as a constituent of the medium BPA. Growth of the Enterobacteria on certain peptones induced resistance to particular antibiotics, although the same strain was sensitive to these same antibiotics after culture on a different peptone-based medium. This has serious implications for the public health sector, as sensitivity tests may sometimes be inaccurate depending on the medium used.

The test species, *S.* ser. Typhimurium, is used throughout this chapter, along with an *E. coli* control strain; *S.* ser. Poona and *Staphylococcus aureus* were only of limited use (i.e. in order to confirm that the observations were not due to abnormal biochemistry of the *S.* ser. Typhimurium and *E. coli*). It should be noted that the axes for each figure may be different as the responses of different organisms and different antibiotics are not directly comparable.

5.5.1. Effect of peptone on the activity of aminoglycosides

The activities of the aminoglycoside antibiotics (amikacin, gentamicin and streptomycin) on *Escherichia coli, Salmonella* ser. Typhimurium, *Salmonella* ser. Poona and

Staphylococcus aureus after growth on batches of BPA media which contained different peptones were recorded using the disk diffusion method.

5.5.1.1. Effect of peptone on activity of amikacin

The peptone used as a component of the medium BPA affected the susceptibility of different organisms to amikacin to varying degrees. Noticeable differences in zones of inhibition surrounding the amikacin disk were recorded for *E. coli* and *S. aureus*, whereas *S.* ser. Typhimurium and *S.* ser. Poona displayed less variation in susceptibility depending on the peptone used. None of the organisms investigated in this work displayed resistance to amikacin; for *S. aureus* an inhibition zone diameter (IZD) of ≤ 18 mm indicated medium induced resistance: this was ≤ 19 mm for the Enterobacteria.

5.5.1.1.1. Effect of peptone on the susceptibility of *Escherichia coli* to amikacin

Peptone used as a component of the medium BPA considerably affected the activity of amikacin on *Escherichia coli* ($P = 0.000$) as shown in Figure 5.1. Inhibition zone diameters measured ranged from 26 mm, noted with peptones C6, M1, S3, Y2 and Y3, to 34 ± 3 mm after growth on G3 peptone-based medium.

Peptones originating from a casein, gelatin or meat source produced diverse values whereas those from a soy or yeast category appeared relatively more consistent; both soy and yeast peptones generated colonies which were less susceptible to amikacin.

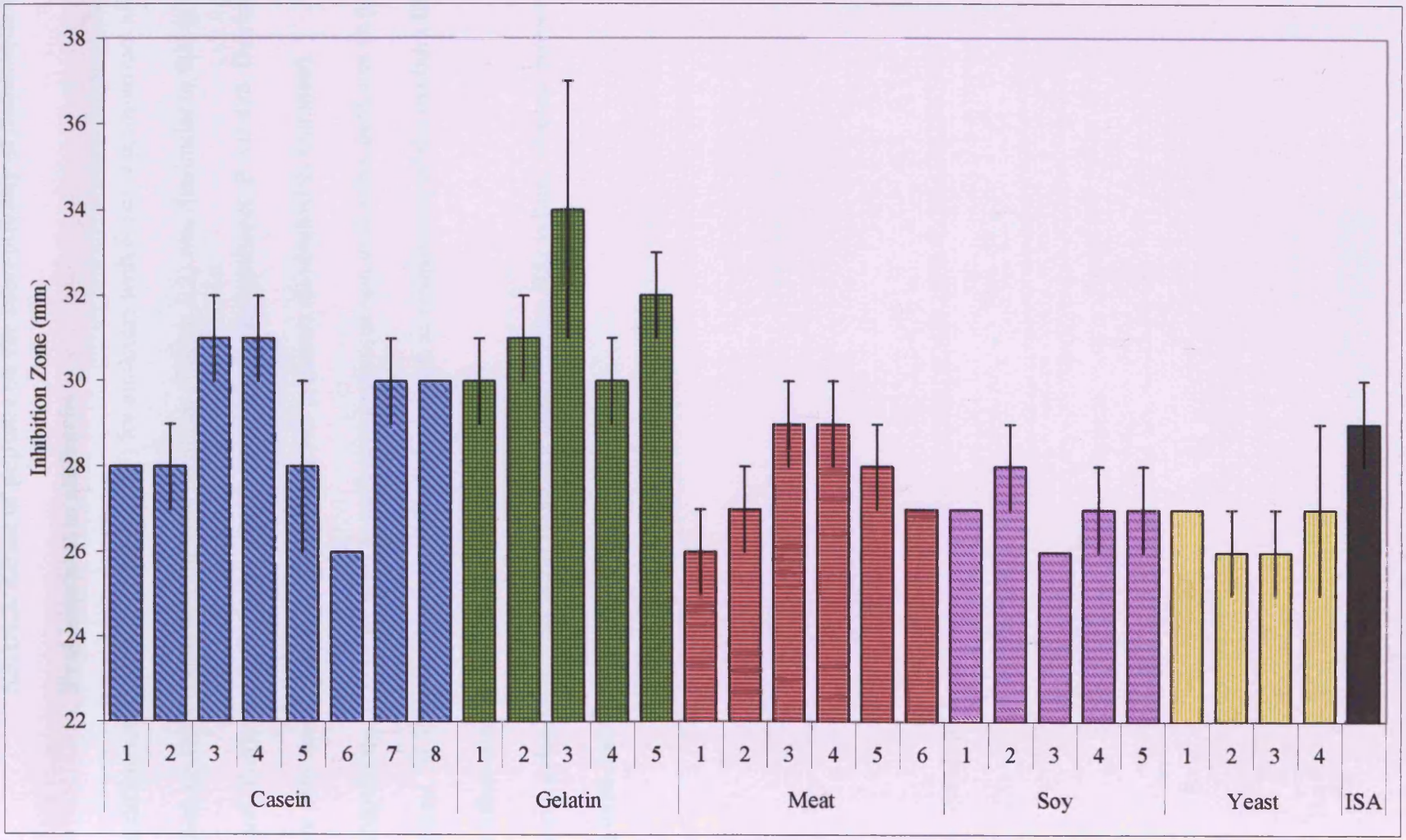

Figure 5.1. Activity of amikacin (30 µg), recorded as the diameter of the zone of inhibition, on *Escherichia coli* after growth on buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.1.1.2. Effect of peptone on the susceptibility of *Salmonella* ser. Typhimurium to amikacin

The difference in inhibition zones (IZD) for amikacin with *S.* ser. Typhimurium after growth on BPA containing variable peptones (Figure 5.2) was dissimilar to that noted for *E. coli*. Furthermore the data were also statistically insignificant: P = 0.438. Deviations were noticeable, growth on G1, G3 and M5 peptones all resulted in increased susceptibility (IZD of 23 mm) to amikacin in comparison with other peptones (e.g. M4 peptone, 28 ± 1 mm); however different peptones as constituents of the medium BPA did not affect antibiotic susceptibility tests.

Casein, gelatin and meat again produced more diverse IZD values, whereas soy and yeast peptones were more consistent by comparison.

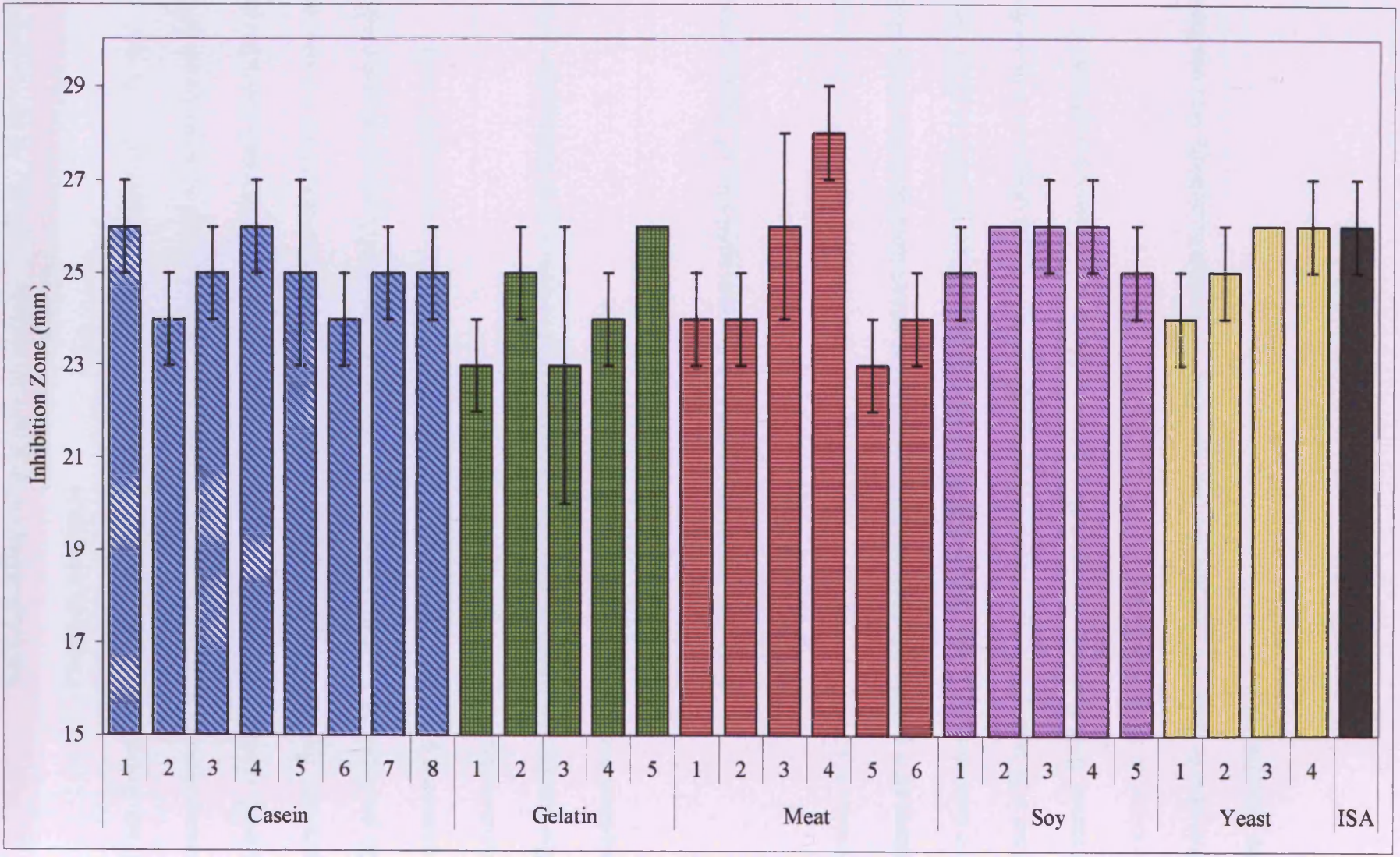

Figure 5.2. Activity of amikacin (30 µg), recorded as the diameter of the zone of inhibition, on *Salmonella* ser. Typhimurium after growth on buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.1.1.3. Effect of peptone on the susceptibility of *Salmonella* ser. Poona to amikacin

S. ser. Poona, in contrast to *S.* ser. Typhimurium, exhibited statistically different susceptibilities to amikacin where different peptones were used as constituents of the growth medium (P = 0.001). However the range of values for IZD was 5 mm for the two serovars: for *S.* ser. Poona peptone Y1 generated an IZD diameter of 29 ± 1 mm, whereas G1 produced 34 ± 1 mm (Figure 5.3 a). Therefore growth of *S.* ser. Poona on medium containing peptone G1 produced organisms which were more susceptible to the antimicrobial.

The statistical differences between the two organisms were due to decreased numbers of peptones tested with *S.* ser. Poona.

5.5.1.1.4. Effect of peptone on the susceptibility of *Staphylococcus aureus* to amikacin

Amikacin activity on *Staphylococcus aureus* was considerably affected by the peptone source (P = 0.023). Media containing peptones C1 or M5 were sensitive to the antibiotic at a diameter of 27 mm, but growth of the organism on S5 based BPA medium was especially susceptible, generating a notable IZD of 40 ± 1 mm (Figure 5.3 b). In this instance, the soy peptone generated *S. aureus* colonies which were exceptionally susceptible to this antibiotic.

This clearly demonstrated that the peptone effect on antibiotic activity was not limited to the Enterobacteria.

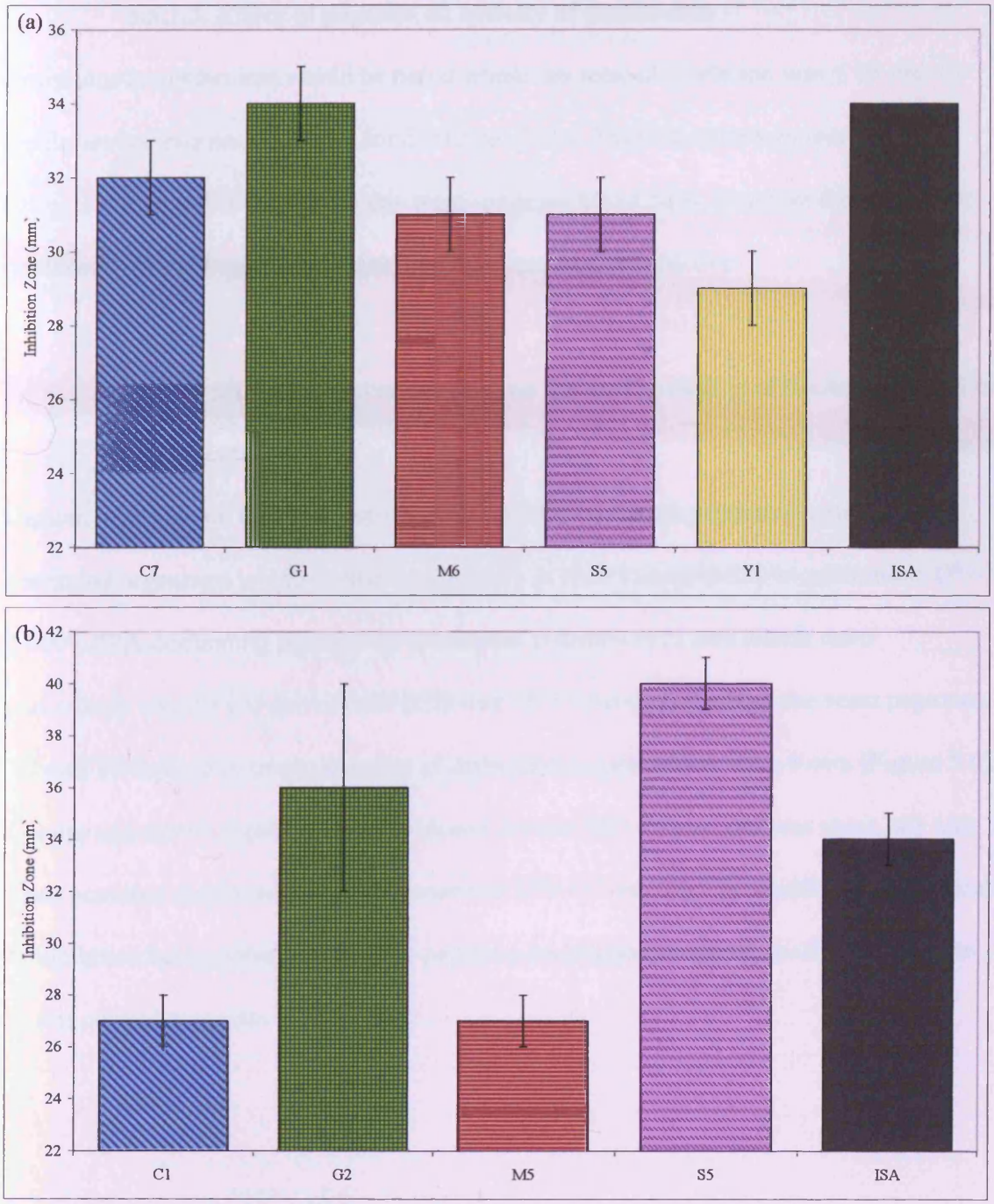

Figure 5.3. Activity of amikacin (30 µg), recorded as the diameter of the zone of inhibition, on (a) *Salmonella* ser. Poona and (b) *Staphylococcus aureus* after growth on selected buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.1.2. Effect of peptone on activity of gentamicin

Resistance to gentamicin would be noted where the zone of inhibition was ≤ 19 mm for the Enterobacteria and ≤ 18 mm for *S. aureus*. *S.* ser. Typhimurium was recorded as being sensitive to gentamicin on one meat- peptone based BPA, however the remaining peptones cultured organisms which were all classified as sensitive.

5.5.1.2.1. Effect of peptone on the susceptibility of *Escherichia coli* to gentamicin

Growth of *E. coli* on BPA medium which contained different peptone constituents generated organisms which varied significantly in their susceptibility to gentamicin (P = 0.000). BPA-containing peptone G3 resulted in colonies of *E. coli* which were particularly sensitive to gentamicin (IZD was 36 ± 0 mm); in contrast the yeast peptones Y2 and Y3 both gave rise to colonies of decreased susceptibility: 22 ± 0 mm (Figure 5.4). Gelatin and casein peptones again produced diverse IZD values, whereas meat, soy and yeast peptones displayed relatively consistent IZD values. Despite significant differences in inhibition being noted, none of the peptones investigated induced levels of resistance in this control organism.

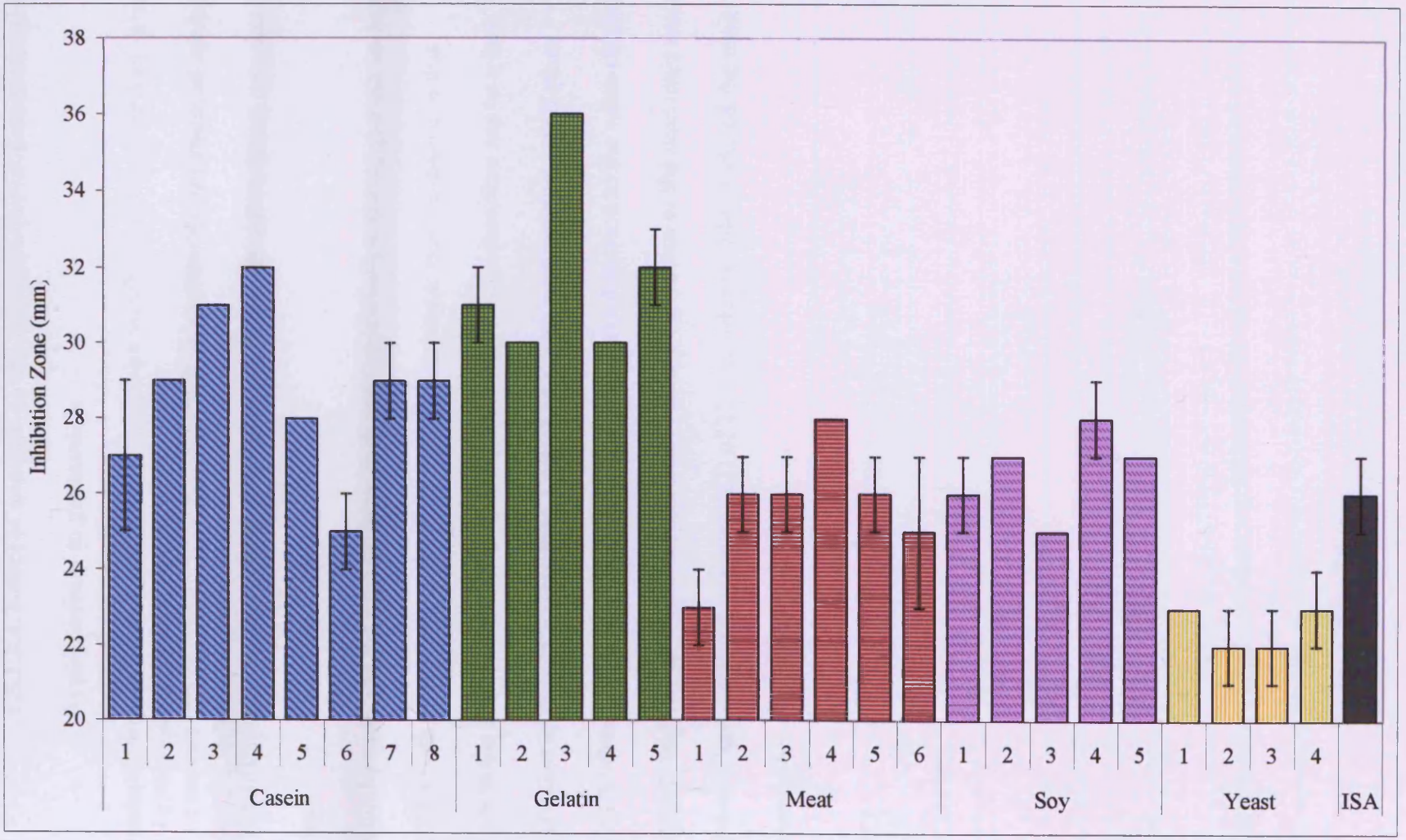

Figure 5.4. Activity of gentamicin (10 µg), recorded as the diameter of the zone of inhibition, on *Escherichia coli* after growth on buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.1.2.2. Effect of peptone on the susceptibility of *Salmonella* ser. Typhimurium to gentamicin

The susceptibility of *S.* ser. Typhimurium to gentamicin after growth on BPA of variable peptone content was noticeably different (Figure 5.5); statistically the zones of inhibition were not different ($P = 0.057$) due a small sample size ($n = 3$) and variation between the replicates.

Of particular note was the susceptibility of *S.* ser. Typhimurium during growth on M3 based BPA with an IZD value of 14 ± 2 mm. This indicates that resistance of this organism to the antibiotic was medium-dependent and would therefore not be a true representation of the strain resistance. Growth of *S.* ser. Typhimurium on all other peptones produced values which were more similar in comparison to the value obtained from growth on M3 peptone. The peptones Y1 and Y4 gave rise to the next most resistant colonies of *S.* ser. Typhimurium with IZD of 21 mm, whereas M4 produced the most sensitive at 28 ± 1 mm.

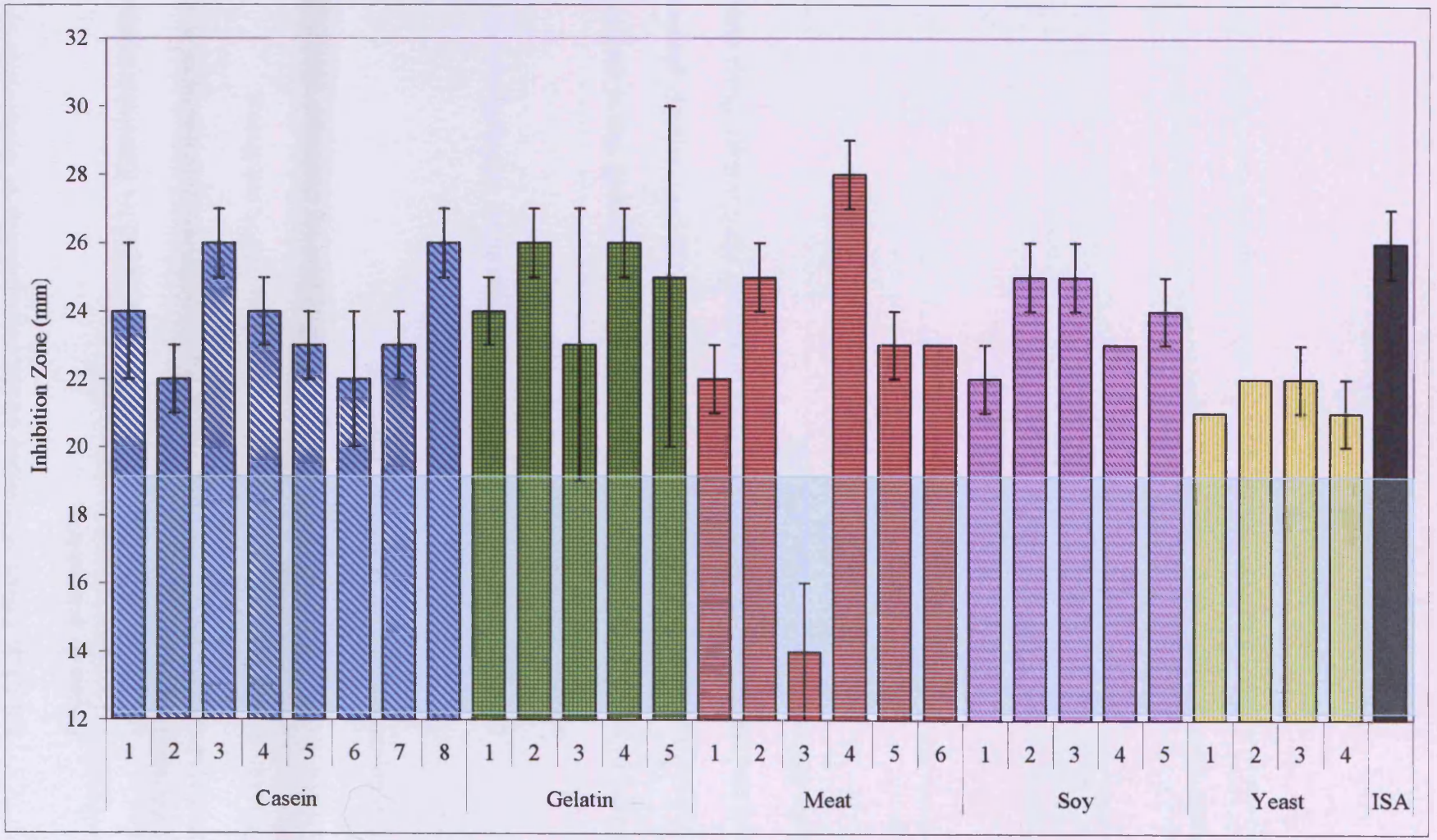

Figure 5.5. Activity of gentamicin (10 µg), recorded as the diameter of the zone of inhibition, on *Salmonella* ser. Typhimurium after growth on buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Below 19 mm, indicated by the transparent blue zone, would confirm resistance. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.1.2.3. Effect of peptone on the susceptibility of *Salmonella* ser. Poona to gentamicin

Cultivation of *S.* ser. Poona on medium containing various peptones generated organisms which produced statistically different IZD values for the antibiotic gentamicin (P = 0.000). The range of IZD was 7 mm, with Y1 peptone-based BPA exhibiting comparatively resistant organisms (26 ± 1 mm) and C7 displaying increased sensitivity to gentamicin dependent on medium (33 ± 1 mm).

5.5.1.2.4. Effect of peptone on the susceptibility of *Staphylococcus aureus* to gentamicin

The susceptibility of *S. aureus* to gentamicin corresponds closely with that of amikacin: gelatin and soy again resulted in especially high values for IZD (37 ± 0 mm), whereas casein and meat both generate relatively resistant organisms (27 ± 0 mm). These data are statistically different with a P value of 0.000.

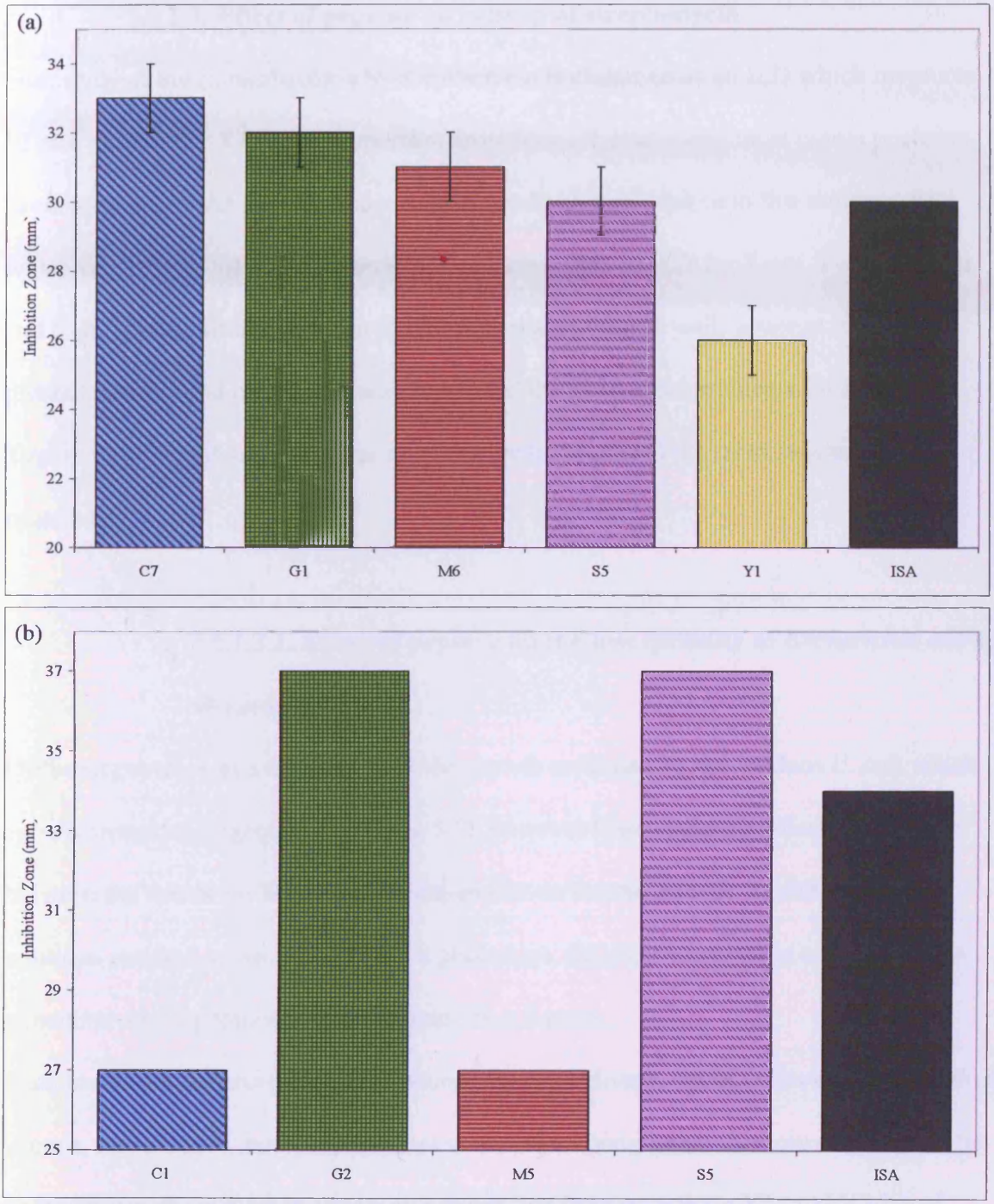

Figure 5.6. Activity of gentamicin (10 µg), recorded as the diameter of the zone of inhibition, on (a) *Salmonella* ser. Poona and (b) *Staphylococcus aureus* after growth on selected buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.1.3. Effect of peptone on activity of streptomycin

Sensitivity of the Enterobacteria to streptomycin is classified as an IZD which measures 12 mm or less. For *S.* ser. Typhimurium growth on all gelatin and most casein peptones, as constituents of the growth medium BPA, resulted in resistance to this antimicrobial which was wholly medium-dependent. The rank orders of IZD for *S.* ser. Typhimurium and *S.* ser. Poona after growth on identical media correlated well, whereas *E. coli* was distinct. Gelatin and casein peptones generated the most resistant lawns for *S.* ser. Typhimurium; contrastingly these identical media produced the most sensitive *E. coli* organisms.

5.5.1.3.1. Effect of peptone on the susceptibility of *Escherichia coli* to streptomycin

Different peptones as a constituent of the growth medium did not produce *E. coli* which were resistant to streptomycin (Figure 5.7), however there was a significant difference between the values for IZD obtained dependent on the medium ($P = 0.000$). Gelatin peptones resulted in the most sensitive phenotype, the most susceptible organisms were generated on G3 peptone-based medium (28 ± 2 mm).

Both casein and gelatin peptones produced the most diverse set of IZD values within their groups; those from a meat, soy or yeast source were comparatively more consistent. The organisms most resistant to streptomycin resulted from growth on Y2- and Y3-based medium (18 ± 0 mm).

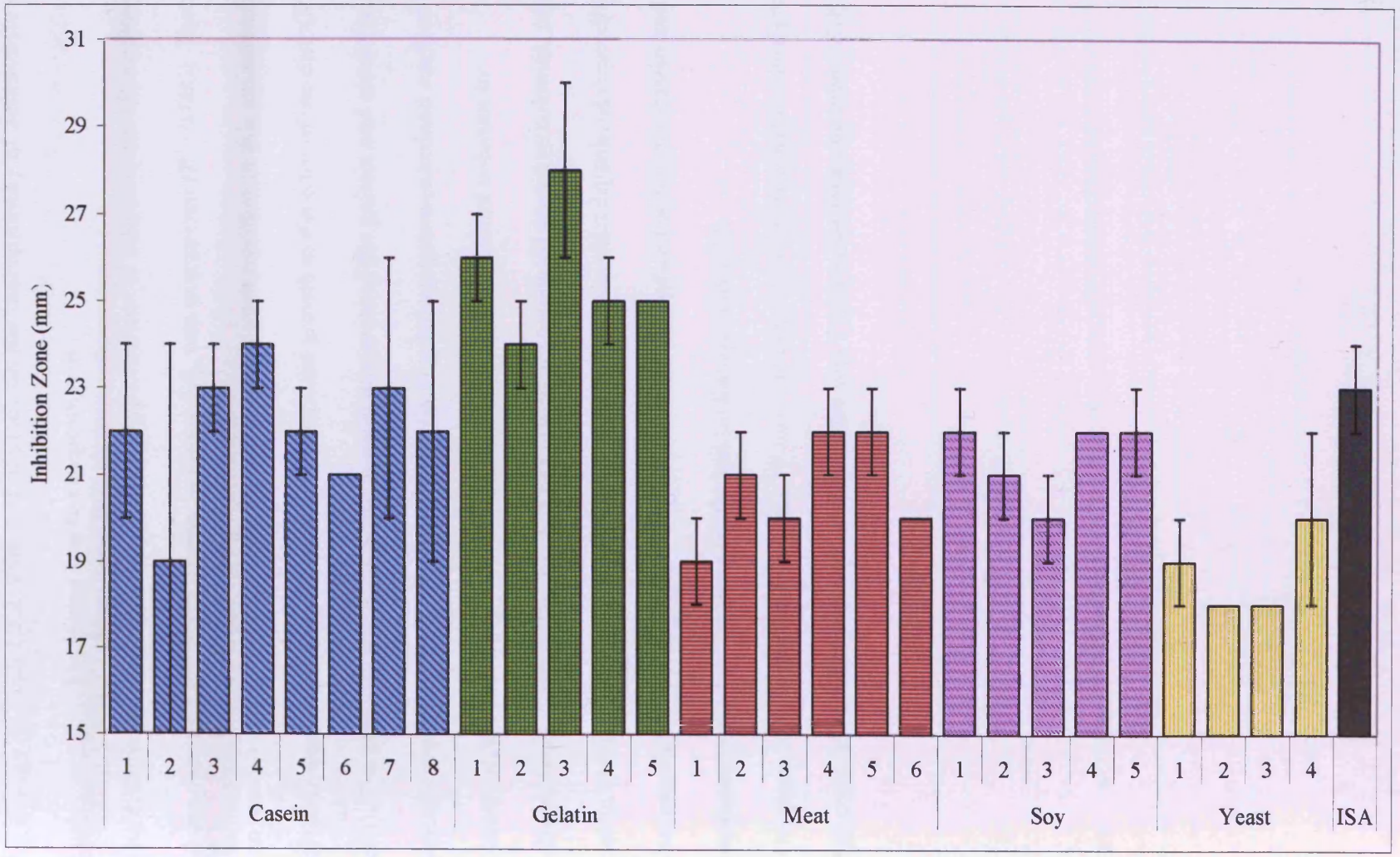

Figure 5.7. Activity of streptomycin (10 μg), recorded as the diameter of the zone of inhibition, on *Escherichia coli* after growth on buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.1.3.2. Effect of peptone on the susceptibility of *Salmonella* ser. Typhimurium to streptomycin

The difference in susceptibility of *S.* ser. Typhimurium to streptomycin after cultivation on the various BPA media, shown in Figure 5.8, was exceptional (P = 0.000). Thirteen of the media investigated generated organisms which were resistant to this antimicrobial; six of these were completely resistant with confluent growth to the edge of the disk (IZD = 6 mm). The peptones which gave rise to resistance were of the gelatin and casein groups, together with one from a soy source (S5). Each gelatin peptone examined resulted in resistance; all casein peptones except C1 also produced resistant colonies to streptomycin. Meat and yeast peptones invariably generated sensitive colonies, whereas casein and soy peptones generated both sensitive and resistant organisms depending on the manufacturer of the peptone. Great diversity was noticed within the casein and meat peptone groups; this was also dependent on the manufacturer.

The range of IZD for this study was 19 mm, the most sensitive organisms were grown on M3 peptone-based medium (25 ± 2 mm, this was 7 mm wider than any other IZD).

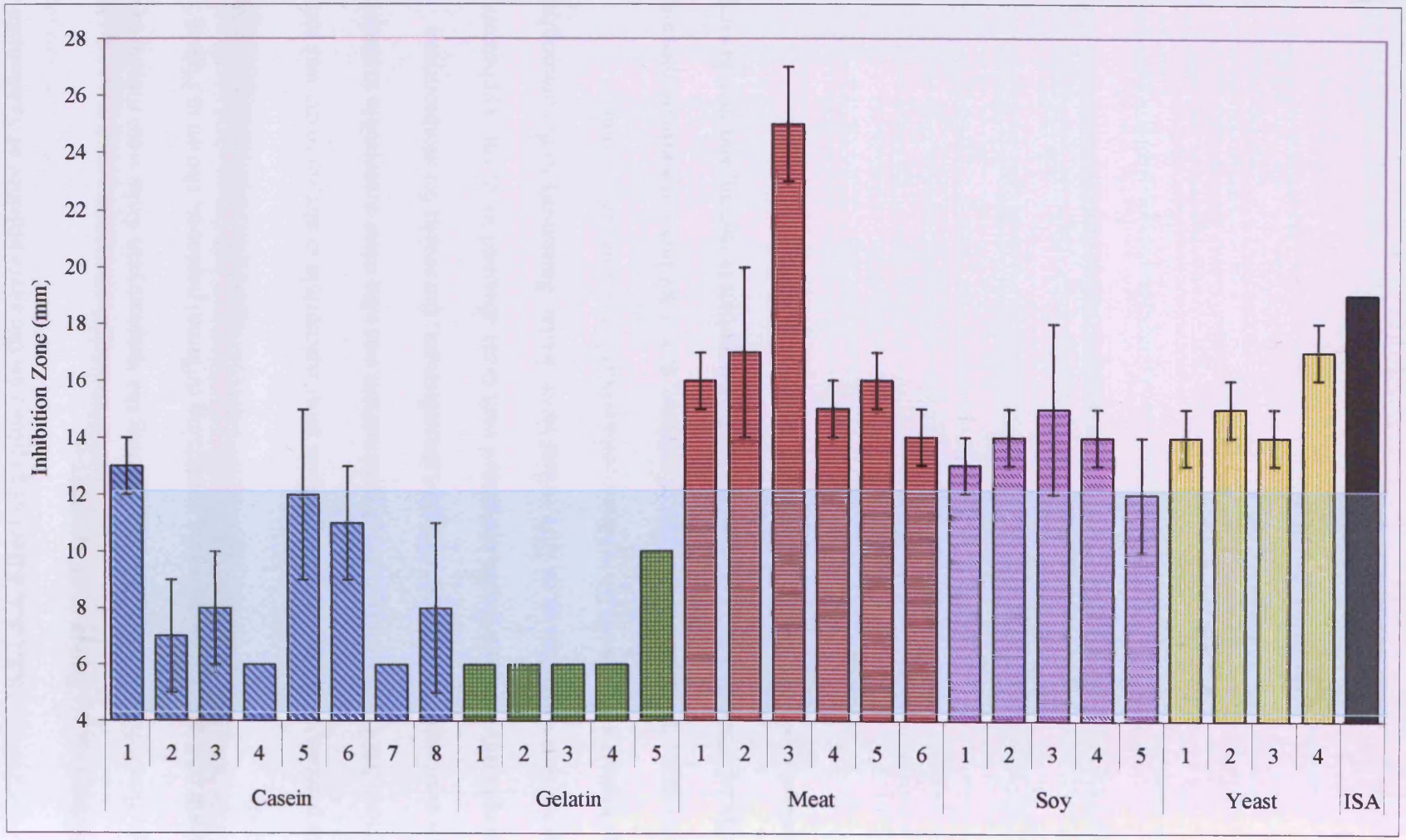

Figure 5.8. Activity of streptomycin (10 μg), recorded as the diameter of the zone of inhibition, on *Salmonella* ser. Typhimurium after growth on buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Below 12 mm, indicated by the transparent blue zone, would confirm resistance. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.1.3.3. Effect of peptone on the susceptibility of *Salmonella* ser. Poona to streptomycin

Significant differences in IZD surrounding the streptomycin disks were measured after growth of *S.* ser. Poona on media containing different peptones, shown in Figure 5.9 (P = 0.000).

The peptone which generated organisms most susceptible to streptomycin was meat peptone M6 (22 ± 1 mm). *S.* ser. Typhimurium was also most susceptible to streptomycin on a meat peptone, but from different manufacturer; the results for streptomycin susceptibility of *S.* ser. Poona correlated with those obtained for *S.* ser. Typhimurium in that the rank orders based on IZD values were similar. Sensitivity to the aminoglycoside was highest after growth on medium containing C7 peptone (18 ± 1 mm).

This again confirmed that the type of peptone, as a constituent of a growth medium, has a profound effect on the outcome of antibiotic susceptibility testing, and that this important conclusion was not limited to a specific serovar or species.

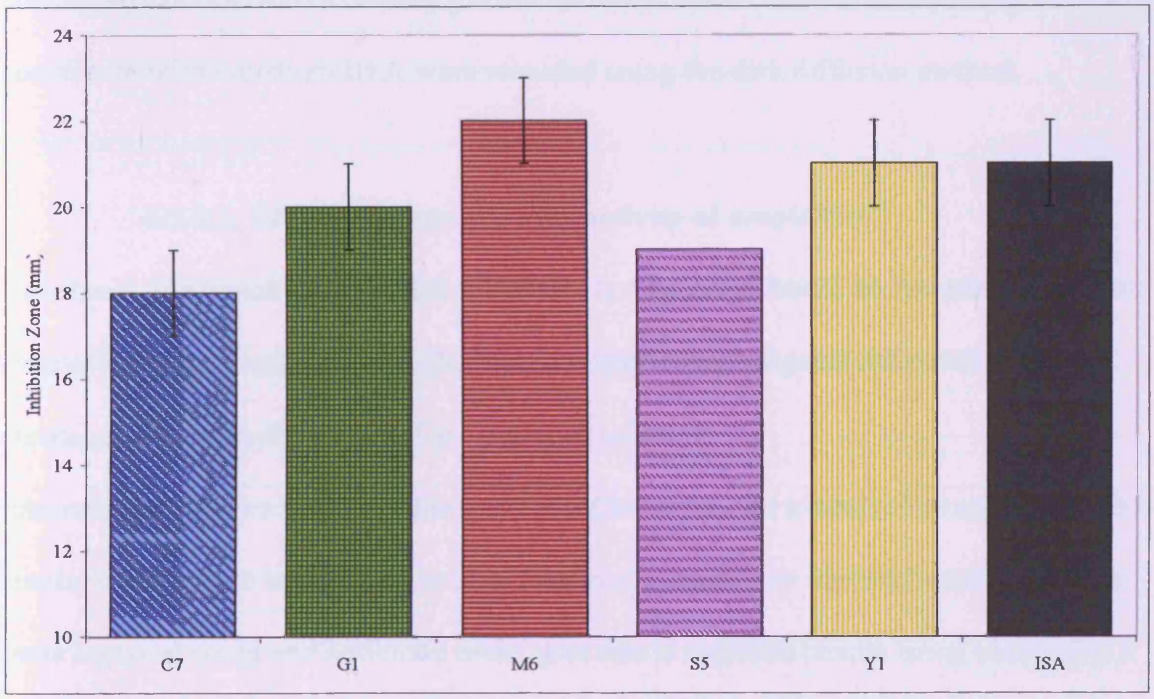

Figure 5.9. Activity of streptomycin (10 µg), recorded as the diameter of the zone of inhibition, on *Salmonella* ser. Poona after growth on selected buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.2. Effect of peptone on the activity of other antibiotic groups

Other groups of antibiotics were examined for possible peptone antagonism to the antimicrobial activity of the respective compound on selected organisms. Those investigated were ampicillin (penicillin or β-lactam), cephalothin (cephalosporin), colistin sulphate (polymixin) and ciprofloxacin (quinolone).

The activities of the these antibiotics, towards several or all of the following organisms, were recorded for *Escherichia coli, Salmonella* ser. Typhimurium, *Salmonella* ser. Poona and *Staphylococcus aureus* during growth on media which contain different peptones as a constituent of the medium BPA were recorded using the disk diffusion method.

5.5.2.1. Effect of peptone on the activity of ampicillin

Resistance of a member of the Enterobacteria to ampicillin would be recorded where the zone of inhibition was ≤ 13 mm; the bacterial species investigated did not display resistance after growth on any of the peptones selected.

Interestingly, relative susceptibilities of the different species to ampicillin appeared to be similar when grown on identical media. Previously, testing on identical media using the same bacterial strain and antibiotic often gave rise to opposite results being obtained (i.e. susceptibility of *E. coli* to streptomycin was greater on gelatin based media; in contrast *S.* ser. Typhimurium was most resistant to streptomycin after growth on gelatin media).

5.5.2.1.1. Effect of peptones on the susceptibility of *Escherichia coli* to ampicillin

Susceptibility of *E. coli* to ampicillin was not significantly altered after growth on media containing different peptones ($P = 0.231$); The IZD for ampicillin with this organism,

grown on BPA containing selected peptones, are shown in Figure 5. 10 a. None of the media tested induced resistance to the antimicrobial; C7 and G1 peptones generated the most susceptible colonies (IZD of 32 mm) whereas Y1 and S2 cultivated organisms were relatively more resistant (27 mm).

5.5.2.1.2. Effect of peptones on the susceptibility of *Salmonella* ser. Typhimurium to ampicillin

Growth of *S.* ser. Typhimurium on the differing BPA, generated ampicillin susceptibility results which were not significantly different (P = 0.200); shown in Figure 5. 10 b. The results were congruent with those generated by *E. coli* on identical media, with C7 and G1 again resulting in growth of the most sensitive organisms (IZD of 31 mm) together with M6 (generating comparatively more resistant colonies, 28 mm). In addition, soy peptone S2 and yeast peptones Y1 and Y4 as constituents of the culture medium produced equally sensitive colonies and, peptone C2 gave rise to an identically resistant lawn of *S.* ser. Typhimurium.

In contrast however, yeast peptones (irrespective of manufacturer) consistently produced *S.* ser. Typhimurium which were relatively highly susceptible to ampicillin compared with the other categories of peptone tested.

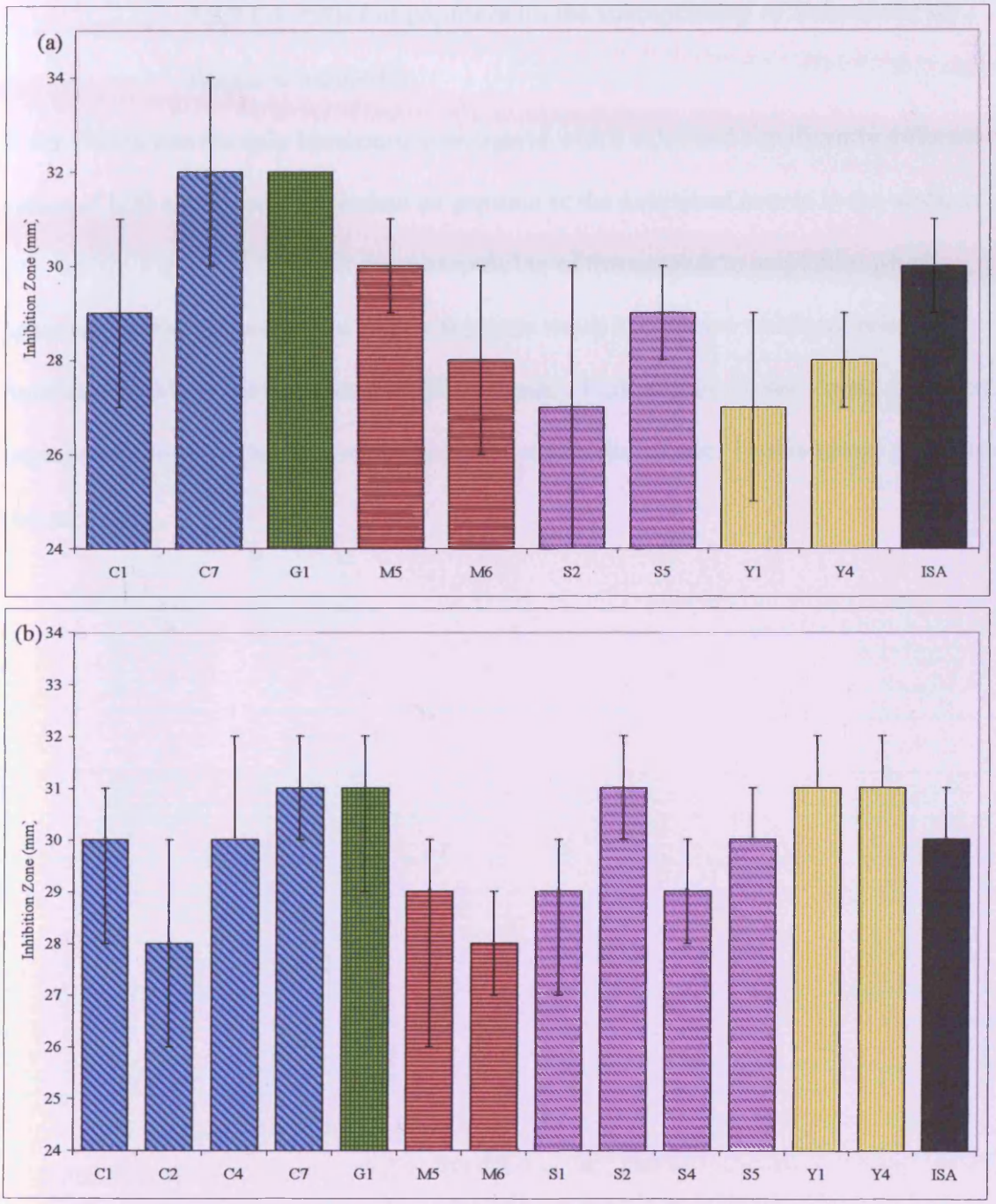

Figure 5.10. Activity of ampicillin (10 μg), recorded as the diameter of the zone of inhibition, on (a) *Escherichia coli* and (b) *Salmonella* ser. Typhimurium after growth on selected buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.2.1.3. Effect of peptones on the susceptibility of *Salmonella* ser. Poona to ampicillin

S. ser. Poona was the only bacterium investigated which exhibited significantly different values of IZD which were dependent on peptone as the nutritional source in the medium ($P = 0.002$). Figure 5.11 depicts the susceptibility of this serovar to ampicillin which again shows that the casein and gelatin peptones result in colonies which are relatively more susceptible to the antimicrobial (IZD 36 mm). Furthermore, *S.* ser. Poona grown on peptone M6 displays the most resistance (32 mm), as does *S.* ser. Typhimurium grown on this peptone.

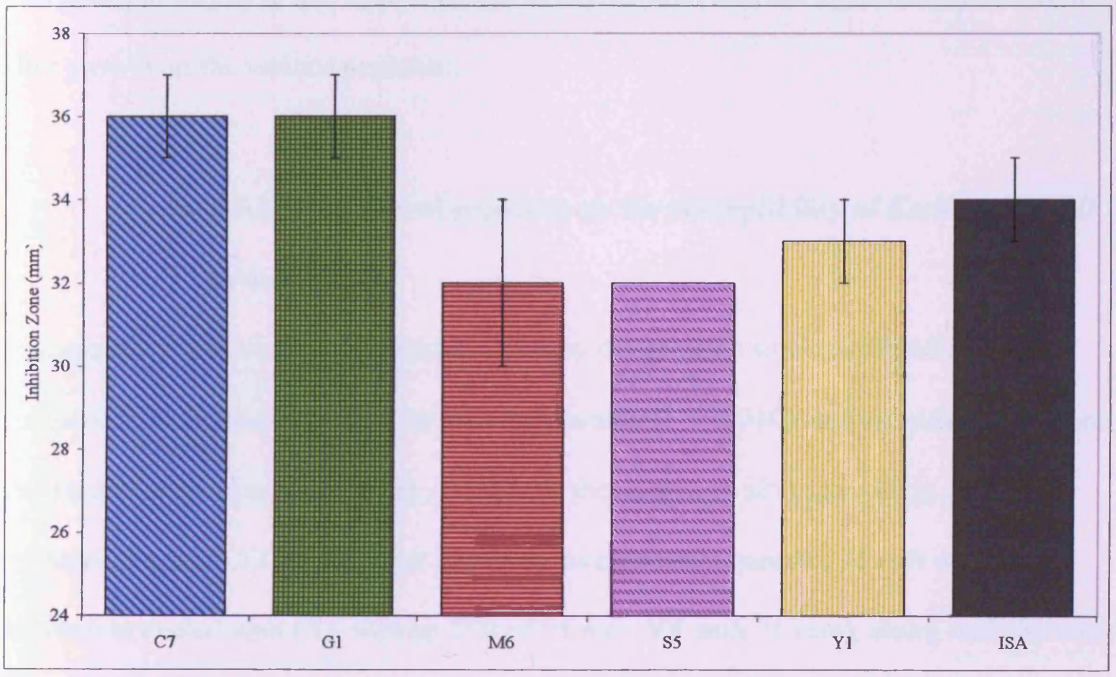

Figure 5.11. Activity of ampicillin (10 µg), recorded as the diameter of the zone of inhibition, on *Salmonella* ser. Poona after growth on selected buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.2.2. Effect of peptone on the activity of cephalothin

For Enterobacteria, an IZD of less than or equal to 26 mm would indicate clinical resistance of the organism. This was noted to be the case for *E. coli* on certain meat and yeast peptones; in contrast, growth of this organism on casein, gelatin, soy and some meat peptones gave rise to resulting colonies being sensitive to cephalothin. Both *E. coli* and *S.* ser. Poona exhibited significant differences in IZD dependent on the growth medium constituent peptone; however *S.* ser. Poona did not show resistance to this antimicrobial. The susceptibility of *S.* ser. Typhimurium to cephalothin was not significantly different after growth on the various peptones.

5.5.2.2.1. Effect of peptones on the susceptibility of *Escherichia coli* to cephalothin

Susceptibility of *E. coli* to cephalothin whereby the peptone source differed as a component of the medium BPA varied considerably ($P = 0.010$) and, of great importance, this control strain was found to be resistant to the antibiotic after growth on certain peptones (Figure 5.12 a). All yeast peptones investigated generated *E. coli* which were resistant to cephalothin (Y1 with an IZD of 24 mm, Y4 with 26 mm), along with the meat peptones M1 (IZD 24 mm) and M6 (26 mm). Another two meat peptones examined cultured *E. coli* which were sensitive to the antimicrobial: M4 and M5 respectively. Peptones C7 and G1 again gave rise to the most sensitive colonies (IZD 33 mm); all soy peptones generated sensitive organisms.

5.5.2.2.2. Effect of peptones on the susceptibility of *Salmonella* ser. Typhimurium to cephalothin

In contrast to *E. coli*, the antibiotic activity of cephalothin against *S.* ser. Typhimurium was not significantly different (P = 0.352) depending on the peptone used as a constituent of the growth medium (Figure 5. 12 b). The IZD values obtained after growth on the differing media were evenly spaced between those media which produced the most sensitive organisms (S2; 35 ± 1 mm), and those that yielded the most resistant (peptones G1 and C2 with 28 ± 1 mm each).

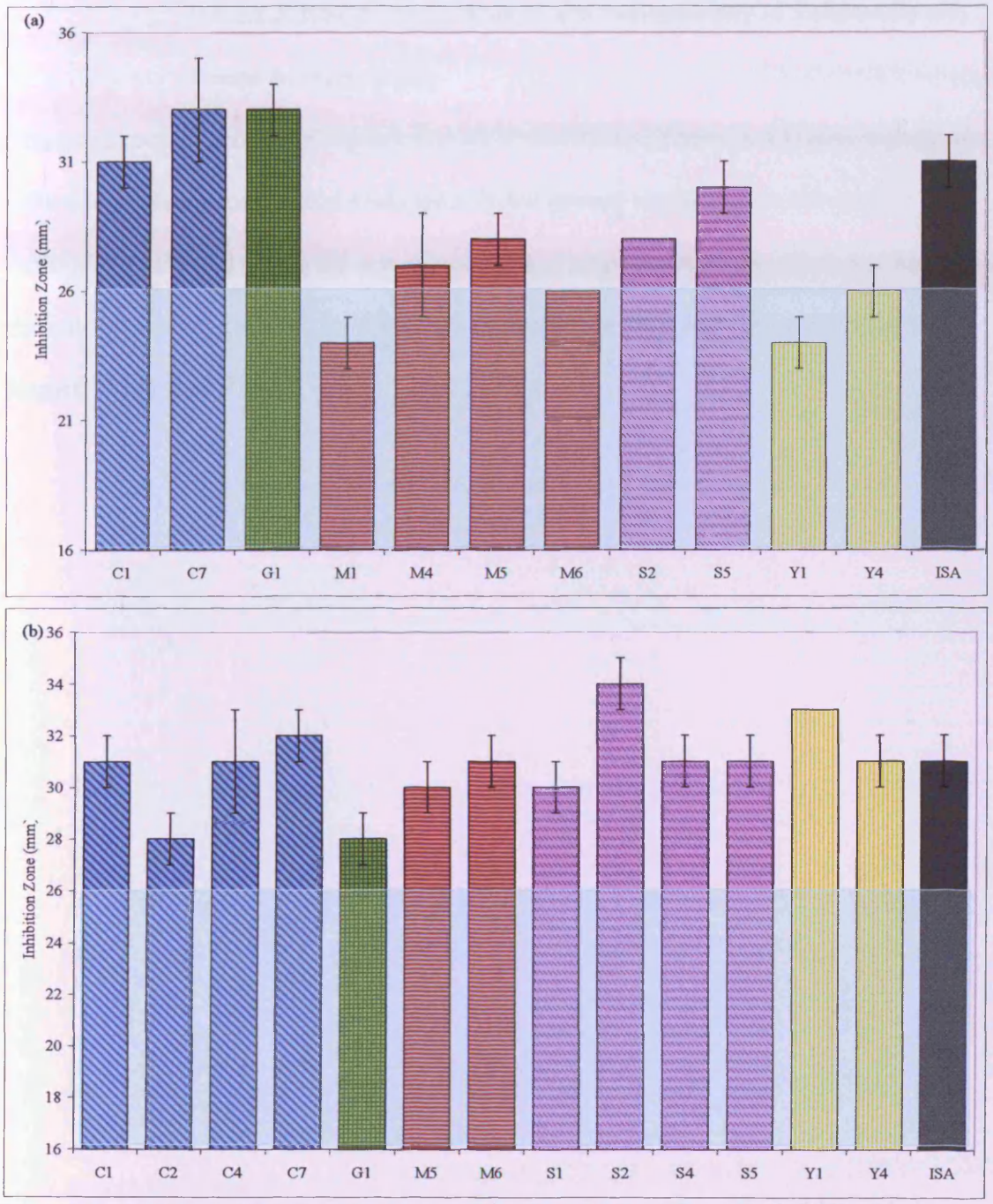

Figure 5.12. Activity of cephalothin (30 µg), recorded as the diameter of the zone of inhibition, on (a) *Escherichia coli* and (b) *Salmonella* ser. Typhimurium after growth on selected buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Transparent blue zones indicate resistance ≤ 26 mm. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.2.2.3. Effect of peptones on the susceptibility of *Salmonella* ser. Poona to cephalothin

The antimicrobial activity of cephalothin on *S.* ser. Poona (Figure 5. 13) gave significant differences when peptone, as a constituent of the growth medium, was altered (P = 0.009). The differing media did not induce clinical resistance and the effect was not as dramatic as with *E. coli*, the smallest IZD being 32 mm (peptones M6 and Y1) and the largest, 35 ± 1 mm (C7).

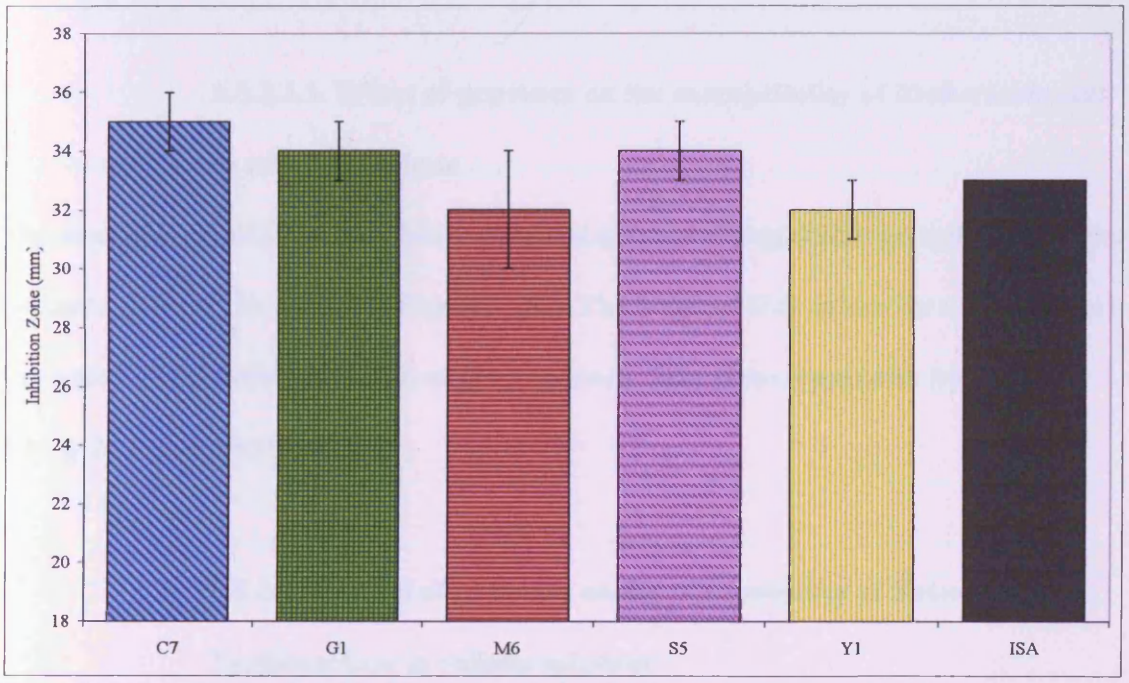

Figure 5.13. Activity of cephalothin (30 µg), recorded as the diameter of the zone of inhibition, on *Salmonella* ser. Poona after growth on selected buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.2.3. Effect of peptone on the activity of colistin sulphate

An inhibition zone of less than or equal to 14 mm would indicate that a member of the Enterobacteriaceae was resistant to colistin sulphate. None of the species examined, in any of the media tested, were found to be resistant to this compound. The IZD values for *E. coli* after growth on the different peptone-based media were not statistically different, whereas *S.* ser. Typhimurium did produce significantly different values when cultured on variable peptone sources.

5.5.2.3.1. Effect of peptones on the susceptibility of *Escherichia coli* to colistin sulphate

The susceptibility of *E. coli* to colistin sulphate was not changed after growth on different peptones ($P = 0.120$); shown in Figure 5.14 a. The range of IZD values for the organisms cultivated on the different growth media was small: 20 ± 0 mm (peptones M6, S2 and Y1) to 24 ± 1 mm (G1 peptone).

5.5.2.3.2. Effect of peptones on the susceptibility of *Salmonella* ser. Typhimurium to colistin sulphate

S. ser. Typhimurium was found to have growth medium dependent susceptibility to colistin sulphate ($P = 0.023$); indicated by Figure 5.14 b which displays organisms grown on casein or gelatin peptone-based BPA to be more sensitive to the antibiotic, in comparison with colonies cultured on meat, soy or yeast peptones. Those with increased resistance (meat, soy or yeast cultivated) generated a mean IZD of 20 mm; the most

sensitive *S.* ser. Typhimurium colonies were grown on C4 peptone-based BPA: 26 ± 2 mm.

5.5.2.4. Effect of peptone on the activity of ciprofloxacin against *Staphylococcus aureus*

S. aureus was found not to be resistant to ciprofloxacin after culture in any of the peptones investigated in this work (≤ 17 mm would indicate resistance); shown in Figure 5. 15. However, the IZD values were significantly different (P = 0.000) with gelatin peptone-based medium generating the most susceptible organisms (IZD of 38 ± 2 mm) and casein peptone giving rise to the most resistant (30 ± 1 mm). Meat peptone produced colonies of a similar sensitivity to casein, whereas soy peptones were similar to gelatin.

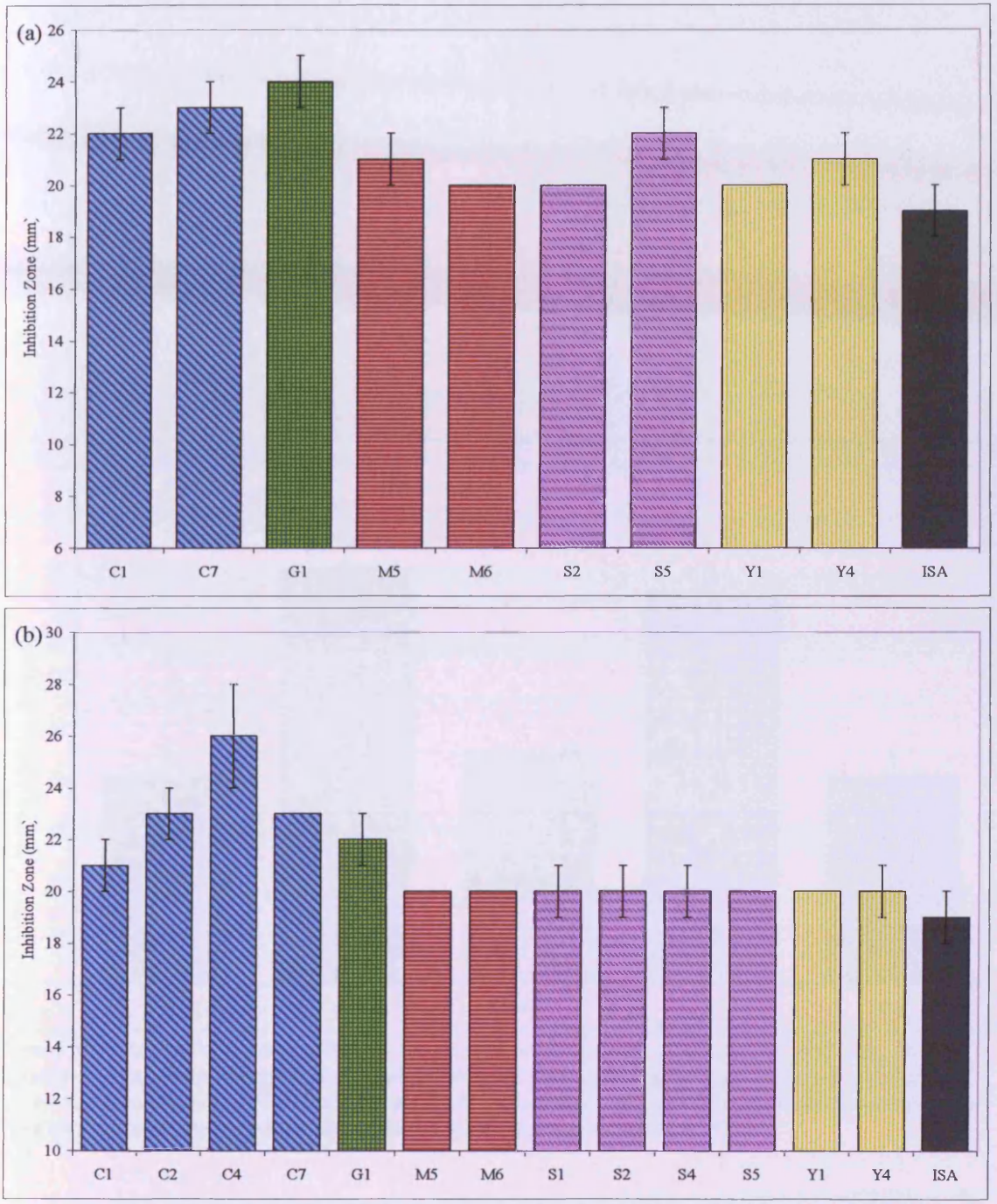

Figure 5.14. Activity of colistin sulphate (25 µg), recorded as the diameter of the zone of inhibition, on (a) *Escherichia coli* and (b) *Salmonella* ser. Typhimurium after growth on selected buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Data bars represent the mean, and error bars the standard deviation where n = 3.

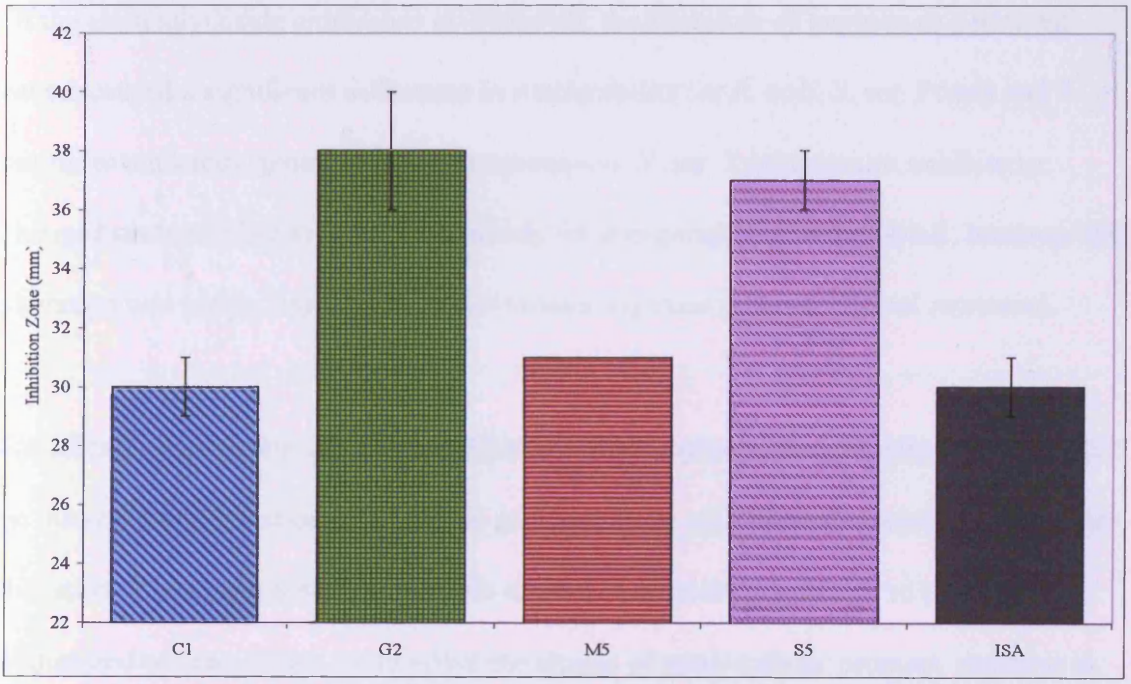

Figure 5.15. Activity of ciprofloxacin (1 μg), recorded as the diameter of the zone of inhibition, on *Staphylococcus aureus* after growth on selected buffered peptone agar containing peptones of different source and manufacturer. A commercially available Iso-sensitest agar (ISA) was included for comparison. Data bars represent the mean, and error bars the standard deviation where n = 3.

5.5.3. Discussion of different peptones used in BPA and their effect on susceptibility to antibiotics

The data described clearly show that peptones as constituents of the growth media considerably affect subsequent antibiotic susceptibility tests. These effects vary with species or strain of organism, the antibiotic being investigated and the peptone used.

5.5.3.1. Effect of peptone on aminoglycoside antibiotics

Of the aminoglycoside antibiotics investigated, the alteration of peptone as a nutrient source caused a significant difference in susceptibility for *E. coli*, *S.* ser. Poona and *S. aureus* to amikacin, gentamicin and streptomycin; *S.* ser. Typhimurium exhibited a changed susceptibility to streptomycin only for this group of antimicrobials, however this alteration was highly significant, as the various peptones induced clinical resistance.

Specifically, *E. coli* was more susceptible to all aminoglycoside antibiotics after growth on the acid hydrolysed casein peptones in comparison with those prepared by enzymatic digestion. It has been noted that the salt content is considerably higher in peptones hydrolysed with acid: this could affect the charge of extra-cellular proteins, resulting in increased binding of the antimicrobial compound and consequently increased susceptibility. The opposite effect was found by Toama *et al.* (1978): here, the addition of inorganic ions decreased the activity of the antibiotics studied.

E. coli also showed a trend whereby those organisms grown on M1 peptone-based media (M1 being meat extract) were always more resistant to the aminoglycoside antibiotics in comparison with *E. coli* cultured on any other meat source peptones. Meat extract is not

prepared by the same method as meat peptones, and the difference in this methodology would affect the peptone composition; this may affect the susceptibility of subsequent cultures.

Similar trends were noticeable for both soy and yeast peptones: soy peptone S3 always produced colonies more resistant to the aminoglycosides relative to the other soy peptones. Yeast peptones Y2 and Y3 generated more resistant *E. coli* compared with peptones Y1 and Y4. In contrast, gelatin peptones always gave rise to sensitive colonies of *E. coli*; peptone G3 especially generated highly susceptible organisms. Gelatin peptones gave considerably slower generation times and lower yields of *E. coli* in comparison to growth on other peptone sources (Chapter 3.4.3); this unusually slow growth may increase susceptibility of the organism to the antibiotic. Lower numbers of cells would be present on the gelatin medium, whilst the concentration of antibiotic was constant for each test; therefore in gelatin media there would be a higher number of antibiotic molecules present for each cell, resulting in increased susceptibility. In contrast, where high cell numbers were attained on yeast media there would be fewer antibiotic molecules per cell, resulting in a relatively more resistant bacterial lawn. Again this shows the diverse composition of peptones despite originating from the same biological source. These unknown distinctions greatly affect susceptibility tests for aminoglycosides with *E.coli*: this is of considerable importance as this strain is recorded as the control strain for these tests.

S. ser. Typhimurium displayed susceptibility zones to all aminoglycosides which were similar and consistent for all soy and yeast peptones, again highlighting that a higher

yield of organisms present on the surface of the culture media may effectively dilute the antibiotic. Within the category of meat peptones, subsequent colonies of *S.* ser. Typhimurium cultured on M3 peptone-based media were irregular with respect to their susceptibility profiles: a normal IZD (in comparison to cultures grown on other peptones) against amikacin, an IZD indicative of clinical resistant against gentamicin and, against streptomycin, organisms which were extremely susceptible. As identical strains were used and media were made using identical constituents, the remarkably different effects which peptone M3 had against the susceptibility of *S.* ser. Typhimurium to amikacin, gentamicin and streptomycin was striking.

The results for the activity of streptomycin against *S.* ser. Typhimurium whereby the culture medium was of variable peptone source or manufacture were interesting; complete resistance was induced for all organisms grown on gelatin peptone-based BPA, and also certain casein and soy peptones. This was in contrast to yeast and meat peptone cultures which were sensitive to the compound.

The limited tests performed utilizing *S.* ser. Poona and *S. aureus* did not show notable differences between rankings of media according to the inhibition zones obtained for the different aminoglycoside antibiotics. These species confirmed that the peptone induced effects were not limited to *E. coli* and *S.* ser. Typhimurium.

5.5.3.2. Effect of peptone on other antibiotic groups

Of the other antibiotic groups, which were not as vigorously investigated as the aminoglycosides were proven to be more susceptible to alteration of peptone source or

manufacturer, the activity of ampicillin was affected by the peptone component of the culture medium for growth of *S.* ser. Poona only. *S.* ser. Typhimurium and *E. coli* both appear to display noticeably different mean values for zones of inhibition but these are not statistically different: probably due to the high standard deviations from the mean. Gelatin peptone G1 produced the most sensitive cultures to the β-lactam antibiotic regardless of bacterial species, with the exceptions of *S.* ser. Typhimurium susceptibility to streptomycin, cephalothin and colistin sulphate. This was a general observation throughout this work.

Cephalothin was strikingly different (from the other non-aminoglycosidic compounds) in its activity against *E. coli* when susceptibility tests were performed on varying peptone sources; in some cases where the peptone utilized in the growth medium was sourced from a meat or yeast origin, clinical resistance was induced. *S.* ser. Poona showed similar trends to *E. coli*, however the differences in IZD values for susceptibility after growth on the various peptones were comparatively slight and no resistant cultures were recorded. In contrast to *S.* ser. Poona and *E. coli*, *S.* ser. Typhimurium exhibited no significant difference in sensitivity to the cephalosporin where peptone as a constituent of the growth medium was altered.

The activity of colistin sulphate dependent on the peptone ingredient of the culture medium was inconsistent between the two bacterial species considered: *S.* ser. Typhimurium susceptibility to the polymixin antimicrobial was significantly different when grown on the variable peptones, whereas the resistance of *E. coli* cultures did not

differ statistically. Unusually, *S.* ser. Typhimurium displayed consistent mean IZD values for peptones originating from meat, soy or yeast sources; the significant differences were due to the casein peptones which were shown once more to be diverse, despite their sharing an identical biological source.

Staphylococcus aureus grown on few selected peptones exhibited considerably different zones of inhibition around the ciprofloxacin disks, this again illustrates that antibiotics belonging to many chemical groups are greatly affected by the components of the growth media, and that this effect is not limited to a single bacterial species.

10

5.5.3.3. Hypotheses for the effect of peptone on antibiotic activity

Explanations for these occurrences are numerous; slow growth may limit uptake of the antibiotic and so allow an increased resistance, in contrast slow growth may result in a lower yield of organisms thus leading to a higher proportion of antibiotic compound molecules to organisms and consequently increased culture sensitivity. The opposite effects would also be possible with faster growth allowing a higher uptake rate of the antibiotic and therefore increased sensitivity. Where synthesis of cellular components is relatively slow (e.g. growth of *Salmonella* in gelatin-based BPW is markedly slower compared with cultures in a soy-based medium) production of antibiotic receptors, for

20 example penicillin binding proteins, would also be slow: thus limiting uptake of the antimicrobial compound.

Where efflux pumps are present in certain bacterial strains, if the growth peptone does not contain an effective energy source, the pumps will remain inactive, thus producing an

organism which, within its host would be resistant yet display a sensitive phenotype *in vitro*. As *E. coli* (NCTC 10418) is a control strain, and based on the assumption that the commercial ISA medium was quality controlled and therefore representative of an *in vivo* response (!), none of the organisms used for this investigation were resistant to the antimicrobials examined and so efflux pumps would not be present.

It is also possible that antagonism or synergism of antibiotic activity with peptone components occurred; antimicrobial compounds may already have been present during raw material harvest for the peptone manufacturing process resulting in a synergistic effect.

NaCl content of the peptone may affect subsequent susceptibility results, as cations (in particular divalent cations) can affect the binding of an antimicrobial (especially the penicillins, polymixins and aminoglycosides) to the external proteins (e.g. penicillin binding proteins). Thus casein peptones which were acid hydrolysed during the manufacturing process would contain substantially higher concentrations of salt and, noticeable in these results, these peptones generally resulted in relatively more susceptible cultures. The single exception to this comment was again *S.* ser. Typhimurium with streptomycin. This organism with this particular antibiotic produced such distinctive results (which were in contrast to all other organisms studied), could indicate that the peptones themselves act directly on the organism. For example, the gelatin or casein peptones may bind to an outer membrane protein specific to this serovar which prevents subsequent binding of the streptomycin; or these peptones may block the uptake channels resulting in no streptomycin being transported into the *Salmonella*. Either of these conditions would result in streptomycin resistance.

5. Public health analyses

This work emphasises the need for a consistent medium, such as ISA, to be used for susceptibility testing at all times. Use of different media can give misleading results. Inappropriate chemotherapy for the treatment of patients may be administered based on these results, thereby wasting time, money and resulting in the patient suffering prolonged or even fatal illness.

This thesis assesses only peptone, it can be expected that blood, serum, malt extract, agar and bile of differing source or manufacture produce similar discrepancies which greatly alter the outcome of scientific research or clinical tests.

5.5.4. Effect of different peptones and tryptones on laboratory-made ISA

The effects of differing peptones, as components of the complex medium ISA (the recommended medium for disk diffusion susceptibility tests), on the antibiotic susceptibility of subsequent cultures of *S.* ser. Typhimurium and a recognized control strain of *E. coli* were investigated. The BSAC standardized disc susceptibility testing method (version 3) (Andrews, 2004) indicates a widely used methodology for testing the antibiotic susceptibility of various organisms; it states necessary controls to include agar thickness and inoculation density. Control strains of bacteria were also noted, and in this work the *E. coli* (NCTC 10418) was used. The medium of choice for this standardized method was ISA; therefore laboratory made ISA was made up (details shown in Chapter 5.3.2.2) using selected peptones in order to examine the possible effects of this material as a constituent of the recommended complex media. A commercially available, dehydrated medium was included for comparison.

5.5.4.1. Effect of different ISA ingredients on antibacterial activity of amikacin

S. ser. Typhimurium cultures exhibited significantly different values for zones of inhibition ($P = 0.020$) where the sources of peptone used in the ISA medium differed in biological origin or source manufacturer (Figure 5.16. a). However, the variation in peptone did not generate clinical resistance to the antimicrobial.

The cultures were most resistant to amikacin on ISA 4 medium, for which the IZD value was 23 mm. ISA 2 medium gave rise to the most sensitive colonies of *S.* ser. Typhimurium. This recipe contained C4 and M4 peptones which both generated relatively sensitive cultures of *S.* ser. Typhimurium to amikacin when utilized as the sole

nutrient source of BPA medium (Figure 5.2). The commercially available medium gave rise to cultures which displayed a similar IZD value in comparison with the laboratory-made preparations.

The control strain of *E. coli* did not show statistically different IZD values (P = 0.070) after culture on the different laboratory prepared ISA media (Figure 5.16. b); data values obtained ranged from 25 ± 1 mm (ISA 3 and 6 media) to 27 ± 2 mm (ISA 5 medium). Nonetheless, trends were noticeable in that those ISA media containing casein peptone C6 (ISA media 3 and 6) showed slightly increased resistance to amikacin; peptone C6 as the sole nutritional compound in BPA medium generated especially resistant *E. coli* cultures in comparison with the other casein peptones (Figure 5.1). The BSAC method states that an acceptable zone range for the *E. coli* (NCTC 10418) strain on ISA with amikacin is 24-27 mm (Andrews, 2004). Therefore all laboratory-prepared ISA media fell within these guidelines; interestingly, the commercially available medium did not, giving an IZD value of 29 mm.

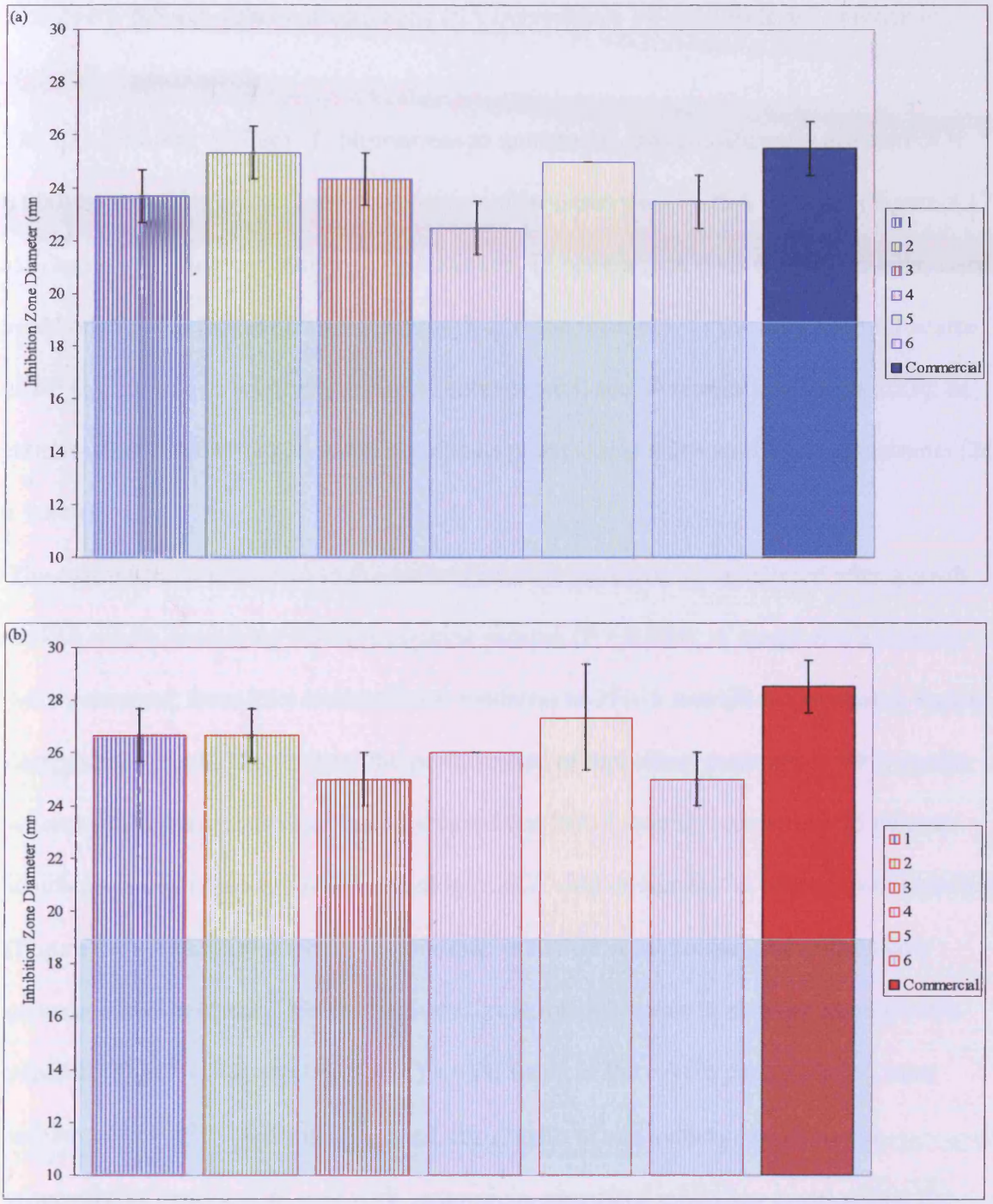

Figure 5.16. Inhibition zone diameters as a response to amikacin (30 µg) activity on (a) *Salmonella* ser. Typhimurium and (b) *Escherichia coli* grown on six different laboratory made Iso-Sensitest agar media whereby the tryptone and peptone ingredients varied. Below 19 mm, indicated by the transparent blue zone, would confirm resistance. Error bars represent standard deviation where n=3.

5.5.4.2. Effect of different ISA ingredients on antibacterial activity of gentamicin

The susceptibility of *S.* ser. Typhimurium to gentamicin was significantly affected (P = 0.000) by the different peptone constituents of laboratory-made ISA medium (Figure 5.17. a). The most resistant organisms to gentamicin, in comparison with the other media, were noted on ISA 4 medium (20 ± 1 mm): both of these peptones, as the only nutrient source of BPA, gave rise to relatively resistant cultures of *S.* ser. Typhimurium (Figure 5.5). In contrast, ISA 5 medium generated organisms of increased susceptibility to gentamicin (26 ± 1 mm).

The susceptibility of *E. coli* to the aminoglycoside gentamicin was altered after growth on ISA media containing variable peptone sources (P = 0.044). A range of IZD values were generated, from 22 ± 0 mm (ISA 6 medium) to 27 ± 1 mm (ISA 5 medium). Again comparisons can be drawn from the performance of individual peptones in BPA media, whereby these peptones were the only variables: ISA 6 medium contained C6 peptone which produced relatively resistant colonies of *E. coli* in contrast to other casein peptones (Figure 5.4). Although peptone G3 was also included in the recipe, and individually generated highly susceptible cultures of *E. coli*, the difference in concentration (casein peptone 11 g l^{-1}, other peptone 3 g l^{-1}) would result in the casein peptone being most influential. All ISA media investigated, for growth of and subsequent antibiotic susceptibility testing of *E. coli* with gentamicin, generated inhibition zones within the stated range: 21-27 mm (Andrews, 2004).

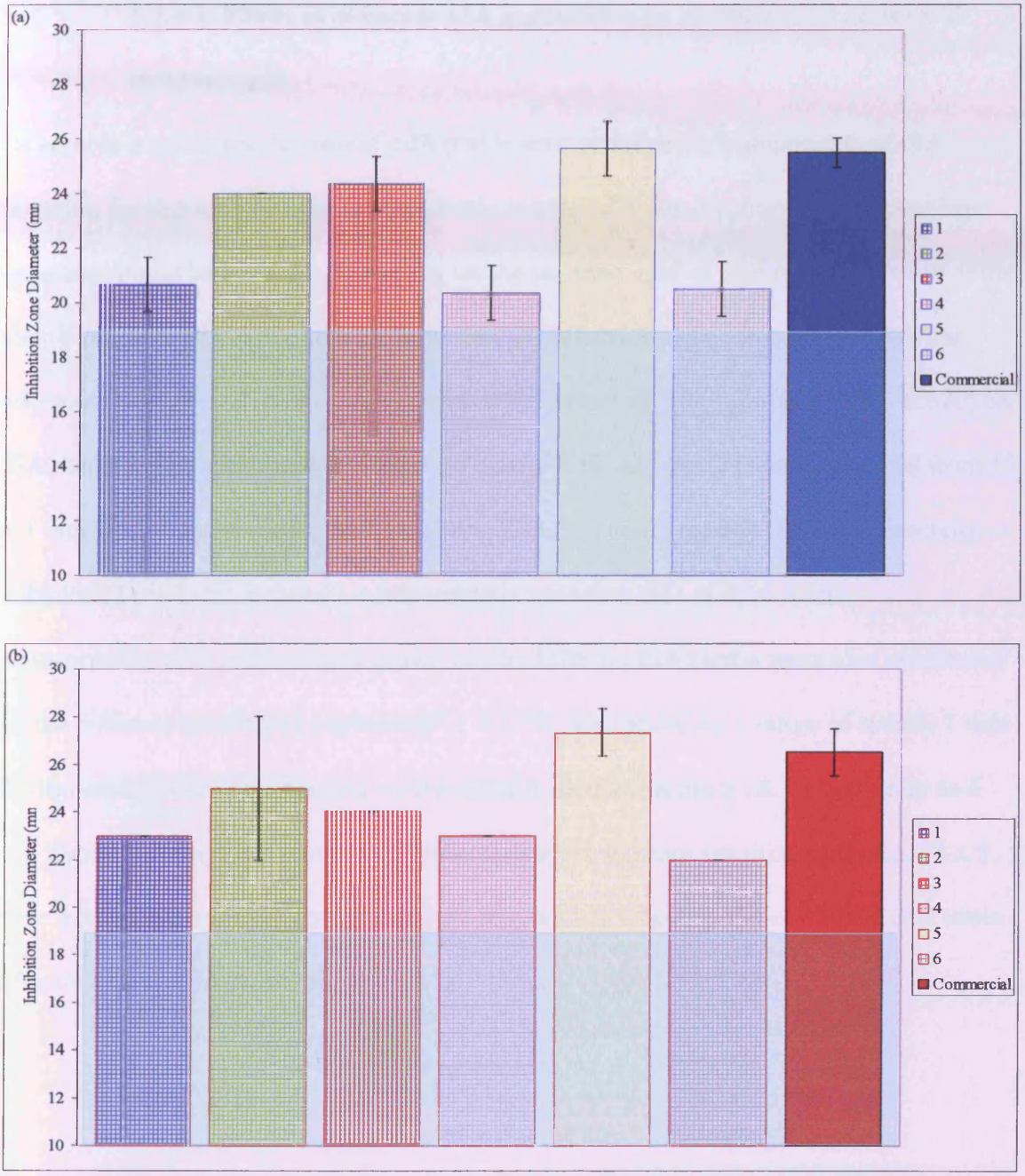

Figure 5.17. Inhibition zone diameters as a response to gentamicin (10 µg) activity on (a) *Salmonella* ser. Typhimurium and (b) *Escherichia coli* grown on six different laboratory made Iso-Sensitest agar media whereby the tryptone and peptone ingredients varied. Below 19 mm, indicated by the transparent blue zone, would confirm resistance. Error bars represent standard deviation where n=3.

5.5.4.3. Effect of different ISA ingredients on antibacterial activity of streptomycin

Of notable interest was the use of different source peptones, as components of ISA medium, for the streptomycin susceptibility testing of *S.* ser. Typhimurium. Complete resistance could be induced, depending on the peptone used as sole nutrient source of the simple medium BPA (Figure 5.8); however, in an increasingly complex medium the effect was suppressed to give no significant difference in inhibition zones ($P = 0.070$) on ISA media containing variable peptones (Figure 5.18. a). The IZD values ranged from 15 ± 1 mm (ISA 4 and 6 media) to 17 ± 1 mm (ISA 2, 3 and 5 media). The commercially dehydrated product produced a comparatively sensitive IZD of 19 ± 1 mm. Susceptibility of *E. coli* cultures grown on the differing ISA media were also unaffected by the different constituent peptones ($P = 0.139$), demonstrating a range of merely 1 mm for the inhibition zones obtained on the variable media (Figure 5.18. b). Similarly to *S.* ser. Typhimurium, the commercial product gave rise to more sensitive colonies: 23 ± 1 mm. Acceptable zone ranges for streptomycin, and its effect on the control *E. coli* strain after culture on ISA, were not given.

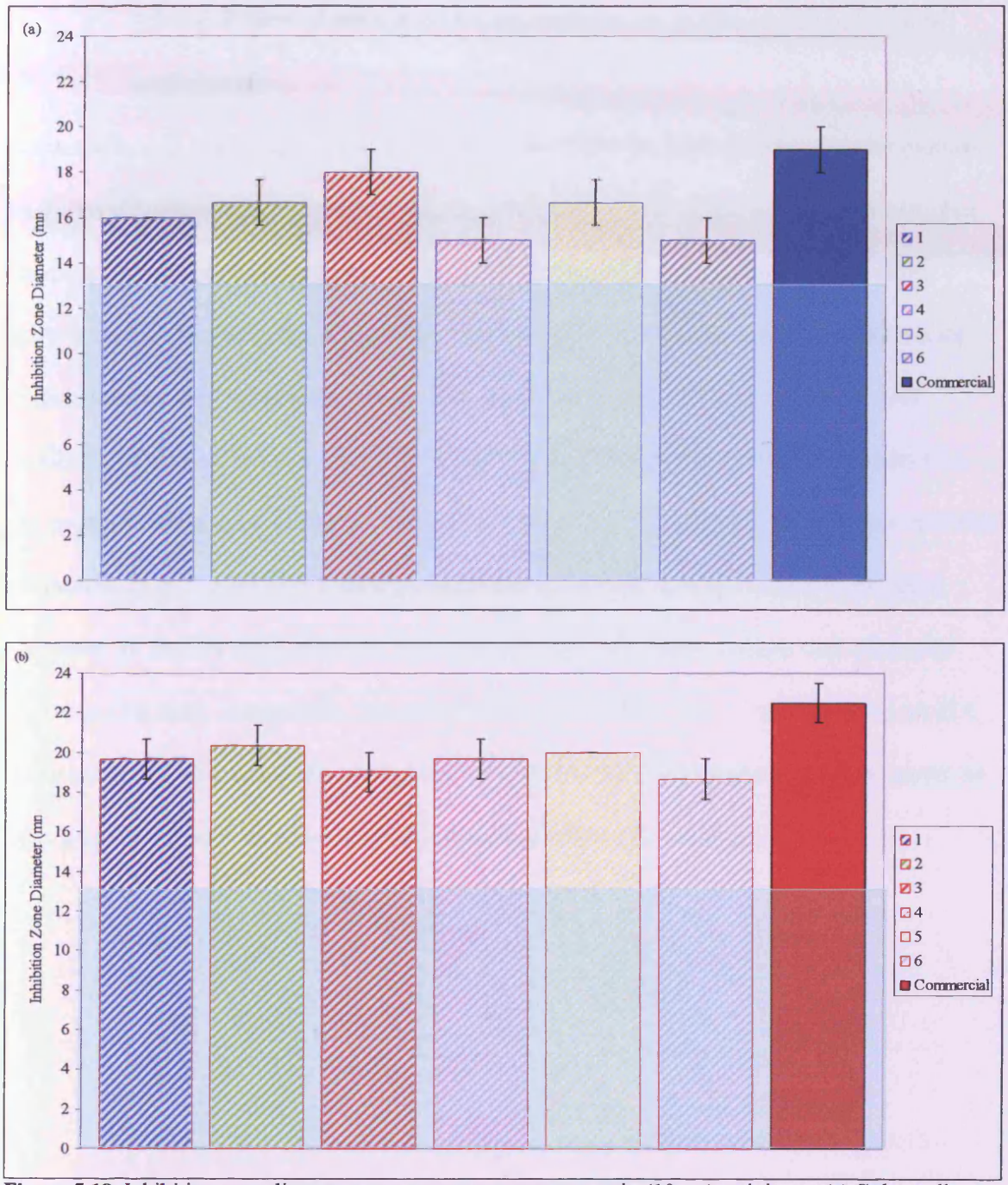

Figure 5.18. Inhibition zone diameters as a response to streptomycin (10 µg) activity on (a) *Salmonella* ser. Typhimurium and (b) *Escherichia coli* grown on six different laboratory-made Iso-Sensitest agar media whereby the tryptone and peptone ingredients varied. Below 12 mm, indicated by the transparent blue zone, would confirm resistance. Error bars represent standard deviation where n=3.

5.5.4.4. Effect of different ISA ingredients on antibacterial activity of cephalothin

Salmonella was not notably affected in its susceptibility to cephalothin whereby peptone, as the sole nutrient source of BPA, was varied and so examination in the more complex medium was deemed unnecessary. The susceptibility of *E. coli* to cephalothin after growth on ISA media containing different peptones was however tested as considerable distinctions existed when cultured on BPA medium (Figure 5.12. a). Again *E. coli* exhibited inhibition zones which were significantly different ($P = 0.026$) dependent on the peptone component of the ISA medium (Figure 5.19). Of greater importance was that all laboratory-prepared ISA media generated cultures which were clinically resistant (between 21 and 24 mm), whereas the commercially available medium was clinically sensitive (26 mm). Acceptable zone ranges for cephalothin with *E. coli* cultured on ISA were not given, and comparisons cannot be made to individual peptone performances as only a small subset was examined for non-aminoglycosidic antibiotics.

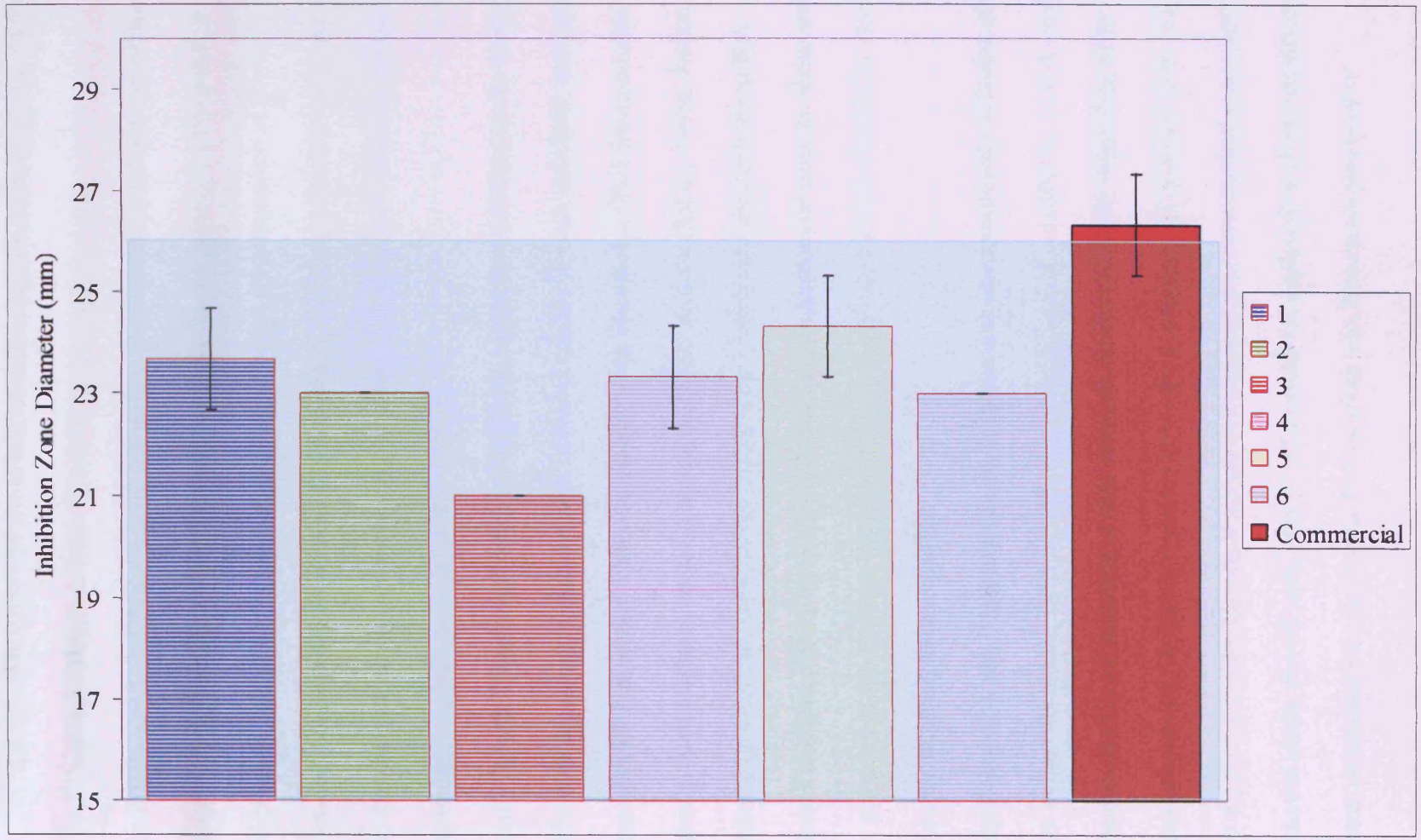

Figure 5.19. Inhibition zone diameters of *Escherichia coli* as a response to cephalothin (30 µg) grown on six different laboratory made Iso-Sensitest agar media whereby the tryptone and peptone ingredients varied. Below 26 mm, indicated by the transparent blue zone, confirmed resistance. Error bars represent standard deviation where n=3.

5.5.4.5. Discussion of the effect of different ISA ingredients on antibiotic susceptibility

Where differences in inhibition zones, dependent on the peptone component of the ISA medium, were noticeable the trends in susceptibility could usually be attributed to the casein peptone constituent: this observation was based on comparison of the respective peptone constituents of both BPA and ISA media. Casein was probably more influential due to this peptone being present at a significantly higher concentration in comparison to the secondary peptone included in the medium.

Generally though it was noted that very different values for inhibition zones were not recorded and the range of IZD for each bacterial species to each antibiotic were not as great as with BPA medium. This was thought to be the result of ISA being a more complex growth medium, i.e. glucose and its polymers provided an energy source, and trace elements vitamins, pyrimidines and purines were freely available from ISA medium. Therefore if the peptones were deficient for certain compounds, these were either provided in the medium or the effects of the deficiency were masked as essential compounds required were available.

Susceptibility of *S.* ser. Typhimurium to streptomycin was remarkably different after culture in the two media: BPA or ISA. Again the increased complexity of ISA medium altered the phenotypic response of this organism to the antibiotic, regardless of its peptone constituent. However this does allow for a fundamental question to be raised: which medium provides the result which correlates with *in vivo* clinical treatment?

The control strain, *E. coli*, exhibited altered results for cephalothin (from sensitive to resistant) dependent on whether the ISA medium was laboratory-prepared or

commercially available; the laboratory-prepared media always fell within the accepted guidelines as published by Andrews (2004) for all other specified organism-antibiotic combinations. As the range of cephalothin values were not notable this was not important, all the IZD values for cephalothin are close to the cut off point for determination of susceptibility.

It can be concluded that if ISA medium is representative of *in vivo* results, and that each batch is quality controlled and produces results within the accepted zones, the use of this complex medium eradicates the effects of variable peptone constituents.

5.6. Conclusion

Where diagnostic tests are being carried out it is of considerable importance that the standardized media, whose complex recipes eliminated the effects of peptone variation, be used (for example ISA for antibiotic susceptibility testing, utilizing the disk diffusion method). However even this may be unsatisfactory in certain cases, as quality control of media for antibiotic susceptibility tests may be flawed; for example acceptable ranges for inhibition zones of control *S. aureus* strains to methicillin and oxacillin are performed on Columbia or Mueller Hinton agar with an additional 2% NaCl. Columbia agar contains 23 g l^{-1} of "special peptone" (a mixture of peptones) which will be equally as variable as the peptones investigated in this work. Variation in performance of Mueller-Hinton agar has been previously discussed; studies on the effect of differing batches and manufacturers of this medium were noted with *P. aeruginosa* which was found to be due to the variable concentration of calcium and magnesium ions (D'Amato *et al.*, 1975). Thymine and thymidine content of the medium also affected subsequent antibiotic

susceptibility testing of bacterial clinical isolates for the compounds sulphamethoxazole and trimethoprim (Ferguson and Weissfeld, 1984). Consequently, health care scientists should be meticulous in their controls, to include reference to known clinical isolates, as what appears to be MRSA, as indicated by susceptibility testing on these variable media, could easily be a methicillin sensitive *S. aureus* and *vice versa*.

It should be noted here that bioassay testing will also be subject to considerable variation due to media components. These tests evaluate the efficacy of an antimicrobial; it may be a patient blood serum level of a prescribed drug and therefore confirmation of correct dosage or the concentration of disinfectant required for successful decontamination of surfaces. Where the medium promotes resistance, the subsequent lawn of microbial growth will show reduced IZD values to both the test and control substances and thus be indicative of unsuccessful drug therapy; this is of considerable importance as the drug dose to a patient may be increased or the prescription altered unnecessarily. The opposite is also true: increased sensitivity of the control organism used to evaluate the efficacy of the antimicrobial used will result in high IZD values. This would indicate a successful drug dosage *in vitro*, however this is not a true indication of circumstances *in vivo*: the infectious bacteria may remain unaffected by chemotherapy and continue to cause complications.

For scientific research, where data are compared between different collaborative laboratories, these results could easily be of an opposing nature due entirely to the difference in growth medium. This chapter highlighted the need for enforced standardized methods in the public health system (many hospital microbiology laboratories do not use the standardized media) and increased consideration of the effects

of different batches of products used when comparing results from differing laboratories or from the literature. The solutions are relatively straight-forward: use of recommended media which are quality controlled before use: the tests should be performed with known resistant and sensitive clinical isolates. Current control strains such as the *E. coli* (NCTC 10418) have been shown during this study to vary considerably in their inhibition zone values depending on the growth medium constituents (particularly for the cephalosporins).

5.7. Acknowledgements

- Merck Chemicals Ltd: Lisa Thompson for generous advice and guidance with the serological methods.
- Cardiff University, School of Biosciences: Paul Seaman and Gareth Williams for their kind donations of *Staphylococcus aureus* and *Escherichia coli* respectively.

CHAPTER SIX

Effects of medium supplements on antibiotic susceptibility

6. Effects of media supplements on antibiotic susceptibility tests

6.1. Summary

The concentration of peptone employed in the medium BPA affected the susceptibility of both *Salmonella* ser. Typhimurium and *Escherichia coli* to the aminoglycosides amikacin, gentamicin and streptomycin; lower concentrations of peptone (1 g l^{-1}) resulted in increased susceptibility to the antibiotic as compared with higher peptone concentrations (20 g l^{-1}).

Additional supplements of either $MgSO_4$ or glucose to the various peptone-based BPA media did, on certain occasions, affect the susceptibility of the Enterobacteria to particular antibiotics; there were no observable trends for these data.

6.2. Introduction

Variation in peptone batch and source as a constituent of the growth medium greatly changed the subsequent results of antibiotic susceptibility testing; these differences were examined further by alteration of peptone concentration within the growth medium and also by the addition of supplements (i.e. glucose and $MgSO_4$).

It was previously shown that the concentration of peptone affected the generation time and yield of the bacteria thereby cultured (Chapter 3.4.6); therefore this variable was also examined for any effect on antibiotic resistance.

It was noted, particularly for resistance of *S.* ser. Typhimurium to streptomycin, that resistance correlated with concentration of carbohydrate present in the medium; i.e. peptones such as casein and gelatin lacked a carbohydrate source and in that case, organisms exhibited resistance to the aminoglycoside. Bryan and Van Den Elzen (1977) showed that both streptomycin and gentamicin required an energy-dependent transport

mechanism operating across the membrane in both Gram positive and negative bacteria; therefore in media which were lacking a suitable energy source this may have been the ultimate cause of resistance. In another study supplemental additives to the medium, for example sugars, inorganic ions, surface active agents and blood, produced organisms which exhibited increased resistance to the antibiotics (Toama *et al.*, 1978). This observation is in keeping with the hypothesis of Bryan and Van Den Elzen.

Peptones with a high NaCl content generally gave organisms which displayed increased sensitivity to antimicrobials in comparison with those with less NaCl. However, divalent cations (Ca^{2+}, Mg^{2+}, Co^{2+} and Mn^{2+}) are known to inhibit accumulation of streptomycin and gentamicin in the organism (Bryan and Van Den Elzen, 1977). This may be due to the two component regulatory system, PhoP-PhoQ, which regulates the organism's response to concentrations of extra-cellular Mg^{2+}. This system has also been shown also to regulate *Salmonella typhimurium* resistance to antimicrobial peptides (Fields *et al.*, 1989; Miller *et al.*, 1989); therefore addition of Mg^{2+} to any of the various peptones may cause differences in the antibiotic susceptibility of the organisms cultured. Similarly, addition of Mg^{2+} to *Pseudomonas aeruginosa* protected the cells from the antimicrobial tobramycin by preventing its initial uptake (Raulston and Montie, 1989).

This chapter aims to increase awareness of the differences between these peptones with respect to antibiotic susceptibility testing.

6.3. Materials and methods

Methods for susceptibility testing and the media used were as previously described in Chapters 2 and 5. Additional supplements were as below:

6. Effects of media supplements on antibiotic susceptibility tests

6.3.1. Effects of peptone concentration

Concentrations of peptone as a constituent of all the media used in this chapter are listed in Appendix 1. Where the effect of peptone concentration, as a constituent of BPA, on antibiotic susceptibility was assessed, the following quantities were used: 1, 5, 7.5, 10 and 20 g l^{-1}. For *Salmonella* ser. Typhimurium varying concentrations of C7 peptone were assessed, whereas *Escherichia coli* were grown on different concentrations of Y1 peptone. Other medium components remained constant.

6.3.2. Medium supplements

The susceptibility of *Escherichia coli*, *Salmonella* ser. Typhimurium and *Salmonella* ser. Poona to different antibiotics, after growth in BPA (containing various peptones) and/or M9 defined medium, with the addition of certain medium supplements was assessed. The recipes for these media are detailed in Appendix 1, and the additional supplements of either glucose or $MgSO_4$ were added where indicated at the following concentrations:

Glucose: 0.5, 1, 2 or 4 % w/v

$MgSO_4$: 1, 2, 4 or 6 mM

6.3.3. Statistical analyses

One-way ANOVA was employed to analyse zones of inhibition produced by the disk diffusion method for determination of antibiotic susceptibility, where data were parametric. Kruskal-Wallis analysis was used for non-parametric data.

6.4. Effect of peptone concentration on antibiotic susceptibility

6. Effects of media supplements on antibiotic susceptibility tests

Work described in Chapter 5.5. highlighted that peptone itself may be affecting the susceptibility of microorganisms to antimicrobial compounds; therefore, if peptone concentrations in the growth medium BPA were altered, this would affect subsequent susceptibility tests. The data below focus on this hypothesis.

6.4.1. Effect of peptone concentration on activities of anti-microbials against *Escherichia coli*

The concentration of peptone Y1 (as a constituent of BPA medium) significantly affected the activity of most antibiotics as shown in Figure 6.1; amikacin was not significantly affected ($P = 0.092$), although mean values still appeared different: large variances within the replicates may have eliminated statistical difference. *E. coli* exhibited statistically-different zones of inhibition dependent on the concentration of peptone with all other antibiotics examined: cephalothin ($P = 0.000$), colistin sulphate ($P = 0.041$), gentamicin ($P = 0.016$) and streptomycin ($P = 0.000$).

Significant differences indicated between the mean values did not always result in a correlation between susceptibility of *E. coli* to the antibiotic and peptone concentration in the growth medium; visual observations of gentamicin and streptomycin curves indicated a negative correlation, in that *E. coli* was more resistant to these antimicrobials at lower concentrations of peptone. The curve for cephalothin displayed a similar correlation, but at 20 g l^{-1} *E. coli* became more sensitive, rather than continuing to show increasing resistance to the cephalosporin. From a concentration of 1 g l^{-1} of peptone to 5 g l^{-1}, *E. coli* showed an increase in resistance to colistin sulphate; further increases in peptone concentration did not have any effect on susceptibility.

6. Effects of media supplements on antibiotic susceptibility tests

An increase in peptone concentration never resulted in increased susceptibility to any of the antibiotics tested.

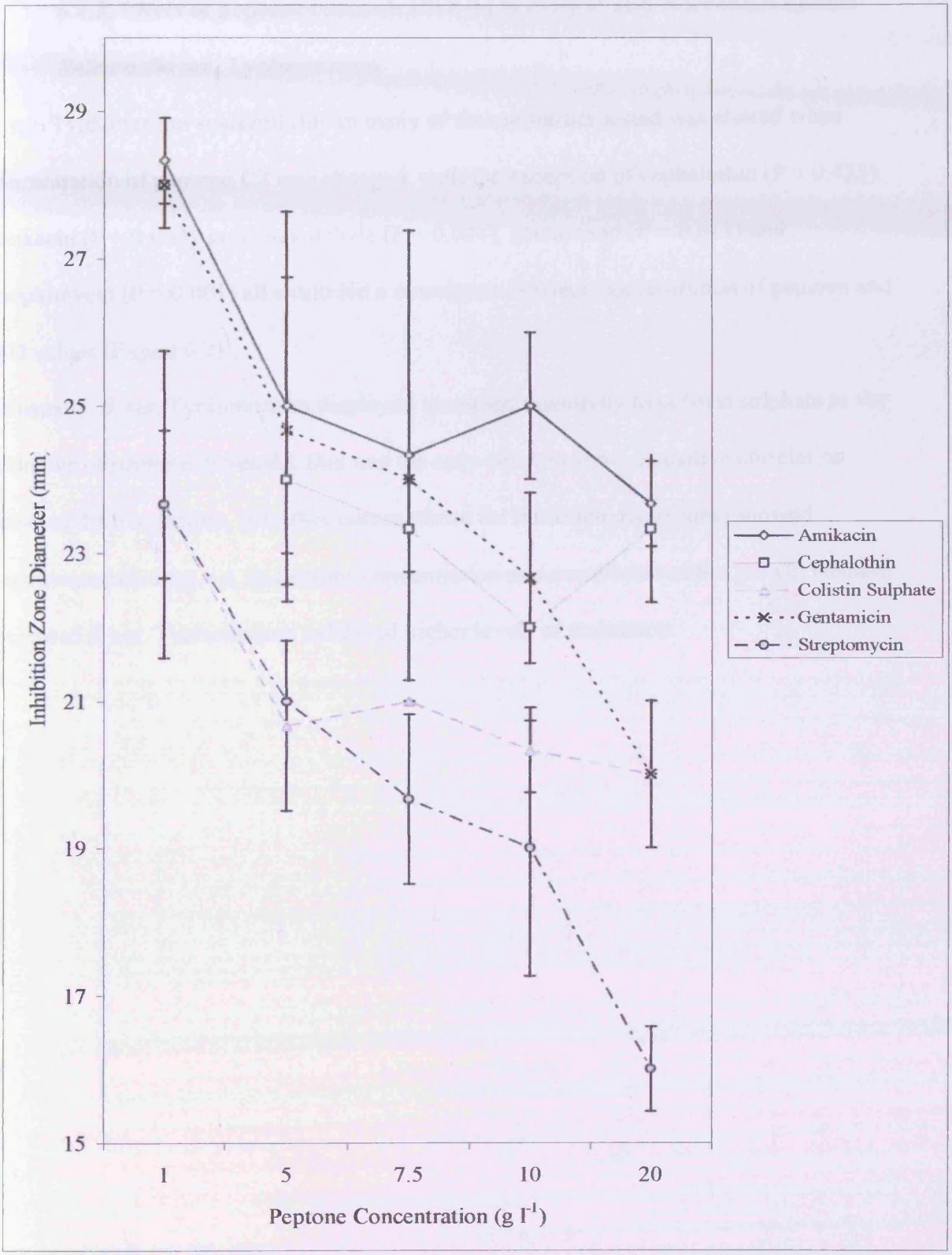

Figure 6.1. Inhibition zone diameters of *Escherichia coli* as a response to antibiotics after growth on different concentrations of Y1 peptone. The antibiotics assessed were amikacin (30 µg), cephalothin (30 µg), colistin sulphate (25 µg), gentamicin (10 µg) and streptomycin (10 µg). Error bars represent standard deviation where n=3.

6.4.2. Effect of peptone concentration on activity of anti-microbials against *Salmonella* ser. Typhimurium

S. ser. Typhimurium susceptibility to many of the antibiotics tested was altered when concentration of peptone C7 was changed, with the exception of cephalothin (P = 0.425); amikacin (P = 0.000), colistin sulphate (P = 0.024), gentamicin (P = 0.013) and streptomycin (P = 0.001) all exhibited a correlation between concentration of peptone and IZD values (Figure 6.2).

Unusually, *S.* ser. Typhimurium displayed increased sensitivity to colistin sulphate as the additions of peptone increased: this was the only curve to show a positive correlation between the two factors. All other curves (those for the aminoglycosides) showed negative correlations, i.e. as peptone concentration as a constituent of the growth medium increased *S.* ser. Typhimurium exhibited higher levels of resistance.

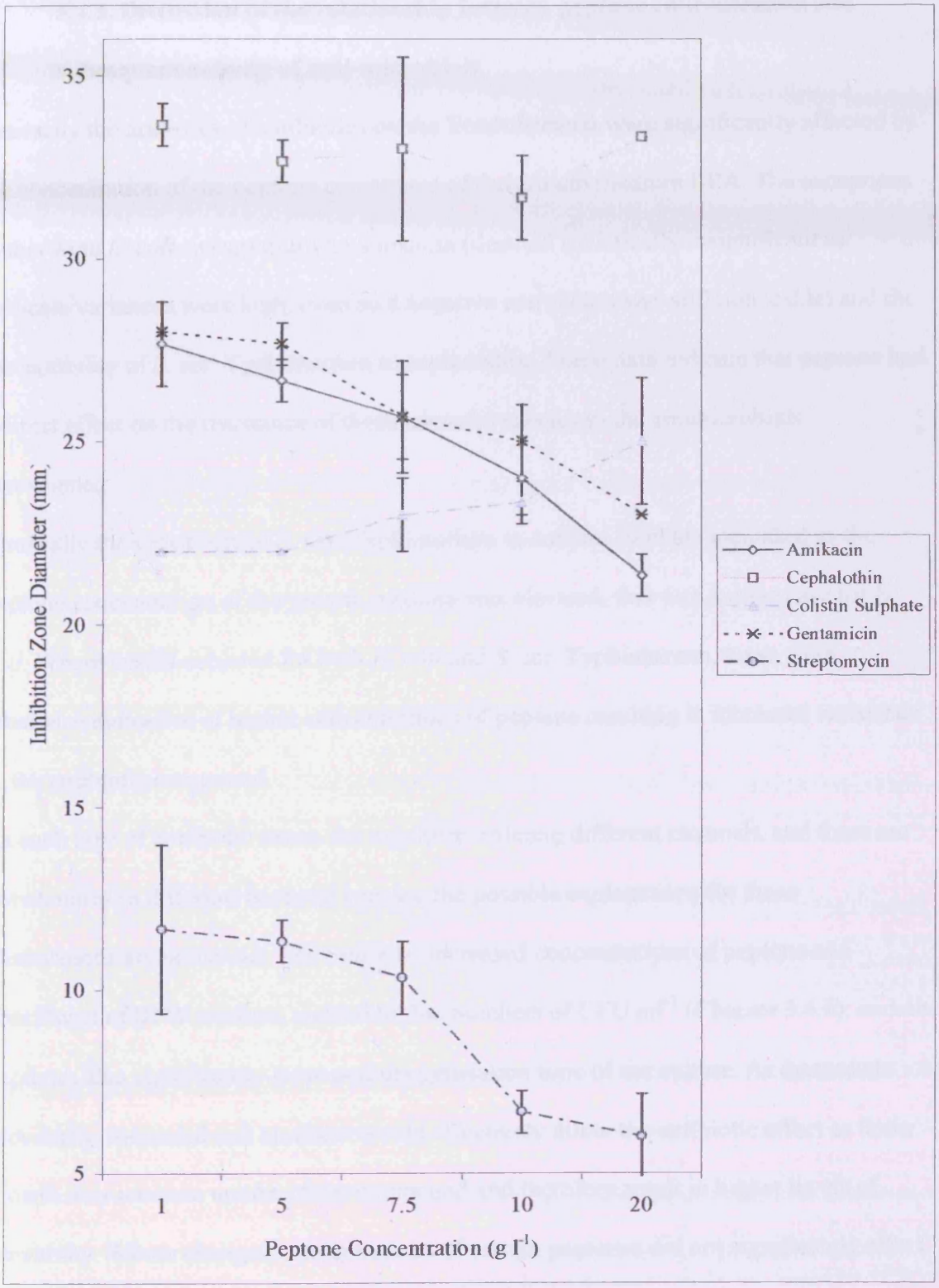

Figure 6.2. Inhibition zone diameters of *Salmonella* ser. Typhimurium as a response to antibiotics after growth on different concentrations of C7 peptone. The antibiotics assessed were amikacin (30 µg), cephalothin (30 µg), colistin sulphate (25 µg), gentamicin (10 µg), shown in graph (a) and streptomycin (10 µg), shown in graph (b). Error bars represent standard deviation where n=3.

6.4.3. Discussion of the relationship between peptone concentration and subsequent activity of anti-microbials

Generally the activities of antibiotics on the Enterobacteria were significantly affected by the concentration of the peptone constituent of the growth medium BPA. The exceptions to this were *E. coli* susceptibility to amikacin (deemed statistically insignificant as replicate variances were high, even so a negative correlation was still noticeable) and the susceptibility of *S.* ser. Typhimurium to cephalothin. These data indicate that peptone had a direct effect on the resistance of these bacterial species to the antimicrobials investigated.

Unusually the sensitivity of *S.* ser. Typhimurium to colistin sulphate increased as the peptone concentration of the growth medium was elevated, this was not the case for *E. coli*. Where trends occurred for both *E. coli* and *S.* ser. Typhimurium, these were otherwise indicative of higher concentrations of peptone resulting in increased resistance to the antibiotic compound.

As each type of antibiotic enters the organism utilizing different channels, and these are not identical in different bacterial species, the possible explanations for these observations are numerous. For example, increased concentrations of peptone as a constituent of BPW medium yielded higher numbers of CFU ml^{-1} (Chapter 3.4.6); certain peptones also significantly increased the generation time of the culture. As discussed previously, increased cell numbers would effectively dilute the antibiotic effect or faster growth may increase uptake of the compound and therefore result in higher levels of sensitivity. Where changed concentrations of certain peptones did not significantly affect generation times but did alter yield (Figure 3.1), uptake may not be affected (as growth would be similar despite the increase in peptone availability) but high cell numbers could

dilute antibiotic concentrations thus leading to increased resistance (as in the cases of negatively correlated curves). The opposite effects can also occur, e.g. generation times can decrease and consequently uptake of the antimicrobial compound will increase with higher concentrations of peptone, but yield does not increase as much in comparison with other peptones thus leading to relatively more susceptible cultures (e.g. *S.* ser. Typhimurium with colistin sulphate).

If the peptone molecules themselves bind to the outer membrane proteins or block the mechanism of entry into the organism, then at higher concentrations this would increase resistance, as antibiotics would then be unable to freely enter the bacterium. This would again explain why higher concentrations of peptone resulted in increased resistance. Where no effect on susceptibility to an antibiotic was recorded with changed peptone concentration, this indicated that there was no antagonism or synergism between peptone and mechanisms of antibiotic activity or access to the cell.

Different peptones gave variable susceptibility results, and at differing concentrations these data were altered again. Naturally, use of the standardized ISA medium would cause the effect to be less pronounced (due to its more complex recipe), but the effect of variable peptone source or manufacturer in combination with peptone concentration may begin to alter results.

6.5. Effect of glucose on antibiotic susceptibility

Previous workers have found contrasting evidence for the effect of additional glucose to the culture media on the resultant organisms subsequently studied for antibiotic susceptibility. Toama *et al.* (1978) demonstrated a decrease in activity of antibiotics (nafcillin, vancomycin, oleandomycin, chloramphenicol, methacycline and novobiocin)

after addition of the carbohydrate, whereas Bryan and Van Den Elzen (1977) showed that active transport was responsible for the uptake of antimicrobials (streptomycin and gentamicin) into the bacterium which would require a substrate. It was generally observed during Chapter 5 that *Salmonella* cultured in media which lacked a carbohydrate source were notably more resistant to streptomycin, than those which were grown on media containing a freely available carbohydrate source. Therefore it was hypothesized that media which lacked this important substrate produced *Salmonella* which were resistant to streptomycin as the antibiotic uptake was diminished. Alternatively *Salmonella* may ferment the glucose present in certain peptones resulting in a lowered medium pH; this would subsequently cause elevated sensitivity (Bridson, 1995).

Controls were M9 defined medium and commercial ISA for comparison. *E. coli* was used as a control organism.

6.5.1. Effect of glucose concentration on activity of anti-microbials against *Escherichia coli*

The susceptibility of *E. coli* to different antimicrobials on BPA, which contained varying concentrations of glucose, was found on occasions to differ in a glucose-dependent manner. This was noted only on certain BPA recipes where the bacteria where cultured on particular peptones as a constituent of the growth medium. Where the glucose concentration of the different peptone-based media significantly affected IZD values for the antimicrobials investigated ($P \leq 0.05$) these are highlighted in blue in Table 6.1. This table shows that the aminoglycosides amikacin and gentamicin were generally more

affected by the glucose content of the medium, particularly during growth on yeast peptone-based BPA, in comparison with cephalothin or streptomycin.

Table 6.1. Statistical P values for the effect of glucose concentration on the activity of various antibiotics, against *Escherichia coli*, after growth on different media. Values ≤ 0.050 indicated that the zone of inhibition was significantly affected by the concentration of glucose present in the medium.

Medium	Antibiotic				
	Amikacin	Cephalothin	Ciprofloxacin	Gentamicin	Streptomycin
M6 BPA	0.067	0.022	–	0.564	0.074
Y1 BPA	0.007	0.072	–	0.012	0.030
Y4 BPA	0.000	0.985	–	0.001	0.078
M9 Agar	0.015	0.171	0.015	–	–
ISA	0.190	0.080	–	0.008	0.262

Specifically, susceptibility of *E. coli* to amikacin on M6 peptone-based medium remained unaltered, irrespective of the additional glucose concentration (Figure 6.3 a). In contrast, growth of *E. coli* on BPA containing the yeast peptones Y1 and Y4, resulted in increased resistance of the subsequent cultures to amikacin only when glucose concentration was elevated. Initially a notable increased resistance was recorded between presence and absence of supplemental glucose; at much higher concentrations there was only a slight increase in resistance to amikacin.

Glucose-mediated effects on the susceptibility of *E. coli* to cephalothin after culture on BPA medium (Figure 6.3 b) were dissimilar to those for amikacin. Values for IZD after growth on M6 peptone-based medium differed significantly, but were irregular. At less than 1 % (w/v), sensitivity decreased, whereas 2 % (w/v) glucose in M6 based BPA resulted in a marked heightening of susceptibility. Sensitivity of *E. coli* increased noticeably with higher concentrations of glucose present in Y1 peptone-based growth medium (these observations were not statistically significant), whereas susceptibility of

E. coli cultured on Y4 peptone-based BPA remained unchanged, despite the inclusion of supplemental glucose.

The activity of gentamicin against *E. coli* on the different BPA media which contained various concentrations of glucose (Figure 6.4 a) was similar to that of amikacin. BPA media containing peptone M6 produced IZD values which were not significantly different dependent on the % (w/v) glucose of the medium. In contrast, both yeast peptones investigated (Y1 and Y4) as constituents of the media produced cultures of *E. coli* for which the susceptibility to gentamicin was altered considerably where glucose concentration differed; a significant increase in resistance to the antimicrobial occured as concentration of glucose in the yeast based media increased.

The addition of glucose to the BPA recipe considerably affected the activity of streptomycin on subsequent cultures of *E. coli* (Figure 6.4 b). Again, M6 peptone as a constituent of the growth medium, containing variable concentrations of glucose, produced IZD values which were not statistically significant; however there was a noticeable difference between presence and absence of glucose, addition of glucose resulted in an increased resistance to streptomycin. Both yeast peptones exhibited similar patterns to those described for M6 peptone.

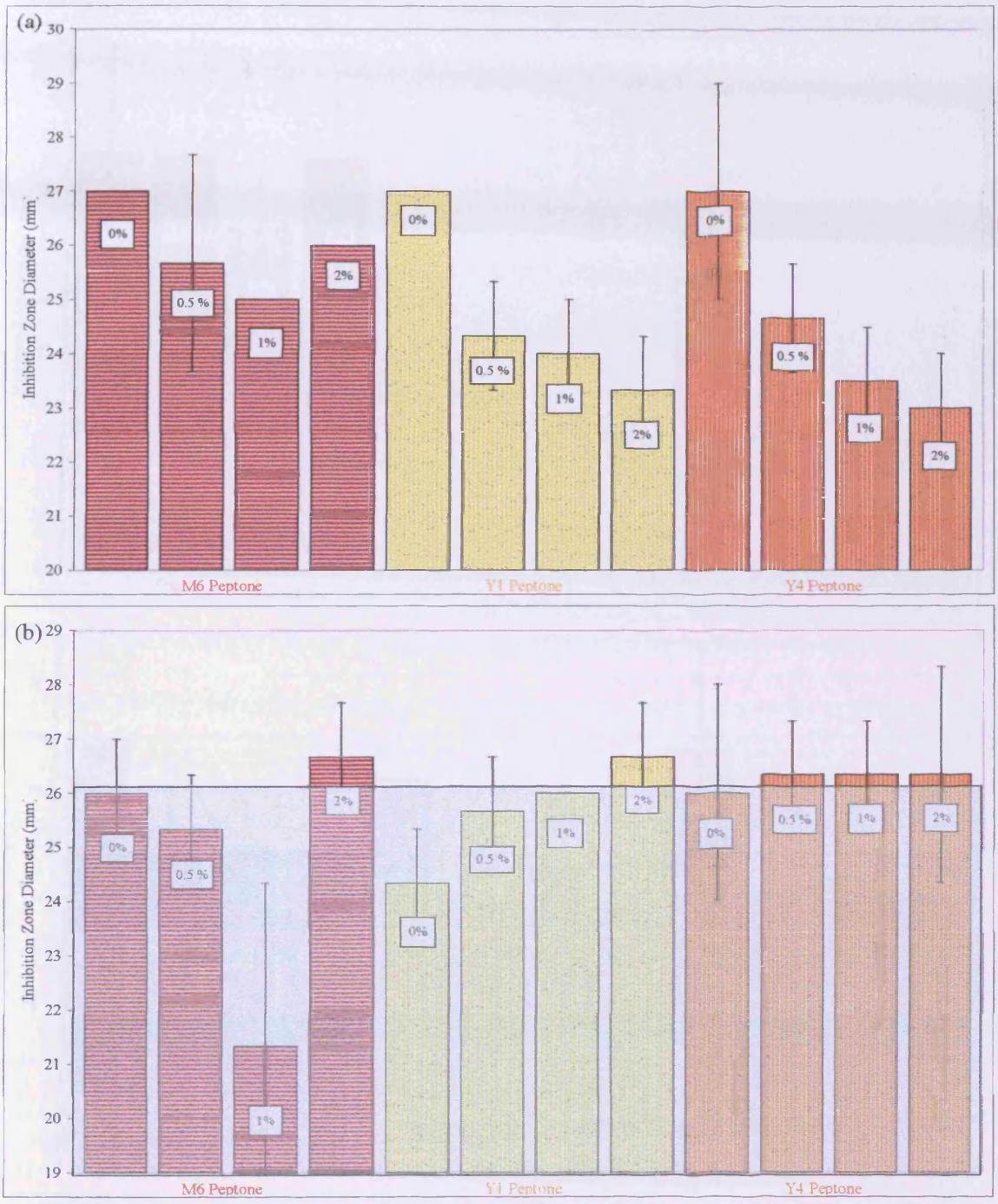

Figure 6.3. Inhibition zone diameters of *Escherichia coli* as a response to (a) amikacin (30 µg) and (b) cephalothin (30 µg) grown on three different buffered peptone agar media whereby the glucose concentration varied from 0 % to 2 %. Resistance to the antimicrobial is indicated by the transparent blue zone. Error bars represent standard deviation where n=3.

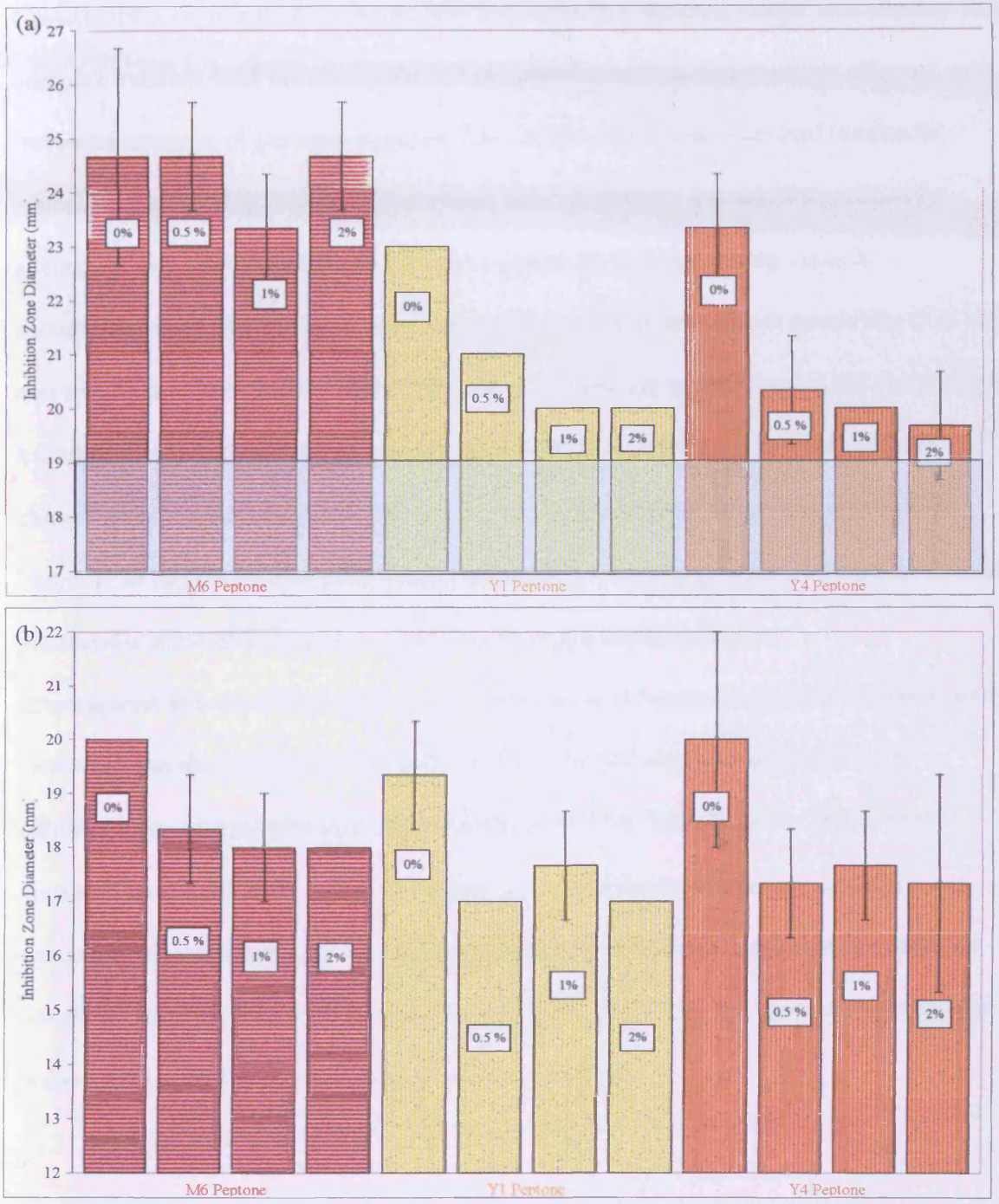

Figure 6.4. Inhibition zone diameters of *Escherichia coli* as a response to (a) gentamicin (10 µg) and (b) streptomycin (10 µg) grown on three different buffered peptone agar media whereby the glucose concentration varied from 0 % to 2 %. Resistance to the antimicrobial is indicated by the transparent blue zone. Error bars represent standard deviation where n=3.

6. Effects of media supplements on antibiotic susceptibility tests

Growth of the organism on commercially available ISA allowed insight into whether this complex medium used for standardized susceptibility testing was similarly affected with increased amounts of glucose (Figure 6.5 a). Of the antibiotics examined (amikacin, gentamicin, streptomycin and cephalothin), only gentamicin exhibited significantly altered activity towards the *E. coli* cultures grown on ISA containing variable concentrations of glucose. In this instance, a considerable increase in sensitivity of *E. coli* was recorded where glucose was added; the concentration of supplement did not produce a marked effect. Of importance was the recorded change from clinical resistance to clinical sensitivity after the addition of glucose as this control strain was noted in the literature to be sensitive to gentamicin. The activity of all other antimicrobials on *E. coli* produced unaltered IZD values, regardless of supplemental glucose.

Investigation into the effect of glucose concentration on antimicrobial susceptibility after growth on the chemically-defined medium M9, showed that both amikacin and ciprofloxacin were significantly affected (Figure 6.5 b). Susceptibility of *E. coli* to amikacin on the defined medium exhibited a trend of decreased resistance as glucose concentration increased; this was in contrast to both cephalothin and ciprofloxacin, for which the former showed unchanged activity towards *E. coli*, and the latter an irregular response towards the different concentrations of glucose.

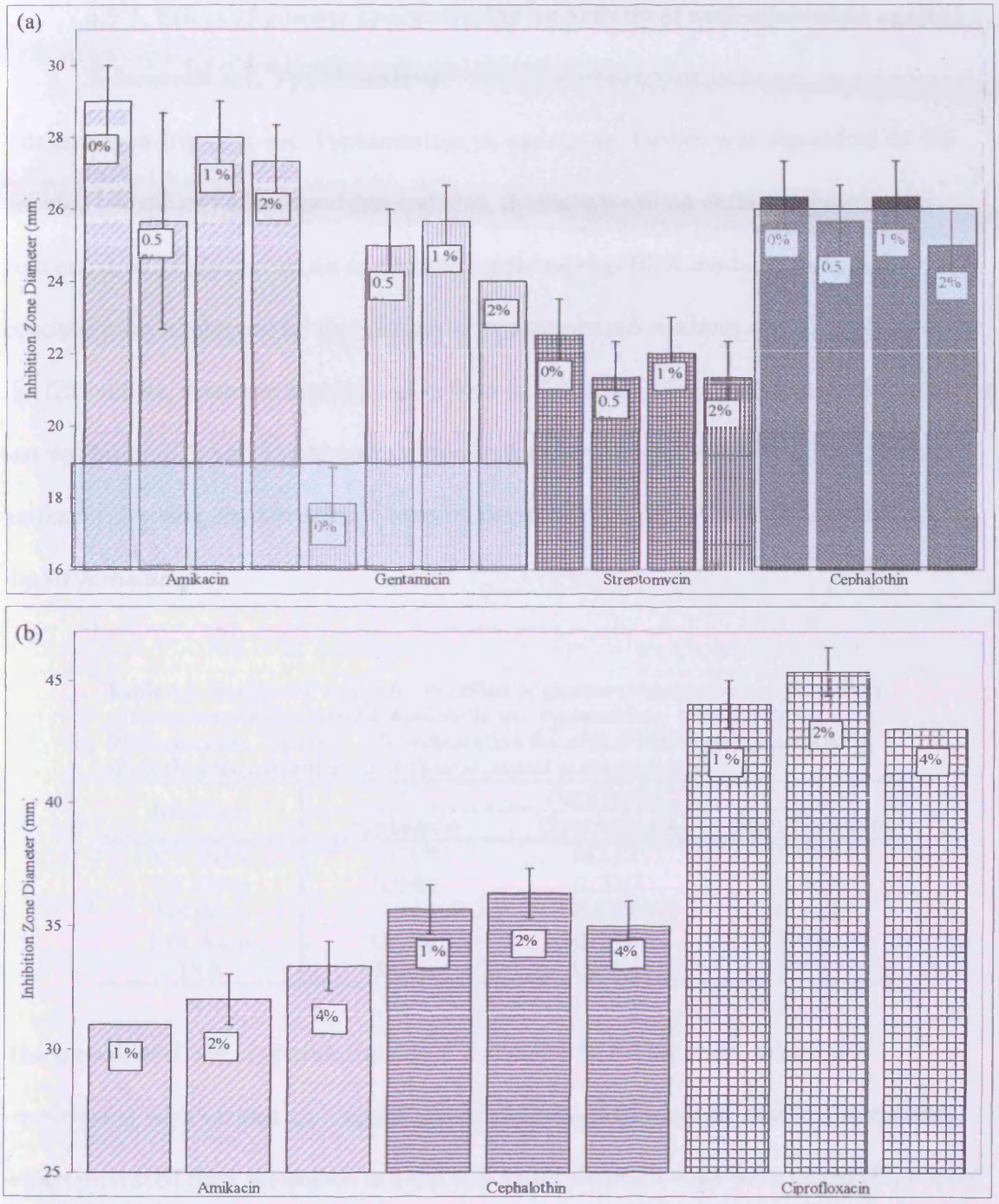

Figure 6.5. Inhibition zone diameters of *Escherichia coli* as a response to various antibiotics after growth on (a) a commercially available Iso-sensitest agar and (b) M9 defined medium whereby the glucose concentration varied from values between 0 % and 4 %. Resistance to the antimicrobial is indicated by the transparent blue zone. Error bars represent standard deviation where n=3.

6.5.2. Effect of glucose concentration on activity of anti-microbials against *Salmonella* ser. Typhimurium

The susceptibility of *S.* ser. Typhimurium to various antibiotics was dependent on the peptone constituent of the medium and also, during growth on certain peptones, the concentration of glucose as an additional supplement to BPA medium. Where the concentration of glucose on that particular peptone-based medium significantly affected the IZD values, these are highlighted in blue in Table 6.2. Glucose concentration affected susceptibility of *S.* ser. Typhimurium to a lesser extent compared with *E. coli*, with only amikacin showing any variance in activity dependent on the additional carbohydrate to the BPA media.

Table 6.2. Statistical P values for the effect of glucose concentration on the activity of various antibiotics, against *Salmonella* ser. Typhimurium, after growth on different media. Values ≤ .050 indicate that the zone of inhibition is significantly affected by the concentration of glucose present in the medium.

Medium	Antibiotic		
	Amikacin	Gentamicin	Streptomycin
C7 BPA	0.013	0.112	0.065
G1 BPA	0.940	0.229	0.060
S5 BPA	0.043	0.029	0.122
M9 Agar	0.729	0.118	1.000
ISA	0.104	0.363	0.003

The growth of *S.* ser. Typhimurium on C7 peptone-based medium, which was investigated with various concentrations of supplemental glucose, resulted in cultures which increased their resistance to amikacin as levels of glucose were raised (Figure 6.6 a). Culture of *S.* ser. Typhimurium on S5 peptone containing variable quantities of carbohydrate also resulted in a significant difference in IZD values. However there was no definite trend; where glucose was present there was an increase in resistance to amikacin compared with no supplement, but the mean IZD value for the 2 %

concentration of the carbohydrate showed a decrease in resistance relative to 0.5 % and 1 %. Gelatin peptone G1 exhibited *S.* ser. Typhimurium cultures which showed no change in susceptibility to amikacin with addition of glucose.

Gentamicin activity against *S.* ser. Typhimurium was found not to be dependent on the glucose concentration of both C7 and G1 peptone-based BPA (Figure 6.6 b). Differences were noticeable for those cultures grown on C7 peptone as glucose concentration changed, but there was no obvious pattern, and the standard deviations were high. Organisms cultured on S5 based BPA which contained variable glucose content produced similar results for gentamicin activity to those obtained for amikacin; generally the addition of glucose caused a decrease in sensitivity of *S.* ser. Typhimurium to the antimicrobial, however 2 % glucose cultured organisms which were relatively susceptible in comparison with the lower percentages of the compound.

The susceptibility of *S.* ser. Typhimurium to streptomycin after growth on different concentrations of glucose as a supplemental addition to BPA medium was notably different in comparison with the other aminoglycoside antibiotics investigated (Figure 6.7). Variable glucose content in C7 peptone-based BPA resulted in subsequent cultures of *Salmonella* remaining resistant to streptomycin, but with noticeable differences in IZD values: media containing 0.5 % and 1 % glucose both exhibited organisms which were more sensitive compared with 0 % and 2 %. The introduction of carbohydrate to G1 peptone-containing media caused cultures to be considerably more sensitive to streptomycin, however the quantity added did not cause further variation; this result was in accordance with the hypothesis that addition of a carbohydrate source would allow uptake of streptomycin and therefore increase sensitivity. This finding was not statistically significant, however this may be due to the large variances between the

replicates. It was interesting to note that the addition of 1 % glucose to the G1 peptone medium caused resultant colonies to be clinically sensitive, whereas all other glucose concentrations examined produced resistant ones. Peptone S5 in combination with different quantities of glucose did not show a statistically significant difference with respect to values of IZD. However, again this may be due to large variances; there was a noticeable decline in sensitivity of *S.* ser. Typhimurium cultures to streptomycin as supplemental carbohydrate levels were elevated. Worthy of note is that all three peptones as constituents of the culture medium, after the addition of variable quantities of glucose, generated *S.* ser. Typhimurium of contrasting susceptibility patterns, i.e. casein peptone produced colonies which were most sensitive at mid-range glucose concentrations, gelatin peptone generated *S.* ser. Typhimurium which were more susceptible at higher levels of glucose, and finally, soy peptone exhibited organisms which showed decreased resistance at lower quantities of the carbohydrate.

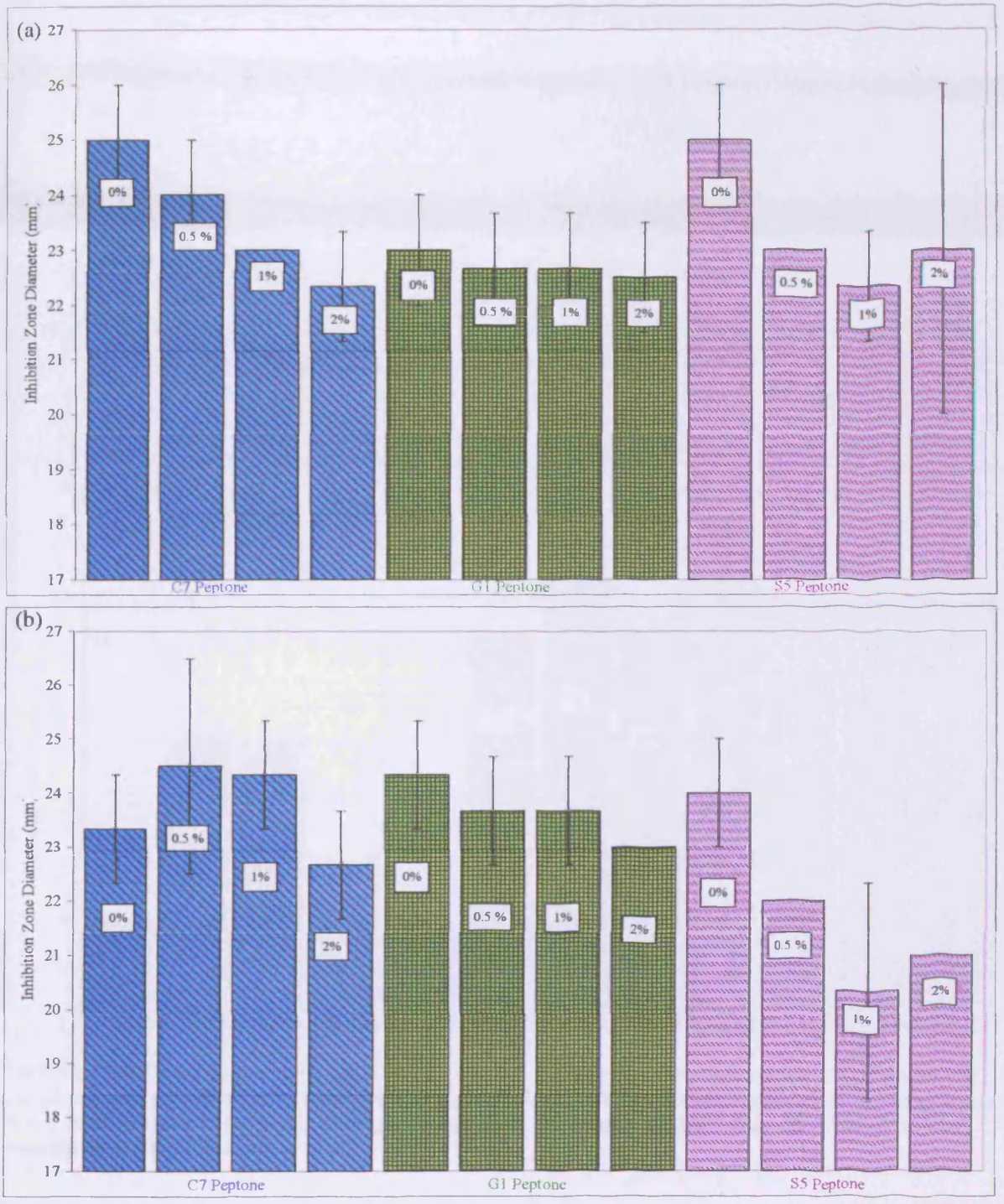

Figure 6.6. Inhibition zone diameters of *Salmonella* ser. Typhimurium as a response to (a) amikacin (30 µg) and (b) gentamicin (10 µg) grown on three different buffered peptone agar media whereby the glucose concentration varied from 0 % to 2 %. Error bars represent standard deviation where n=3.

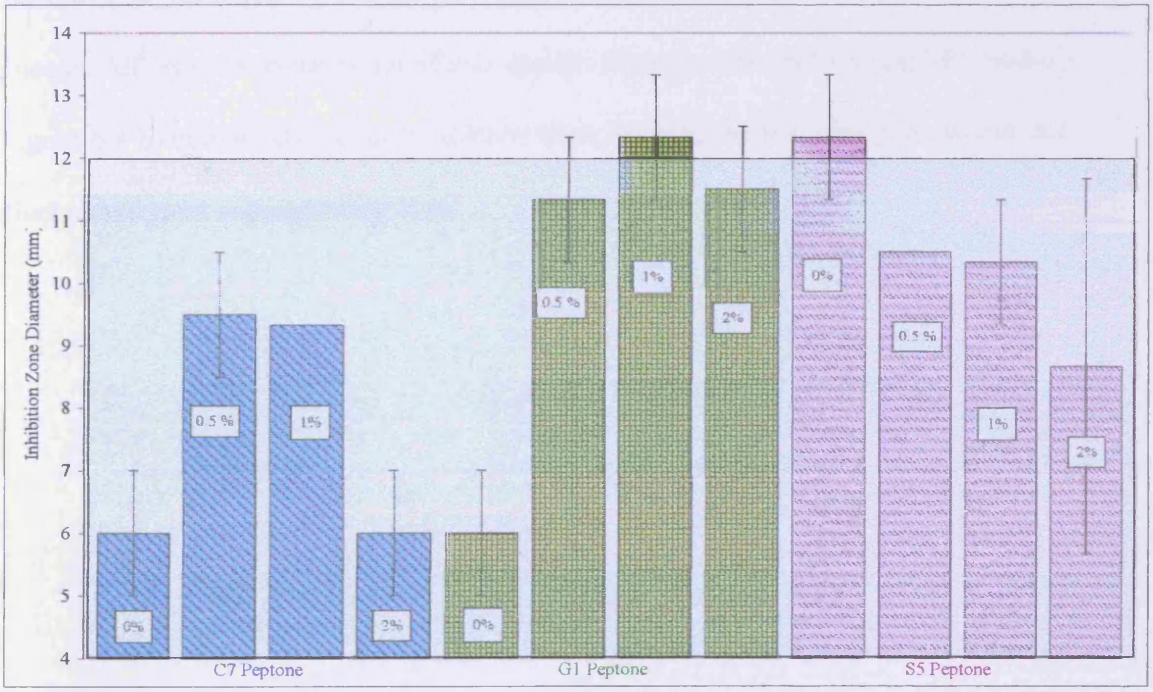

Figure 6.7. Inhibition zone diameters of *Salmonella* ser. Typhimurium as a response to streptomycin (10 µg) grown on three different buffered peptone agar media whereby the glucose concentration varied from 0 % to 2 %. Resistance to the antimicrobial is indicated by the transparent blue zone. Error bars represent standard deviation where n=3.

6. Effects of media supplements on antibiotic susceptibility tests

Cultures of *S.* ser. Typhimurium were grown on ISA and M9 medium containing different concentrations of glucose for comparison: ISA was commercially tested for quality control before purchase and M9 was of a defined recipe (Figure 6.8). Susceptibility testing of the aminoglycosides on these media generally showed that concentration of glucose did not affect the results; the exception was the activity of streptomycin against the organism grown on ISA with variable glucose content (Figure 6.8 a). These data displayed an increase in *Salmonella* sensitivity as glucose levels were raised to 1 %, followed by a slight decrease in susceptibility to streptomycin at 2 % glucose. All other investigations of this species after growth on ISA and M9 media (Figure 6.8 b) containing variable carbohydrate content showed that glucose did not affect subsequent susceptibility tests.

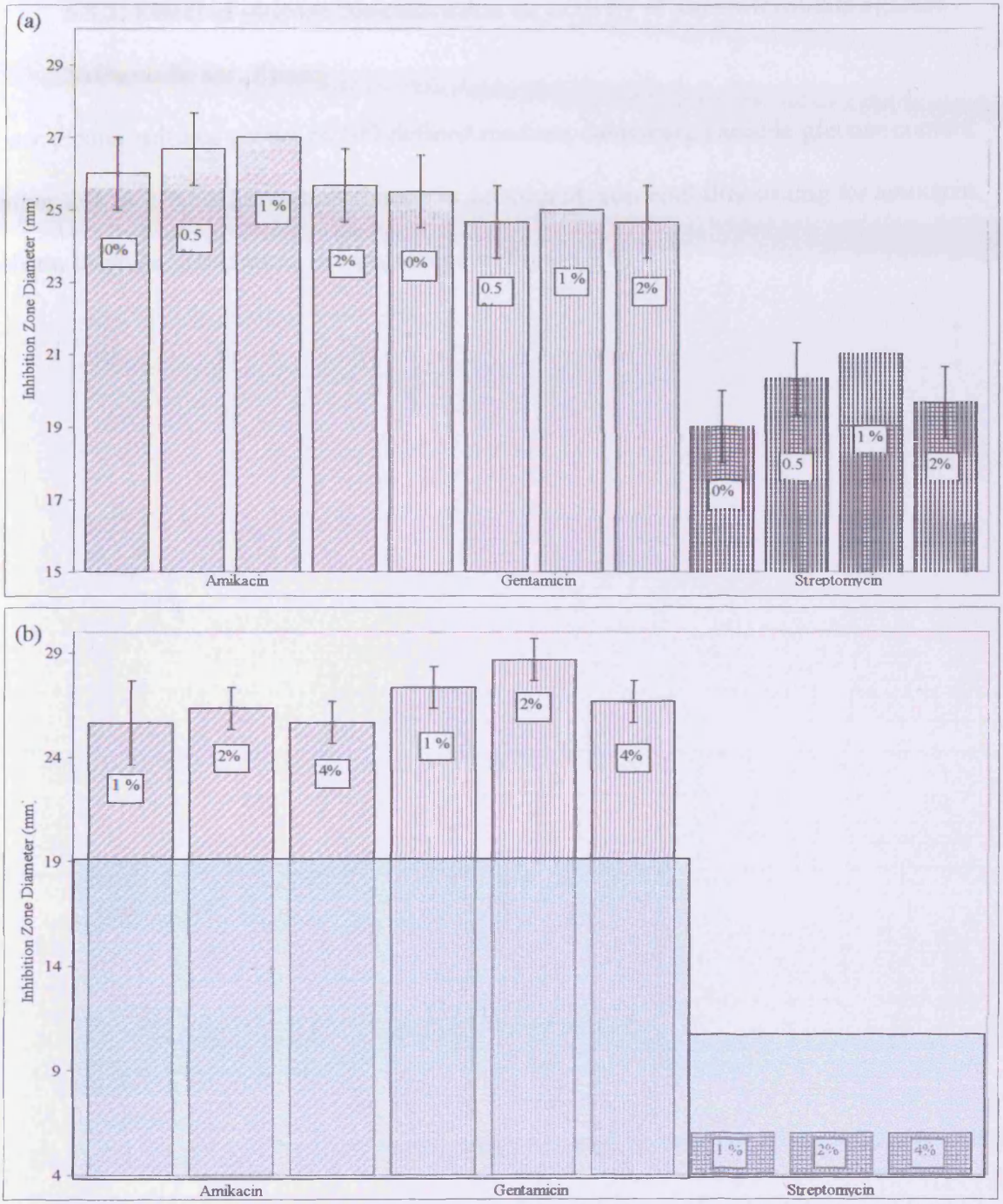

Figure 6.8. Inhibition zone diameters of *Salmonella* ser. Typhimurium as a response to various antibiotics after growth on (a) a commercially available Iso-sensitest agar and (b) M9 defined medium whereby the glucose concentration varied from values between 0 % and 4 %. Resistance to the antimicrobial is indicated by the transparent blue zone. Error bars represent standard deviation where n=3.

6.5.3. Effect of glucose concentration on activity of anti-microbials against *Salmonella* ser. Poona

S. ser. Poona cultures grown on M9 defined medium containing variable glucose content (either 1 % or 2 %) exhibited no change in subsequent susceptibility testing for amikacin, colistin sulphate, gentamicin or streptomycin (Figure 6.9).

6. Effects of media supplements on antibiotic susceptibility tests

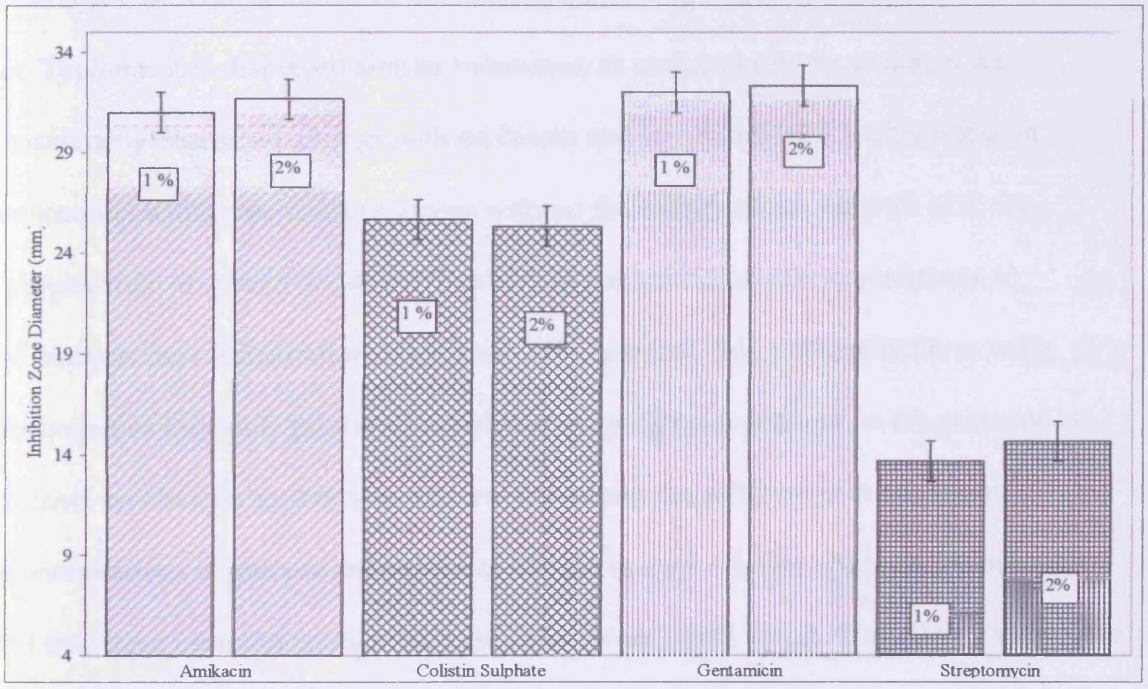

Figure 6.9. Inhibition zone diameters of *Salmonella* ser. Poona as a response to various antibiotics after growth on M9 defined medium whereby the glucose concentration was 1 % or 2 %. Error bars represent standard deviation where n=3.

6.5.4. Possible effects of glucose concentration on activity of anti-microbials against the Enterobacteria

Previous literature noted that the addition of glucose decreased the activity of the antibiotics studied (Toama *et al.*, 1978) and the addition of glucose to BPA medium containing certain peptones showed this to be true; for example yeast peptones as constituents of the growth medium produced *E. coli* which exhibited increased resistance to all three aminoglycosides where supplemental glucose was added to the BPA. In all of these cases, the concentration of the carbohydrate did not greatly affect the IZD values. *S.* ser. Typhimurium displayed similar behaviour, as susceptibility to amikacin was considerably decreased after growth on casein and soy based BPA with glucose in comparison with those cultures grown without the carbohydrate. Growth of *S.* ser. Typhimurium on casein peptone C7 exhibited a notable decrease in resistance to amikacin as the concentration of glucose was increased. Soy peptone cultures were dissimilar, as they only produced variable susceptibility dependent on the presence of glucose; no obvious pattern was discerned between the different concentrations. Where addition of glucose caused the antibiotic to have a lesser effect on the organisms this may have been due to increased growth rate and yield which diluted the antibiotic to organism ratio or, if efflux pumps were present, glucose would provide an increased energy source for this process.

In contrast to these observations were those cases in which, after growth on specified peptones as constituents of the growth medium, a significant difference in IZD values was exhibited, whereby the activity of the antimicrobial increased with higher concentrations of glucose. Fewer examples showing this relationship were observed: *E.*

coli became more susceptible to gentamicin where glucose was added to ISA medium. This supplement caused the resultant cultures to become clinically-susceptible, whereas ISA which lacked additional carbohydrate, produced clinically-resistant organisms. Susceptibility of *E. coli* to amikacin after growth on M9 medium with a higher percentage of glucose also showed a slight increase in comparison with organisms cultured on media of lower carbohydrate concentrations. *S.* ser. Typhimurium showed only one example of increased antimicrobial activity which was dependent on glucose in the media studied: i.e. a dramatic increase from complete resistance to streptomycin on G1 peptone-based BPA to borderline levels of susceptibility after growth on media which contained glucose. Explanations for these data may be that relatively fast generation times were achieved in media with additional glucose in comparison to those without supplements; consequently in the poorer media (gelatin based and M9 media) this caused more rapid growth, and therefore quicker uptake of extra-cellular components including the antibiotics. This relatively rapid growth caused increased uptake of the antimicrobial, but did not produce a yield of organisms sufficient to dilute the antibiotic. Alternatively it is known that the transport of streptomycin and gentamicin is dependent on active transport (Bryan and Van Den Elzen, 1977), thus in a gelatin based medium the addition of glucose may enable these antibiotic compounds to be taken up into the organism; such organisms cultured on these growth media would exhibit increased sensitivity in comparison with those cultured without the glucose supplement. Peptones of greater nutritional content (e.g. soy and yeast) were already capable of producing a high yield independent of additional glucose, and so bacteria subsequently cultured on these media did not display this trend.

The most common outcome was that the addition of glucose to the growth medium did not significantly affect the susceptibility of the Enterobacteria thereby cultured. For example, where it was noted that peptones as constituents of the media did not significantly affect susceptibility of *S.* ser. Typhimurium to both amikacin and gentamicin (Figures 5.2 and 5.5 respectively) the inclusion of glucose in the gelatin peptone-based medium did not subsequently alter the resistance of the cultures generated. Therefore, increased availability of energy sources did not affect the susceptibility of the Enterobacteria on those peptones.

Of note was that *E. coli* cultured on M9 agar medium, with different concentrations of supplemental glucose, displayed a significant difference in susceptibility with the aminoglycoside amikacin. Thus it is not surprising that susceptibility to amikacin was altered by glucose concentration as a supplement to the BPA media investigated. The same was true of *S.* ser. Typhimurium with streptomycin.

6.6. Effect of magnesium on antibiotic susceptibility

The addition of $MgSO_4$ to the culture medium was expected to decrease the activity of antibiotics, as numerous studies have previously found this to be the case (Bryan and Van Den Elzen, 1977; Toama *et al.*, 1978; Raulston and Montie, 1989). Again varying concentrations were added in order to determine the effect of $MgSO_4$ on susceptibility of Enterobacteria to various antibiotics, whereby the sole nutrient source (peptone) was of different biological origin or manufacturer.

6. Effects of media supplements on antibiotic susceptibility tests

6.6.1. Effect of $MgSO_4$ concentration on activity of anti-microbials against *Escherichia coli*

$MgSO_4$ as a constituent of the growth medium did not markedly affect the subsequently cultured *E. coli* with respect to their susceptibility to the antimicrobials investigated. Where the medium was BPA of variable peptone source only one peptone-antibiotic combination (*E. coli* grown on peptone M6 based BPA with streptomycin) produced different IZD values dependent on addition of $MgSO_4$ (Table 6.3). However two out of three antimicrobials tested produced significantly different activity dependent on concentration of $MgSO_4$ added to ISA medium, a complex medium recommended for use in the standardized disk diffusion method.

Table 6.3. Statistical P values for the effect of $MgSO_4$ concentration on the activity of various antibiotics, against *Escherichia coli,* after growth on different media. Values ≤ 0.050 indicate that the zone of inhibition is significantly affected by the concentration of $MgSO_4$ present in the medium.

Medium	Antibiotic				
	Amikacin	Cephalothin	Ciprofloxacin	Gentamicin	Streptomycin
M6 BPA	0.370	–	–	0.476	0.026
Y1 BPA	0.330	–	–	0.132	0.467
Y4 BPA	0.092	–	–	0.370	0.265
M9 Agar	0.141	0.946	0.042	–	–
ISA	0.012	–	–	0.330	0.019

The susceptibility of *E. coli* which had been cultured on BPA media of differing peptone content (peptones M6, Y1 and Y4) to the aminoglycosides amikacin (Figure 6.10 a), gentamicin (Figure 6.10 b) and streptomycin (Figure 6.11) was generally not altered by the addition of $MgSO_4$. However, growth of this organism on M6 peptone-based BPA generated cultures which exhibited lower sensitivity to streptomycin, where $MgSO_4$ was added.

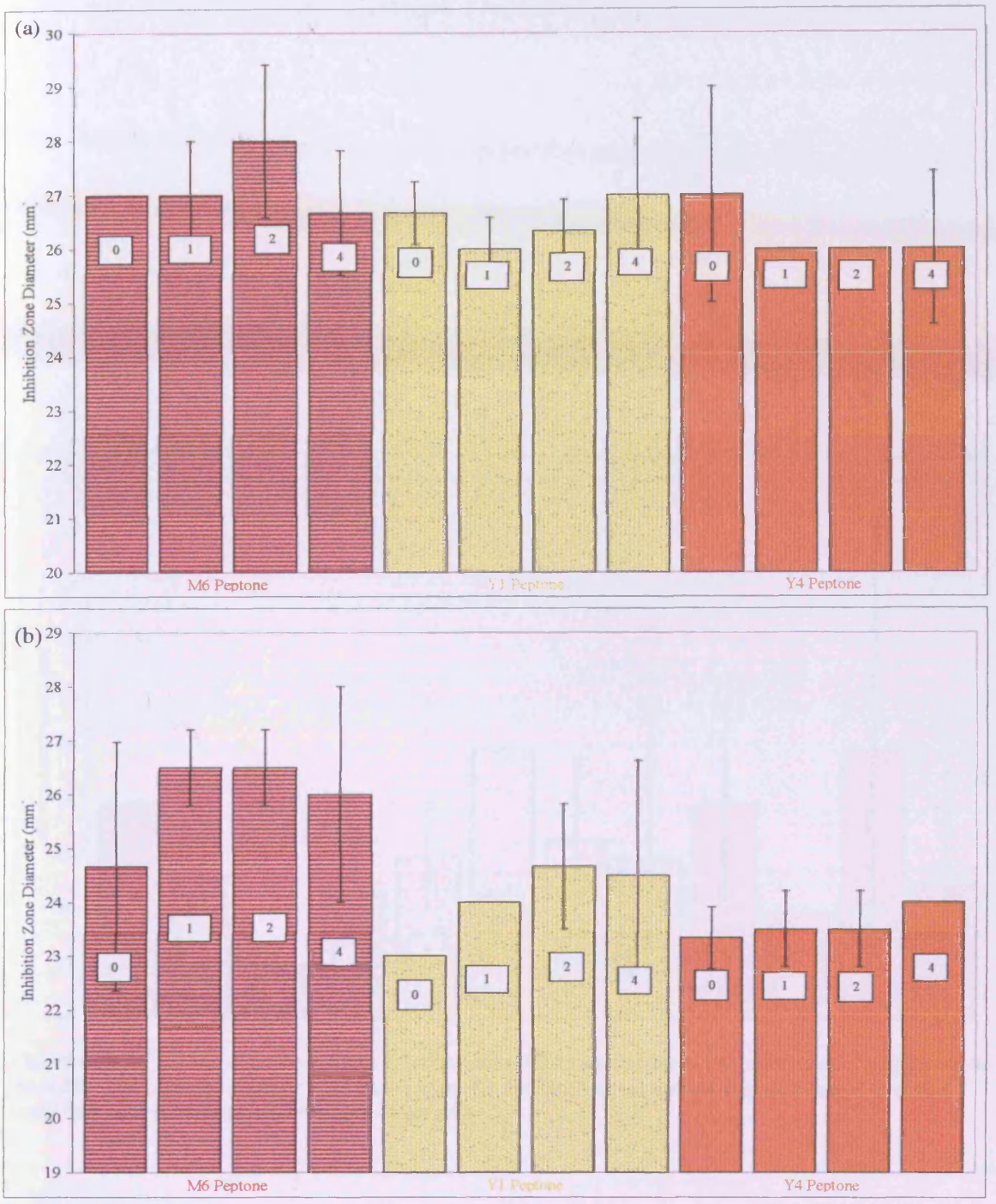

Figure 6.10. Inhibition zone diameters of *Escherichia coli* as a response to (a) amikacin (30 µg) and (b) gentamicin (10 µg) grown on three different buffered peptone agar media whereby the $MgSO_4$ concentration varied from 0 to 4 mM. Error bars represent standard deviation where n=3.

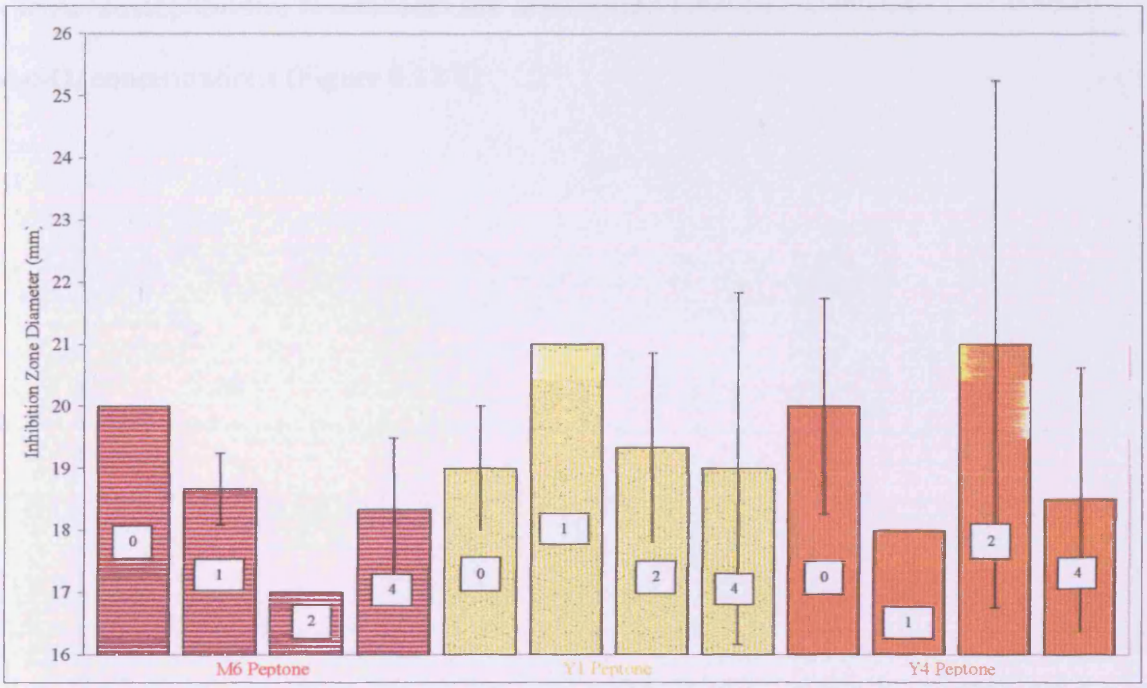

Figure 6.11. Inhibition zone diameters of *Escherichia coli* as a response to streptomycin (10 μg) grown on three different buffered peptone agar media whereby the $MgSO_4$ concentration varied from 0 to 4 mM. Error bars represent standard deviation where n=3.

6. Effects of media supplements on antibiotic susceptibility tests

The addition of $MgSO_4$ to ISA was performed for comparison, and in these studies the activity of both amikacin and streptomycin on *E. coli* was decreased as the concentration of this additive was increased. In contrast to this observation was the activity of gentamicin in the presence of variable $MgSO_4$ concentrations: the antimicrobial was not affected by these alterations (Figure 6.12 a).

Again the defined medium M9 acted as a control, the addition of divalent ions affected the susceptibility of *E. coli* to ciprofloxacin, only after growth on this minimal medium, whereas susceptibilities to amikacin and cephalothin remained unchanged by increased $MgSO_4$ concentrations (Figure 6.12 b)

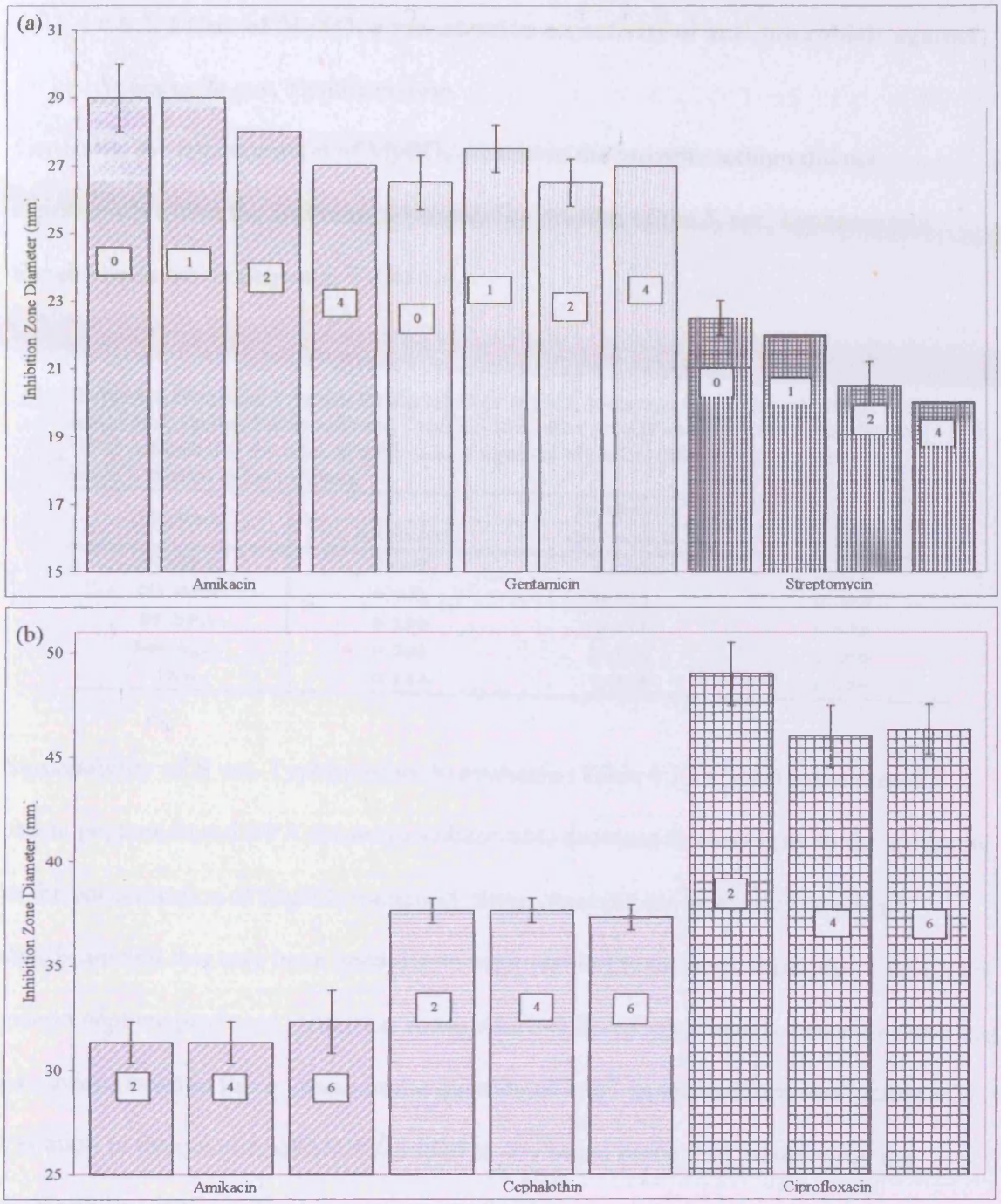

Figure 6.12. Inhibition zone diameters of *Escherichia coli* as a response to various antibiotics after growth on (a) a commercially available Iso-sensitest agar and (b) M9 defined medium whereby the $MgSO_4$ concentration varied from 0 to 6 mM. Error bars represent standard deviation where n=3.

6. Effects of media supplements on antibiotic susceptibility tests

6.6.2. Effect of $MgSO_4$ concentration on activity of anti-microbials against *Salmonella* ser. Typhimurium

Generally, the concentration of $MgSO_4$ present in the growth medium did not significantly affect the antibiotic susceptibility profiles of the *S.* ser. Typhimurium thereby cultured; as shown in Table 6.4.

Table 6.4. Statistical P values for the effect of $MgSO_4$ concentration on the activity of various antibiotics, against *Salmonella* ser. Typhimurium, after growth on different media. Values ≤ 0.050 indicate that the zone of inhibition is significantly affected by the concentration of $MgSO_4$ present in the medium.

Medium	Antibiotic		
	Amikacin	Gentamicin	Streptomycin
C7 BPA	0.095	0.002	0.392
G1 BPA	0.041	0.311	0.392
S5 BPA	0.136	0.241	0.449
M9 Agar	0.008	0.136	1.000
ISA	0.114	0.267	0.976

Susceptibility of *S.* ser. Typhimurium to amikacin (Table 6.13 a) after growth on C7 casein peptone-based BPA showed an observable decrease in sensitivity to the antibiotic as the concentration of $MgSO_4$ increased; these observations were not statistically significant and this may have been due to high variability between replicates. Growth on gelatin peptone produced inhibition zones which differed significantly however there was no obvious trend in these values as the quantity of Mg^{2+} in the medium was increased. Variation in the quantity of $MgSO_4$ added to soy based media had no effect on the susceptibility of *S.* ser. Typhimurium to amikacin.

The activity of gentamicin on this organism with varying Mg^{2+} content of the culture medium was also dependent on the type of peptone employed (Table 6.13 b). Casein based-BPA produced considerably different IZD values when $MgSO_4$ was added to the medium, however increasing the concentration of this compound did not cause a notable

difference. Cultures produced on gelatin peptone generated a similar pattern; however these values displayed high standard deviations and so were not found to be statistically significant. Again soy peptone medium with variable quantities of supplemental Mg^{2+} did not generate a significant difference in inhibition zones surrounding the gentamicin disks. Streptomycin activity remained unchanged irrespective of the Mg^{2+} content in the different peptone-based media (Figure 6.14). Casein and gelatin peptone-based BPA continued to generate resistant organisms whereas *S.* ser. Typhimurium grown on soy peptone produced borderline IZD values. These soy based media of variable $MgSO_4$ concentrations cultured organisms which marginally decreased in susceptibility to the antimicrobial as Mg^{2+} increased, however this was barely noticeable with the lowest IZD value being 10.7 ± 1.2 mm (4 mM) and the highest 12.3 ± 1.5 mm (0 mM).

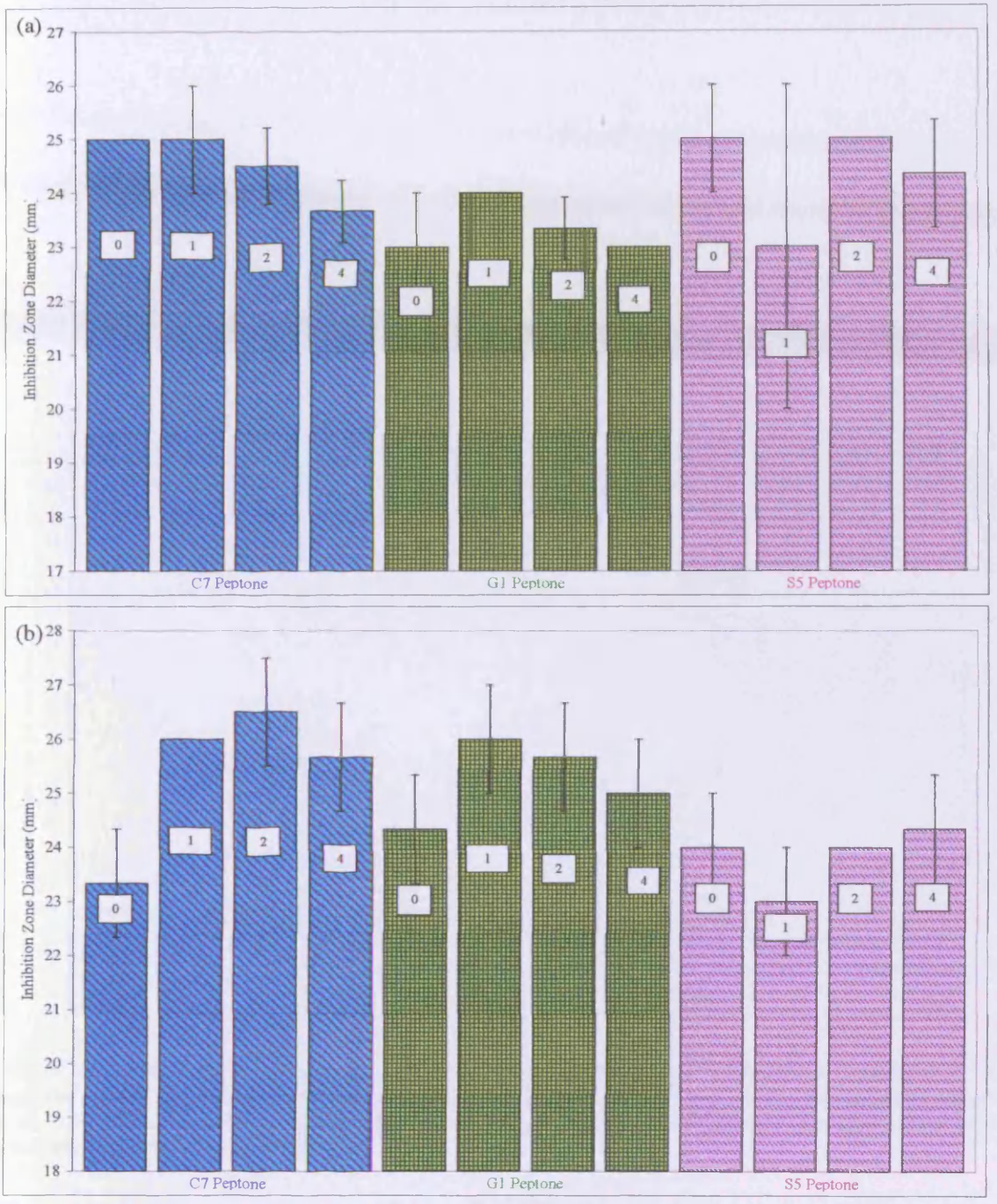

Figure 6.13. Inhibition zone diameters of *Salmonella* ser. Typhimurium as a response to (a) amikacin (30 μg) and (b) gentamicin (10 μg) grown on three different buffered peptone agar media whereby the $MgSO_4$ concentration varied from 0 to 4 mM. Error bars represent standard deviation where n=3.

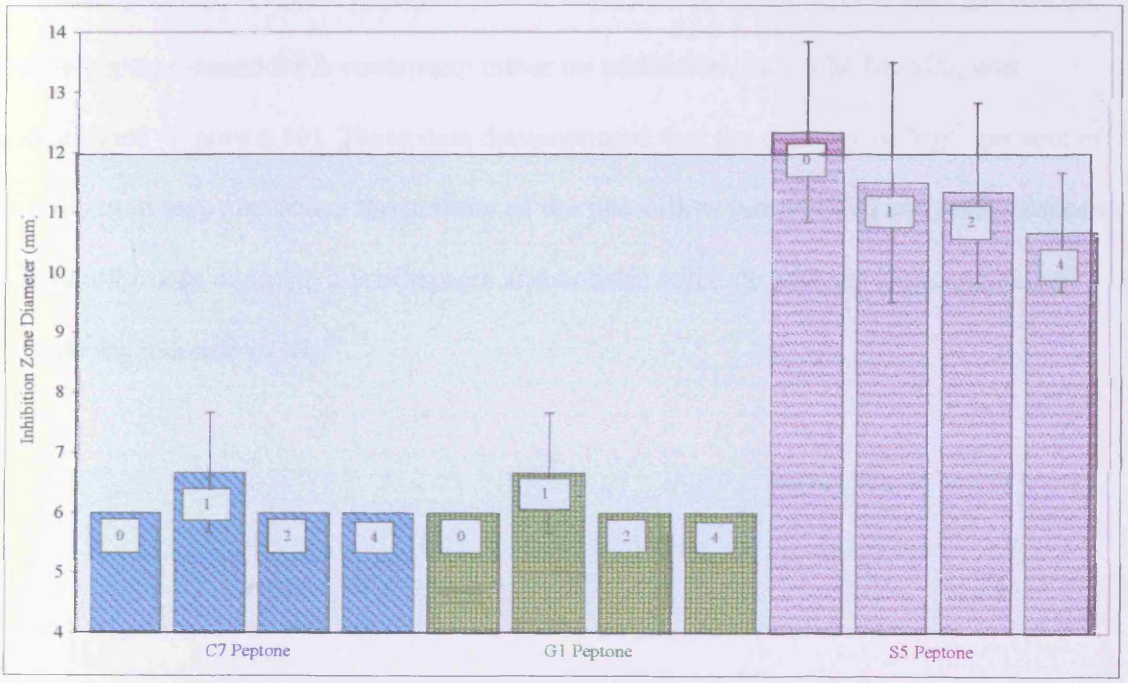

Figure 6.14. Inhibition zone diameters of *Salmonella* ser. Typhimurium as a response to streptomycin (10 µg) after growth on three different buffered peptone agar media whereby the $MgSO_4$ concentration varied from 0 to 4 mM. Resistance to the antimicrobial is indicated by the transparent blue zone. Error bars represent standard deviation where n=3.

6. Effects of media supplements on antibiotic susceptibility tests

The addition of Mg^{2+} (at concentrations between 0-4 mM) to the commercially available ISA medium did not affect the susceptibility of *S.* ser. Typhimurium to any of the aminoglycoside antibiotics investigated (Figure 6.15 a). Defined medium M9 produced similar results for gentamicin and streptomycin (i.e. $MgSO_4$ supplements at the given concentrations did not affect the disk diffusion method) with the exception of amikacin: this antibiotic exhibited an increase in activity from 2 mM to 4 mM and then a slight decrease from 4 mM to 6 mM which was determined to be significant (Figure 5.15 b). The susceptibility of *S.* ser. Typhimurium to other antimicrobial agents after growth on casein peptone-based BPA containing either no additional, or 2 mM $MgSO_4$, was investigated (Figure 6.16). These data demonstrated that the quantity of Mg^{2+} present in this medium may also affect the activity of the penicillins (ampicillin) and cephalosporins (cephalothin). In contrast, ciprofloxacin and colistin sulphate activity were unchanged despite the increase in Mg^{2+}.

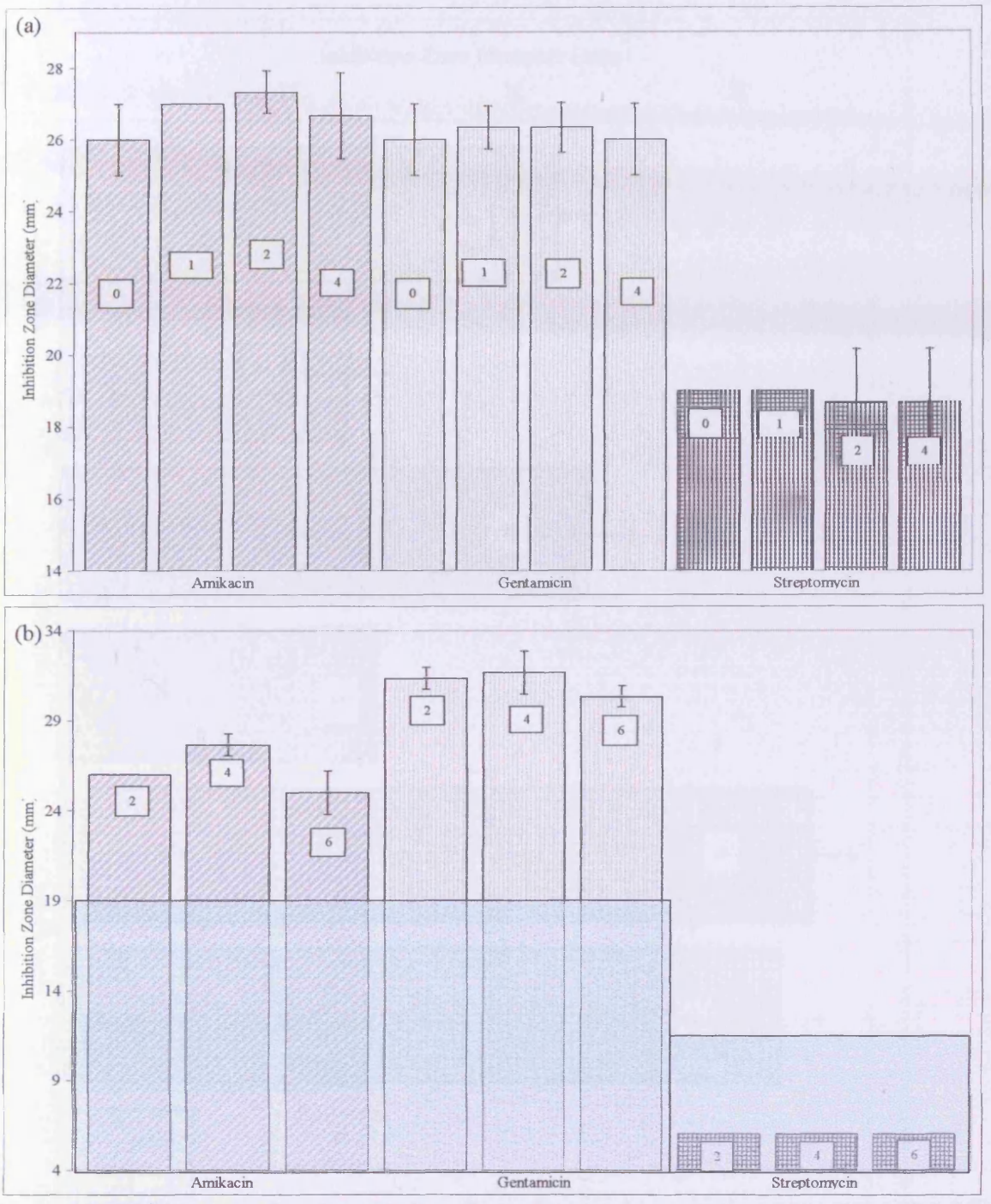

Figure 6.15. Inhibition zone diameters of *Salmonella* ser. Typhimurium as a response to various antibiotics after growth on (a) a commercially available Iso-sensitest agar and (b) M9 defined medium whereby the $MgSO_4$ concentration varied from 0 to 6 mM. Resistance to the antimicrobial is indicated by the transparent blue zone. Error bars represent standard deviation where n=3.

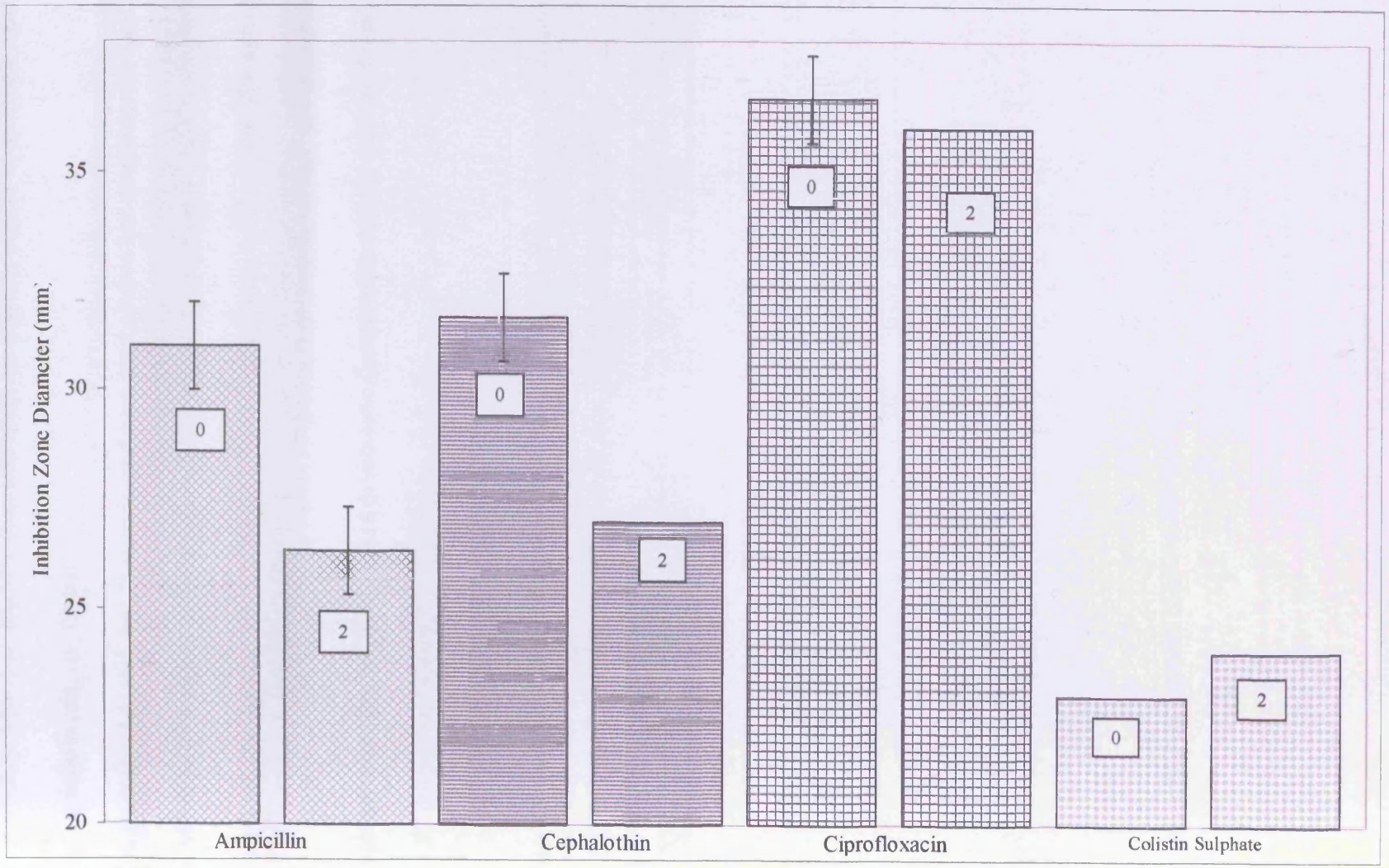

Figure 6.16. Inhibition zone diameters of *Salmonella* ser. Typhimurium as a response to various antimicrobials after growth on C7 based buffered peptone agar medium whereby the $MgSO_4$ concentration was either 0 or 2 mM. Resistance to the antimicrobial is indicated by the transparent blue zone. Error bars represent standard deviation where n=3.

6.6.3. Effect of $MgSO_4$ concentration on activity of anti-microbials against *Salmonella* ser. Poona

The susceptibility of *S.* ser. Poona to amikacin, cephalothin and streptomycin after culture on M9 defined medium was not significantly altered by additional $MgSO_4$ supplements to the growth medium (2 mM – 6 mM) (Table 6.5 and Figure 6.17). Ampicillin activity was significantly decreased as Mg^{2+} content of the medium increased and, in contrast to *S.* ser. Typhimurium, this was also true of gentamicin (gentamicin activity remained unaltered for *ST* despite an increase in $MgSO_4$).

Table 6.5. Statistical P values for the effect of $MgSO_4$ concentration on the activity of various antibiotics, against *Salmonella* ser. Typhimurium, after growth on M9 agar. Values ≤ 0.050 indicate that the zone of inhibition is significantly affected by the concentration of $MgSO_4$ present in the medium.

Medium	Antibiotic				
	Ampicillin	Amikacin	Cephalothin	Gentamicin	Streptomycin
M9 Agar	0.036	0.145	0.329	0.048	1.000

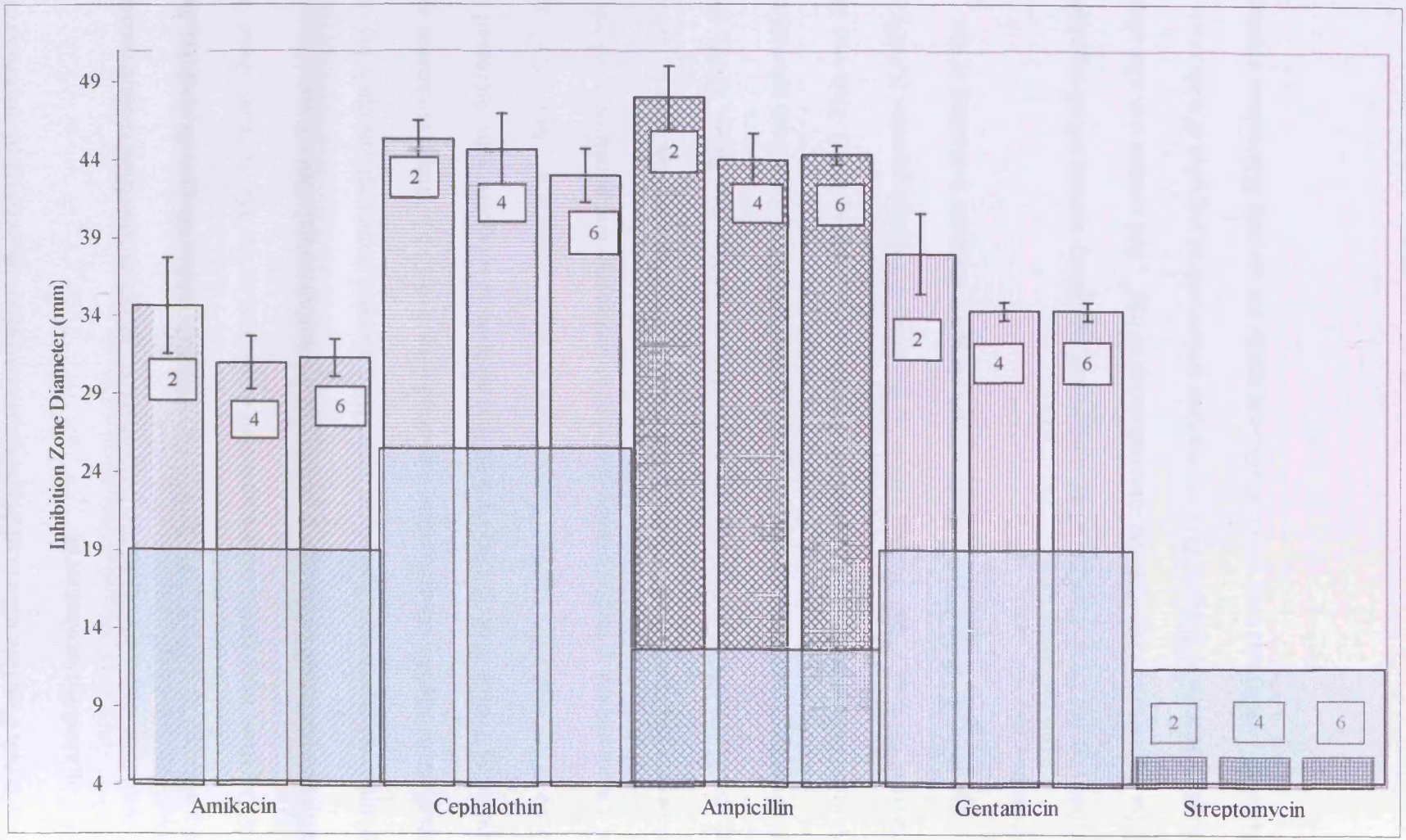

Figure 6.17. Inhibition zone diameters of *Salmonella* ser. Poona as a response to various antimicrobials after growth on M9 medium whereby the $MgSO_4$ concentration varied from 2 to 6 mM. Resistance to the antimicrobial is indicated by the transparent blue zone. Error bars represent standard deviation where n=3.

6. Effects of media supplements on antibiotic susceptibility tests

6.6.4. Possible effects of $MgSO_4$ concentration on activity of anti-microbials against Enterobacteria

The antibiotic susceptibility of the Enterobacteria grown on specified culture media was rarely affected by differing concentrations of $MgSO_4$ within the growth medium. Where the IZD values were statistically significant dependent on the Mg^{2+} content these data mostly demonstrated a decrease in activity of the antimicrobial. Only *S.* ser. Typhimurium grown on C7 peptone-based BPA showed decreased susceptibility to gentamicin as $MgSO_4$ concentration increased (Figure 6.13 b). This is consistent with the literature as Toama *et al.* (1978) showed that addition of inorganic ions decreased the activity of the antibiotics studied; furthermore divalent cations (Ca^{2+}, Mg^{2+}, Co^{2+} and Mn^{2+}) were proven to inhibit accumulation of streptomycin and gentamicin, which require energy dependent transport across the membrane, in many bacteria to include Gram positive and Gram negative organisms (Bryan and Van Den Elzen, 1977). Finally addition of Mg^{2+} to *Pseudomonas aeruginosa* protected this species from tobramycin by preventing initial uptake of the antibiotic (Raulston and Montie, 1989). This was thought to occur as binding of the divalent cations to the outer-membrane proteins (OMP) altered the ionic strength of the OMP's which in turn caused a decrease in binding of the antibiotics.

Alternatively the PhoP-PhoQ is a two component regulatory system which regulates the cellular response to extra-cellular concentrations of Mg^{2+}; this system was also shown to regulate resistance of *Salmonella typhimurium* antimicrobial peptides (Fields *et al.*, 1989; Miller *et al.*, 1989) and may also contribute towards the varying IZD values obtained.

6.7. Conclusion

The effect of differing peptone on antibiotic susceptibility was demonstrated in Chapter 6, in addition this chapter shows that the concentration of both peptone material and additional supplements (in this case glucose and Mg^{2+}) would also have a significant effect on resultant values, particularly if these components were altered simultaneously. There is a need therefore, to assess quantities of all standard media components as well as consistent sources; the media recipe which performs the best for a given task may contain a different ratio of components to that presently used.

CHAPTER SEVEN

Chemical determination of peptone constituents

7. Chemical determination of peptone constituents

7.1. Summary

Chemical analysis of the peptones investigated gave insight into the composition of each of these complex samples. Fragmentation and respective molecular weights were linked to growth properties and principle component analysis highlighted the samples which were similar to one another.

7.2. Introduction

To this point the peptones subject to investigation have only been examined with regard to their effect on microbial growth, as constituents of the culture medium, and the consequent effects this change in growth characteristics has on bacterial morphology and phenotype. Therefore analytical chemistry techniques have been utilized in order to gain a more detailed understanding of the differences and similarities between the chemical fragments of each peptone. Thus this chapter deals not with complex chemical data analysis and identification, but compares the peptone fractions and relates them to their known ability to support microbial growth.

The first analysis employed was high performance liquid chromatography (HPLC) for which a size-exclusion technique was used. This method, also known as gel filtration has previously been used to determine the molecular weight of proteins (Whitaker, 1963; Andrews, 1964). In addition, bovine proteins were successfully analysed using size-exclusion-chromatography (SEC) HPLC (Elgar *et al.*, 2000). As the peptone fragments were of unknown size, SEC was used to obtain this information, in order relate

generation times or yields of bacteria to the fragment sizes; perhaps smaller fragments were more rapidly taken up into the cell and thus aided faster growth.

A second technique used to indicate fragment size of each peptone was mass spectrometry (MS). Zhou *et al* (2002) describe the use of electrospray ionisation (ESI) with internal calibration for protein identification. ESI MS has also been applied to the identification of other biological compounds to include multiple antibiotics, for example erythromycins (Morgan *et al.*, 1998), aminoglycosides, β-lactams, tetracyclines, antifungals and glycopeptides (Morgan *et al.*, 2001). Detailed discussions for the applications of MS in biology are provided by Lloyd (1993) and Baldwin (2005).

7.3. Materials and methods

7.3.1. Statistical analysis

Principle component analysis (PCA) was used to analyse mass spectra of the peptones, this chemometric analysis had previously been used for MS data of tryptically digested proteins (Bryant *et al.*, 2001) and this technique is well summarised by Davies (2005).

7.4. Results

The raw spectra for both HPLC and MS are given in Appendix 3, these are referred to throughout this section.

7.4.1 PCA analysis of electrospray ionization mass spectrometry

The principle of ESI MS is based on fragments being ionized more softly, under atmospheric pressure, in contrast to the usual harsh ionization in a vacuum. This application prevents increased fragmentation and so is more useful when analysing larger biological compounds. Figure 7.1. shows a PCA plot of the MS data obtained for each peptone; the variables plotted on the axes are called principle components (PC) and they essentially represent the variability within the data set. Therefore Figure 7.1. depicts the similarities and differences between the mass spectrum of each peptone. The MS spectrum for each peptone is shown Appendix 3.2.

Specifically, the PCA analysis noted that there were two distinct groups for each peptone source with the exception of yeast; yeast peptones, despite differences in manufacturer and batch processes, were also the only peptone class which produced consistent results with respect to growth.

Casein peptones were divided with C1, C2 and C5 exhibiting very different fragment size from C3, C4 and C6; peptone C8 is closer to the 3, 4, 6 group. Close to the casein C1, 2, 3 group were the soy peptones S5 and S2; these two soy peptones were also distinctly separate from the remaining peptones of soy origin, S1, S4 and S3, which were adjacent to the yeast peptone cluster. Within this yeast-soy assemblage were meat peptones M6, M2 and M3; with the residual members of the meat peptone group neighbouring the casein C3, C4, C6 group which made up a large central cluster. At the base of the central group were gelatin peptones G1, G2 and G4; whereas peptones G3 and G5 were distinctly separate from all other peptones.

Observations of the raw MS spectra (Appendix 3.2) shows that C6 and C4 contain a higher quantity of material per unit mass.

7. Chemical determination of peptone constituents

In summary, all peptones of the same origin were split into two distinct groups after analysis by PCA with the single exception of yeast peptones. Great diversity was also displayed for generation times and yields within all these same peptone categories; the only consistent group was yeast and this trend continues throughout the chemical analysis.

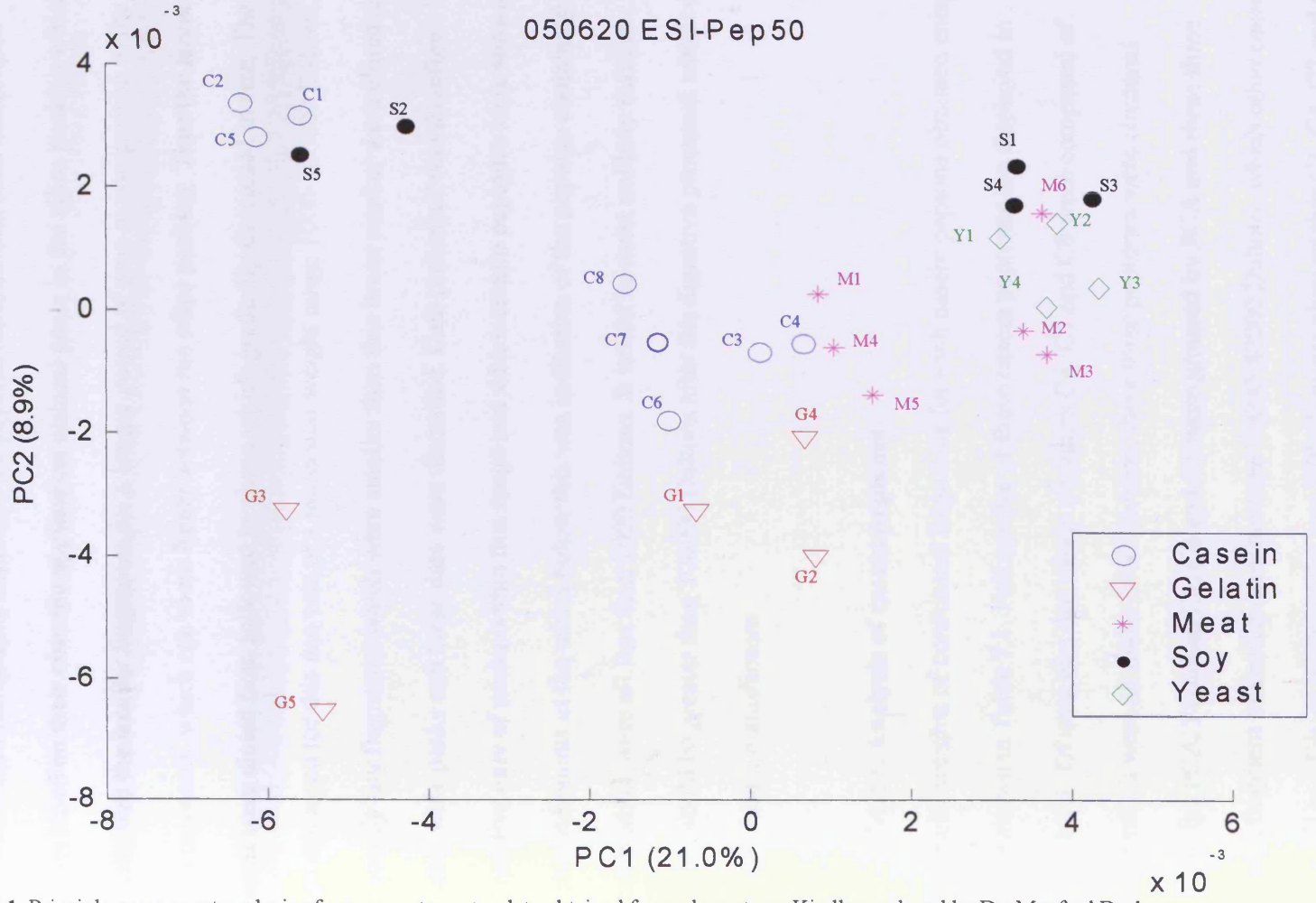

Figure 7.1. Principle component analysis of mass spectrometry data obtained for each peptone. Kindly produced by Dr. Manfred Beckman.

7.4.2 Analysis of HPLC and ESI MS spectra

Size exclusion chromatography separated the peptone samples into their constituent fragments based on their size: the application utilized pores in the silica packing which essentially trap the smaller fragments for a longer period of time in comparison to the larger components, which run more freely between the silica packing. Thus the larger fragments were eluted first, followed by those which gradually decreased in size. The column employed for this use was of a molecular weight range 1000-300000 Daltons, consequently any fragments which were smaller than this lower marker were eluted as noticeably wide peaks and these data were discarded. Data presented was therefore representative only of peaks within this range and where certain peptones were shown to have no components in the tables below this was indicative of the peptone containing fragments which were all less than 1000 Daltons. It would be most unlikely that any fragments would be greater than 300000 Daltons after the digestive processes which were performed during manufacture.

7.4.2.1. Analysis of casein peptones

The molecular weights of constituent fragments for each casein peptone obtained using HPLC are shown in Table 7.1. Full spectra of these casein peptones are displayed in Appendix 3.1.1. Of note was that casein peptones C4, C7 and C8 were composed of fragments which were all under 1000 Daltons; these three peptones were clustered together in the PCA. Peptones C1, C2 and C5 were grouped by PCA and these three displayed a fragment of similar size, between 36000-40000 Daltons, which other casein peptones did not. Observations of the raw HPLC spectra showed that C3, C4, C7 and C8

all gave rise to a single, sharper peak at a retention time between 7.10 and 7.32 min; These peptones were all relatively close together on the PCA and were all digested by acid hydrolysis. The remaining casein peptones (C1, C2, C5 and C6) were digested by enzymatic processes and depict wider, double or triple peaks of a retention time between 4 and 5 min.

Observations of the raw MS spectra (Appendix 3.2) showed that C6 and C4 contain a higher quantity of material per unit mass.

A wide range of generation times were produced by bacteria cultured on casein peptone-based media and this is reflected is in the diverse make up of the individual examples. Of note was that C6 peptone, which contained the highest number of fragments in the casein group and a high quantity of material per unit mass, always produced the highest yield of bacteria grown on a casein peptone-based medium. In contrast, those casein peptones which did not contain many fragments, particularly C8, generated notably low yields in comparison with the other casein-based media.

7.4.2.2. Analysis of gelatin peptones

Again there was good correlation between the PCA for MS and the HPLC results (Table 7.2): peptones G3 and G5 both contained a larger constituent fragment at ~45000 Daltons and these samples were close together on PCA. This was in contrast to gelatin peptones G1, G2 and G4, also grouped together by PCA, which exhibited a relatively smaller fragment at 41000-42000 Daltons. Generally the gelatin peptones were similar in constitution; containing two main fragments within the weight range measured. Yields of bacteria grown on gelatin based media differed significantly however the HPLC analysis

did not reflect this. However, the raw MS spectrum for G3 (Appendix 3.2.2.3) showed that the peak of m/z ratio 175.46, present on all other gelatin peptone spectra, was absent. This lack of this peak distinguishes G3 from the other peptones. All peaks eluted using this method were within the weight range of the column (Appendix 3.1.2) whereas peptones originating from different sources exhibited large quantities of smaller components; the lack of these smaller components, thought to be amino acids, may cause slower uptake of nutrients due to extra-cellular processes required to digest the larger molecules before uptake.

Table 7.1. Molecular weights for each fragment of the casein-based peptones as determined by high performance liquid chromatography. Data are the mean ± standard deviation where n = 3.

Peptone		Number of fragments	Mean Molecular Weight $\times 10^3 \pm$ Standard Deviation
Casein	1	2	40.2 ± 0.6 7.9 ± 0.0
	2	2	62.7 ± 0.6 39.6 ± 0.3
	3	1	3.2 ± 0.0
	4	0	
	5	2	36.4 ± 0.5 17.4 ± 2.8
	6	3	66.1 ± 0.8 29.3 ± 0.4 4.9 ± 0.4
	7	0	
	8	0	

Table 7.2. Molecular weights for each fragment of the gelatin-based peptones as determined by high performance liquid chromatography. Data are the mean ± standard deviation where n = 3.

Peptone		Number of fragments	Mean Molecular Weight $\times 10^3 \pm$ Standard Deviation
Gelatin	1	2	42.6 ± 0.0 35.4 ± 0.6
	2	2	41.4 ± 0.0 32.7 ± 0.0
	3	2	45.4 ± 0.0 30.4 ± 2.2
	4	2	41.6 ± 0.3 29.8 ± 2.5
	5	2	45.4 ± 0.0 32.2 ± 2.0

7.4.2.3. Analysis of meat peptones

The diverse meat peptones all generated between two and three fragments on HPLC analysis, however these fragment weights did differ noticeably (Table 7.3). All examples exhibited a fragment at approximately the 40000 Dalton mark; in addition peptones M1 and M2 produced components of a higher weight (51000 and 46000 Daltons respectively) and M4 and M5 displayed relatively low molecular weight masses (4000 and 2000 respectively). Interestingly number of fragments did not substantially differ, ranging between two or three. The raw HPLC spectra for all meat peptones varied considerably, confirming the diversity of the group. Of note was peptone M6 (Appendix 3.1.3.6) which was noticeably different relative to the other meat peptones producing two distinctly different, sharp peaks. For the raw MS spectra M4 (Appendix 3.2.3.4) was noted to be particularly different in comparison with the remaining samples: it contained markedly fewer substantial peaks. This was of importance though as peptone M4-based media consistently cultured poor yields at 24 h, whereas the remaining meat peptone samples, as constituents of growth media, generated yields which were relatively closer in value.

7.4.2.4. Analysis of soy peptones

HPLC analysis (Table 7.4) and raw spectra (Appendix 3.1.4) of soy peptones did not obviously match the PCA for MS. Here, peptones S2 and S4 both gave rise to relatively lower component fragments (three and four) in comparison with S1, S3 and S5 which generated six or seven component weights each. However PCA grouped together S2 and S5, and then separately clustered S1, S3 and S4; this does not reflect the MS data. Peptone S3 does not display the higher weight fragments, 70000-50000 Daltons which the other soy samples possess. The HPLC raw spectrum for peptone S3 (Appendix

3.1.4.3) indicates very little material per unit mass, which again is in contrast to the ESI MS raw spectrum (Appendix 3.2.4.3). The MS spectra do however show a great likeness between composition and quantity of S2 and S5, matching the PCA; here both these peptones appear to have relatively fewer peaks in comparison to S1, S3 and S4 (forming a separate group after PCA).

Soy peptones as constituents of growth media all produced good yields in comparison with peptones of other origins; however within the group S2 consistently gave rise to the highest yields and S3, the lowest. This was unexpected as S2 contains the fewest fragment sizes.

10

7.4.2.5. Analysis of yeast peptones

Yeast peptones exhibited great similarity based on PCA and each yeast peptone produced fragments at approximately 61000, 42000 and 5000 Daltons as shown in Table 7.5 (peptone Y4 generated an 8000 Dalton fragment rather than a 5000). Peptone Y1 produced a relatively different spectrum in comparison with the other yeast peptones as this example contained only three fragments whereas the remainder of the group constituted 6-7 different component weights. Surprisingly peptone Y1 was the best growth nutrient of all the yeast peptones with respect to yield at 24 h, and Y2 based media gave rise to the lowest yields; the differences are not notable in the raw spectra for

20 HPLC or MS.

Table 7.3. Molecular weights for each fragment of the meat-based peptones as determined by high performance liquid chromatography. Data are the mean ± standard deviation where n = 3.

Peptone		Number of fragments	Mean Molecular Weight $\times 10^3$ ± Standard Deviation
Meat	1	3	51.3 ± 0.0 43.4 ± 0.0 5.9 ± 2.8
	2	3	45.6 ± 5.2 39.0 ± 1.4 12.4 ± 5.4
	3	3	40.2 ± 0.6 38.7 ± 0.0 9.5 ± 3.7
	4	2	39.7 ± 2.8 4.1 ± 4.4
	5	3	41.3 ± 0.6 11.5 ± 3.5 2.0 ± 4.3
	6	3	40.6 ± 0.0 37.1 ± 0.5 6.9 ± 4.1

Table 7.4. Molecular weights for each fragment of the soy-based peptones as determined by high performance liquid chromatography. Data are the mean ± standard deviation where n = 3.

Peptone		Number of fragments	Mean Molecular Weight $\times 10^3$ ± Standard Deviation
Soy	1	6	66.2 ± 0.0
			62.3 ± 0.0
			56.4 ± 1.1
			45.9 ± 0.8
			32.0 ± 0.0
			6.7 ± 0.6
	2	3	37.5 ± 0.0
			28.8 ± 1.7
			14.0 ± 3.1
	3	6	62.2 ± 1.0
			45.7 ± 3.5
			21.8 ± 1.7
			16.6 ± 0.6
			10.7 ± 1.4
			5.2 ± 2.5
	4	4	74.4 ± 2.2
			51.3 ± 6.5
			33.9 ± 0.0
			10.9 ± 1.8
	5	7	65.2 ± 3.3
			59.7 ± 1.6
			50.6 ± 0.8
			45.4 ± 0.0
			29.6 ± 0.0
			10.3 ± 0.0
			3.5 ± 0.4

Table 7.5. Molecular weights for each fragment of the yeast-based peptones as determined by high performance liquid chromatography. Data are the mean ± standard deviation where n = 3.

Peptone		Number of fragments	Mean Molecular Weight $\times 10^3$ ± Standard Deviation
Yeast	1	3	61.0 ± 1.3 42.3 ± 0.9 5.8 ± 5.2
	2	7	81.6 ± 1.8 61.6 ± 1.1 46.4 ± 2.8 42.6 ± 0.0 25.3 ± 0.6 12.3 ± 0.0 5.6 ± 1.4
	3	7	78.8 ± 0.0 60.7 ± 2.7 52.1 ± 0.6 44.6 ± 0.0 41.8 ± 0.0 18.4 ± 5.3 8.2 ± 2.1
	4	6	62.6 ± 0.9 54.7 ± 0.4 44.8 ± 1.1 19.3 ± 3.3 10.1 ± 1.1 5.6 ± 1.0

7.5. Conclusion

In depth analysis of these data, in order to fully understand the large quantity of information available, would greatly contribute to the process of defining an improved growth medium. It was clear that a higher number of different sized fragments was not always beneficial, as in the case of yeast peptone Y1 (containing the fewest fragments). Peptones which contained smaller constituents, below the capabilities of this HPLC system, generally gave rise to better growth; suggesting that smaller molecules were more easily taken up into the organism and so aided rapid growth. Low quantities of peptone per unit weight also caused peptones to be poorer growth medium constituents; such as meat peptone M4 (Appendix 3.2.3.4).

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CHAPTER EIGHT

Review

8. Review and further work

8.1. Review

The aims of this thesis were to record the effects of variable peptone, as a constituent of the culture medium, on the growth of microorganisms and investigate possible hypotheses for these observations. The peptones were of variable biological origin, purchased from different manufacturers and had been produced under individual batch conditions during production. The medium chosen to evaluate these peptones was buffered peptone water due to its simple recipe which contained no other undefined ingredients. Therefore *Salmonella* was the organism selected as BPW was a pre-enrichment medium for the species.

The initial growth profiles performed displayed marked differences in generation times and yields of the organisms cultivated whereby the medium contained variable peptone; not only between the different peptone groups but also within each group. Chapter three discusses these results, showing that the generation time (Table 3.3) for *S.* ser. Typhimurium ranged from 14.8 ± 3.1 min to 61.0 ± 6.8 min, these data were both obtained during growth on casein peptone-based BPW. Similarly the yield of this organism at 24 h (Table 3.4) was considerably different dependent on the nutrient source (peptone) provided: 0.49×10^9 CFU ml^{-1} in a meat peptone-based BPW and 7.04×10^9 CFU ml^{-1} in a yeast based medium. These observations were not limited to one strain or to the genus *Salmonella*: *S.* ser. Poona, *Escherichia coli* and *Staphylococcus aureus* also exhibited comparable results.

Investigation into the cause of these differences was initiated, beginning with the presence of growth inhibitors in the peptones themselves: however increasing the

concentration of peptone did not decrease the growth rate (Figures 3.1 and 3.2), indicating that this was not the case.

Subsequently Chapter 4 addressed the alteration in morphology which occurred after growth in the different peptone based media. These included EM and swim migration techniques to determine the presence of flagella and subsequent motility. Again the diversity exhibited both between peptone groups and within groups was considerable with certain media (particularly gelatin and casein) producing aflagellate, non-motile organisms; whereas *Salmonella* which were of normal, peritrichous morphology and fully motile were noted in other peptone based-media.

The lack of synthesis of flagella caused doubt with respect to the process of obtaining a complete and accurate identification of the *Salmonella* genus: which relies on the serotyping of H antigens presented on the flagella. Thus it was recorded that those organisms cultured in BPW media constituting a poorer peptone did not generate the correct serotype according to the Kauffmann-White scheme of identification. Antibiotic susceptibility tests were also observed to be greatly affected by the peptone component of the growth medium, to include the alteration of the breakpoint from sensitive to resistant with the antibiotics streptomycin and cephalothin.

The extent to which peptone, the nutrient component of the medium, affected the growth and consequently the morphology, serology and susceptibility is of considerable importance. Chemical analysis was performed to give insight into the differences in composition of the peptones investigated and found that a lack of certain amino acids, a low quantity of material per unit weight and the presence of

carbohydrates were the most common reasons to affect the growth of the subsequently cultured bacteria.

Selection of culture medium is of little realised importance: its knock on effects contributing to the resultant outcome of the scientific work. This study investigated only the complex ingredient peptone, yet surely similar effects would be found if the subject of investigation were blood, serum, malt extract, egg yolk, bile salts or any other biological ingredient included in the growth medium recipe. The differences in agar gels were well documented, due to the variable nature of the red algal seaweeds harvested for the purpose; one effect of these different agars was variation in antibiotic susceptibilities dependent on the agar component of the test medium (Toama *et al.*, 1978).

This work has outlined the importance of complex ingredients on microbial culture and growth; however it is likely that cell tissue culture would also be greatly affected by variable components of the growth medium. It would therefore be of great interest to ascertain whether these effects were substantial, and if further research into tissue culture growth media would be of great benefit.

8.2. Further work

Aflagellate *S.* ser Enterica were shown to be attenuated in murine models of infection (La Ragione *et al.*, 2003) as the organisms were less able to invade host cells in comparison to the wild type; Other studies recorded aflagellate Salmonella to be less adhesive (Robertson *et al.*, 2000). Observations of aflagellate organisms, cultured on gelatin peptone-based medium, in an infection model would be of great interest. However transfer from a nutrient poor medium to a nutrient rich medium

resulted in the synthesis of flagella, and this would probably also occur upon inoculation into the host animal.

The question of viable but non-culturable bacteria and the need to recover and culture these organisms (Bloomfield *et al.*, 1998; Lloyd and Hayes, 1995) was relevant to the observation of longer generation times in autoclaved media in comparison to filter sterilized media. In addition media which successfully enumerated healthy *Salmonella* did not necessarily produce good growth of heat-shocked organisms. These points highlight the importance of selecting media specific to the species and its present condition: viable but non-culturable organisms may indeed grow *in vitro* if provided with the right conditions, nutrients and perhaps microbial flora?

The literature is rich in articles which focus on the use of different chemical compounds in the search for a better selective enrichment broth for *Salmonella* (van Schothorst and Renaud, 1985; Patil and Parhad, 1986; Reissbrodt *et al.*, 1996) however none focus on the use of different sources of the complex ingredients. Are selective enrichment broths such as Rappaport's, Muller-Kauffmann tetrathionate or selenite F, to name a few, affected by the peptone constituent? Would the selective differential media, XLD or HE, be equally affected by the various peptones available?

For this thesis specifically the addition of supplements such as glucose and tyrosine to the migration plate assays would have been of great benefit. Supplements of various concentrations would have quantified the concentration required for motility whereas the EM procedures, with addition of supplements, were qualitative only.

8. Review and further work

Chemical analyses lack an in depth interpretation; MS spectra could be used to identify certain compounds and determine the exact composition of each peptone. However this work in itself would provide the material for an entirely separate thesis and thus the analysis is minimal and merely for basal comparison.

I have enjoyed (almost) all aspects of this work and feel privileged to have had such a wonderful opportunity and experience. Whatever future scientific projects I partake in, I will always tailor-make my media to the requirements of the work (to the best of my abilities) and purchase a batch large enough to last/as big as possible. Where batches are interchanged, quality assurance tests would be performed to ensure experimental continuity. These are the author's suggestions to all scientists using any complex ingredient in any project.

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APPENDICES

Appendix 1

1.1. Initial peptone pools from which experimental peptones were selected

The following tables show the original choice of peptones from which the twenty-nine experimental peptones, used throughout this work, were selected.

1.1.1. Comprehensive list of casein peptones

Code	Substrate: Digest	Product	Source	Product Number
-	Casein: Pancreatic	Casein	Merck	1.07213
-		Tryptone	Merck	1.10213
-		Tryptone	Merck	1.10213
C1		**Casein**	**Merck**	**1.07213**
-		Tryptone	LabM	MC5
-		Bacto Casitone	Becton-Dickinson	0259-17-9
-		Bacto Tryptone	Becton-Dickinson	211705
-		Bacto Tryptone	Becton-Dickinson	211705
-		Casein	Merck	1.07213
-		Casein Peptone II	Biotecnica	148-G
C2		**Casein I**	**Hi-Media**	**RM014**
C3	**Casein: HCl**	**Casein Technical**	**Hi-Media**	**RM013**
C4		**Vitamin Free Casein**	**Merck**	**1.02238**
-	Casein: Pancreatic	Casein	Merck	1.07213
-		Casein	DMV	CE 90 M
C5		**Tryptone**	**Organotechnie**	**19553**
-		Tryptone	Organotechnie	19553
-		Tryptone	Organotechnie	19553
-		Casein Peptone	Biospringer	HCP301
C6		**Casein Peptone**	**Merck**	**1.07213**
-		Casein Peptone	DMV	CE 90 M
-		Tryptone	Oxoid	L42
-		Tryptone	Oxoid	L42
-		Casein	Merck	1.07213
-		Casein	DMV	CE 90 M
-		Casein	DMV	CE 90 M
C7	**Casein: HCl**	**Casein**	**DMV**	**CE 90 ML**
-	Casein: Pancreatic	Casein	DMV	CE 90 M
-		Casein	Merck	1.07213
-		Casein	Merck	1.07213
-		Casein	Merck	1.07213
-		Casein	DMV	CE 90 M

Appendix 1

C8	Casein: HCl	Casein	DMV	CE 90 ML
-	Casein: Pancreatic	Casein	DMV	CE 90 M
-		Casein	Merck	1.07213
-		Casein	Merck	1.07213
-		Casein	Merck	1.07213
-		Casein	DMV	CE 90 M
-		Casein	Merck	1.07213
-		Casein	Merck	1.07213

1.1.2. Comprehensive list of gelatin peptones

Code	Substrate: Digest	Product	Source	Product Number
G1	**Gelatin: Pancreatic**	**Gelatin**	**Merck**	**1.07284**
G2		**Gelatin**	**Merck**	**1.07284**
G3		**Gelatin**	**Hi-Media**	**RM020**
G4		**Gelatin**	**Merck**	**1.07284**
G5		**Gelatone**	**Becton-Dickinson**	**265710**

1.1.3. Comprehensive list of meat peptones

Code	Substrate: Digest	Product	Source	Product Number
M1	Meat: Proteolytic	**Meat Extract**	**Merck**	**1.03979**
-	**Meat: Pancreatic**	Meat Extract	Merck	1.03979
-		Meat Extract	Merck	1.03979
M2		**Meat Peptone (Bovine)**	**Biotecnica**	**102**
-	Meat: Peptic	Meat Peptone	Merck	1.07224
-		Meat Peptone	Merck	1.07224
M3	**Meat: Pancreatic**	**Meat Peptone**	**Merck**	**1.07214**
-		Peptone from Poultry	Merck	1.10245
M4		**Meat Peptone (Porcine)**	**Biotecnica**	**307**
-	Beef Extract	Beef Extract	Becton-Dickinson	211520
-	Meat: Peptic	Meat Peptone	Solabia	2.80246
-	Meat: Pancreatic	Meat Peptone	Merck	1.07214
-	Meat: Peptic	Meat Peptone	Merck	1.07224
-	**Meat: Pancreatic**	Meat Peptone	Merck	1.07214
-		Meat Peptone	Merck	1.07214
-		Meat Peptone	Merck	1.07214
-		Meat Peptone	Biotecnica	307
M5		**Meat Peptone**	**Biotecnica**	**307**
-		Meat Peptone	Biotecnica	307
-		Meat Peptone (Porcine)	Merck	1.09827
-		Meat Peptone (Porcine)	Merck	1.09827
-		Meat Peptone (Porcine)	Merck	1.09827
M6	**Not Specified**	**Liver Powder**	**Merck**	**1.05347**

Appendix 1

1.1.4. Comprehensive list of soy peptones

Code	Substrate: Digest	Product	Source	Product Number
-	Soymeal: Papain	Soy Peptone	Merck	1.07212
S1		**Soy Peptone**	**Solabia**	**A1601**
S2		**Soytone**	**Becton-Dickinson**	**243620**
S3		**Soy Peptone**	**Biospringer**	**HSP-A**
S4		**Neutralised Soya**	**Oxoid**	**L44**
S5		**Soy Peptone**	**Solabia**	**A1601**
-		Soy Peptone	Solabia	A1601
-		Soy Peptone	Solabia	A1601
-		Soy Peptone	Solabia	A1601

1.1.5. Comprehensive list of yeast peptones

Code	Substrate: Digest	Product	Source	Product Number
-	Yeast Extract: Autolysed	Yeast Extract	Merck	1.03753
Y1		**Yeast Extract**	**Merck**	**1.03753**
Y2		**Bacto Yeast Extract**	**Becton-Dickinson**	**212750**
-		Yeast Extract	Merck	1.03753
-		Yeast Extract	Merck	1.03753
Y3		**Yeast Extract**	**Merck**	**1.03753**
-		Yeast Extract	Oxoid	L21
-		Yeast Extract	Merck	1.03753
Y4		**Yeast Extract**	**Merck**	**1.03753**

1.1.6. Comprehensive list of other peptones

Code	Substrate: Digest	Product	Source	Product Number
-	Egg: Pancreatic	Egg Peptone	Merck	1.1382
-	Meat/Casein	Bacteriological Peptone	LabM	MC24
-	Protein: Enzymatic	Bacto Peptone	Becton-Dickinson	211677

Appendix 1

1.2. Commercially available dessicated culture media

1.2.1. Unselective

- Nutrient Agar CM3 (Oxoid)

Formula	Concentration (g l^{-1})
Lab lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0

- Nutrient Broth CM1 (Oxoid)

As NA but without addition of agar.

- Tryptone Soya Agar CM131 (Oxoid)

Formula	Concentration (g l^{-1})
Tryptone	15.0
Soya peptone	5.0
Sodium chloride	5.0
Agar	15.0

- Tryptone Soya Broth CM129 (Oxoid)

As TSA but without addition of agar.

Appendix 1

- M9 Defined Medium (Neidhardt *et al.* 1974)

Component	Concentration (mM)
KH_2PO_4	22
Na_2HPO_4	42
NH_4Cl	19
$MgSO_4$	1
$CaCl_2$	0.09
NaCl	9

- Buffered Peptone Water

Component	Concentration ($g\ l^{-1}$)
Peptone	10.0
NaCl	5.0
Na_2HPO_4	3.5
KH_2PO_4	1.5

- Buffered Peptone Agar

As BPW but with additional agar: 0.3% for "sloppy" agar and 1.5% for solid agar.

Appendix 1

1.2.2. Selective differential media

- Xylose Lysine Desoxycholate Medium CM469 (Oxoid)

Salmonella and *Edwardsiella* appear as red colonies with a black centre on the red/pink agar.

Formula	Concentration ($g\ l^{-1}$)
Yeast Extract	3.0
L-Lysine HCl	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Sodium desoxycholate	1.0
Sodium chloride	5.0
Sodium thiosulphate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.08
Agar	12.5

Appendix 1

- Brilliant Green Agar (Modified) CM329 (Oxoid)

Salmonella colonies are most likely red-pink-white in colour and opaque, surrounded by zones of brilliant red in the agar.

Formula	Concentration ($g\ l^{-1}$)
"Lab-lemco" powder	5.0
Peptone	10.0
Yeast Extract	3.0
Disodium hydrogen phosphate	1.0
Sodium dihydrogen phosphate	0.6
Lactose	10.0
Sucrose	10.0
Phenol red	0.09
Brilliant green	0.0047
Agar	12.0

Appendix 1

- Hektoen Enteric Agar CM419 (Oxoid)

Green-blue colonies on the blue agar, with or without black centres, are possibly *Salmonella*.

Formula	Concentration ($g\ l^{-1}$)
Proteose peptone	12.0
Yeast Extract	3.0
Lactose	12.0
Sucrose	12.0
Salicin	2.0
Bile salts No. 3	9.0
Sodium chloride	5.0
Sodium thiosulphate	5.0
Ammonium ferric citrate	1.5
Acid fuchsin	0.1
Bromothymol blue	0.065
Agar	14.0

1.2.3. Specific application

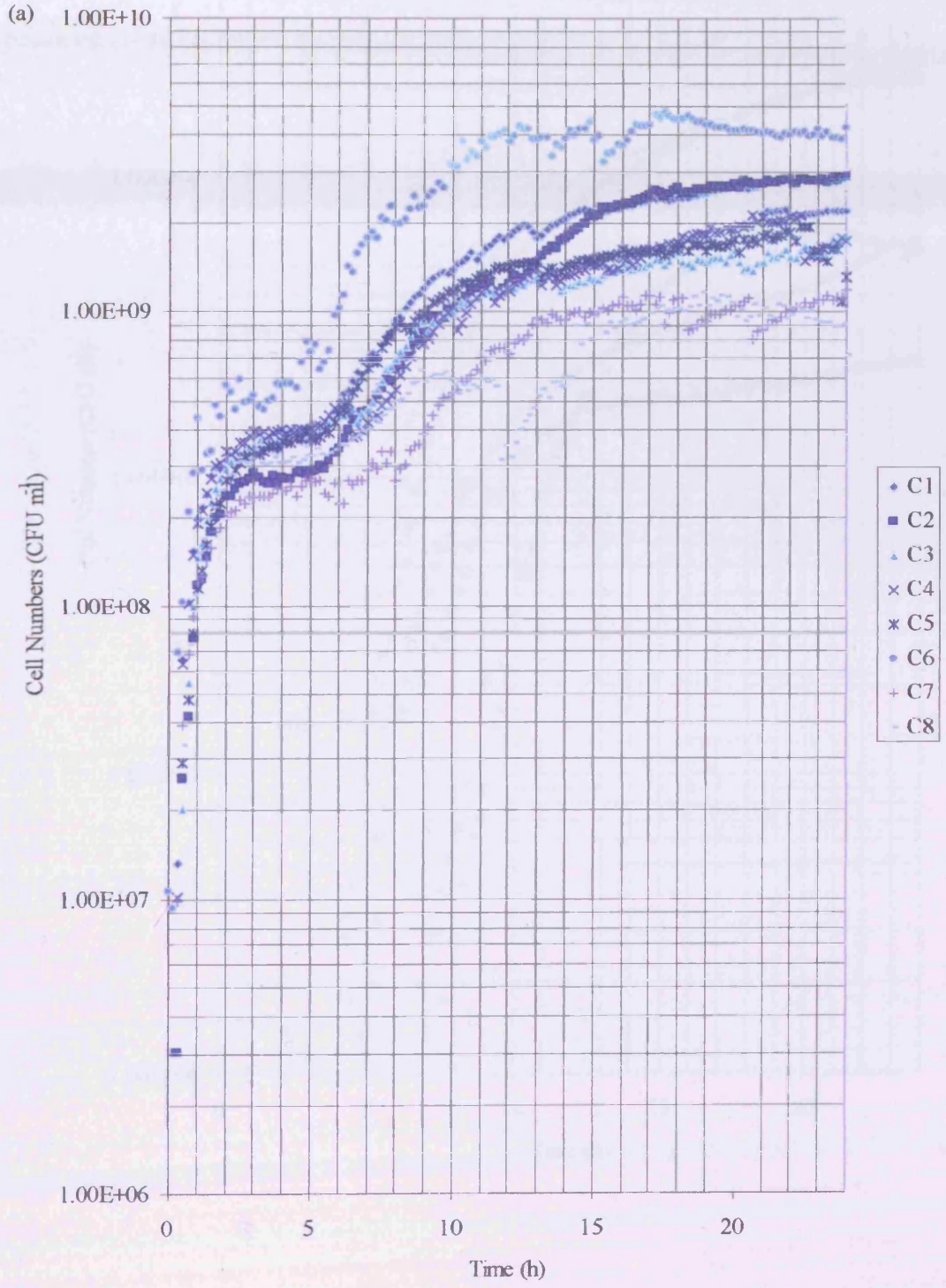
- Iso-Sensitest Agar

Formula	Concentration (g l^{-1})
Tryptone	11.0
Peptones	3.0
Glucose	2.0
Sodium chloride	3.0
Soluble starch	1.0
Disodium hydrogen phosphate	2.0
Sodium actetate	1.0
Magnesium glycerophophate	0.2
Calcium gluconate	0.1
Cobaltous sulphate	0.001
Cupric sulphate	0.001
Zinc sulphate	0.001
Ferrous sulphate	0.001
Manganous chloride	0.002
Menadione	0.001
Cyanocobalamin	0.001
L-Cysteine hydrochloride	0.02
L-Tryptophan	0.02
Pyridoxine	0.003
Pantothenate	0.003
Nicotinamide	0.003
Biotin	0.0003
Thiamine	0.00004
Adenine	0.01
Guanine	0.01
Xanthine	0.01
Uracil	0.01
Agar	8.0

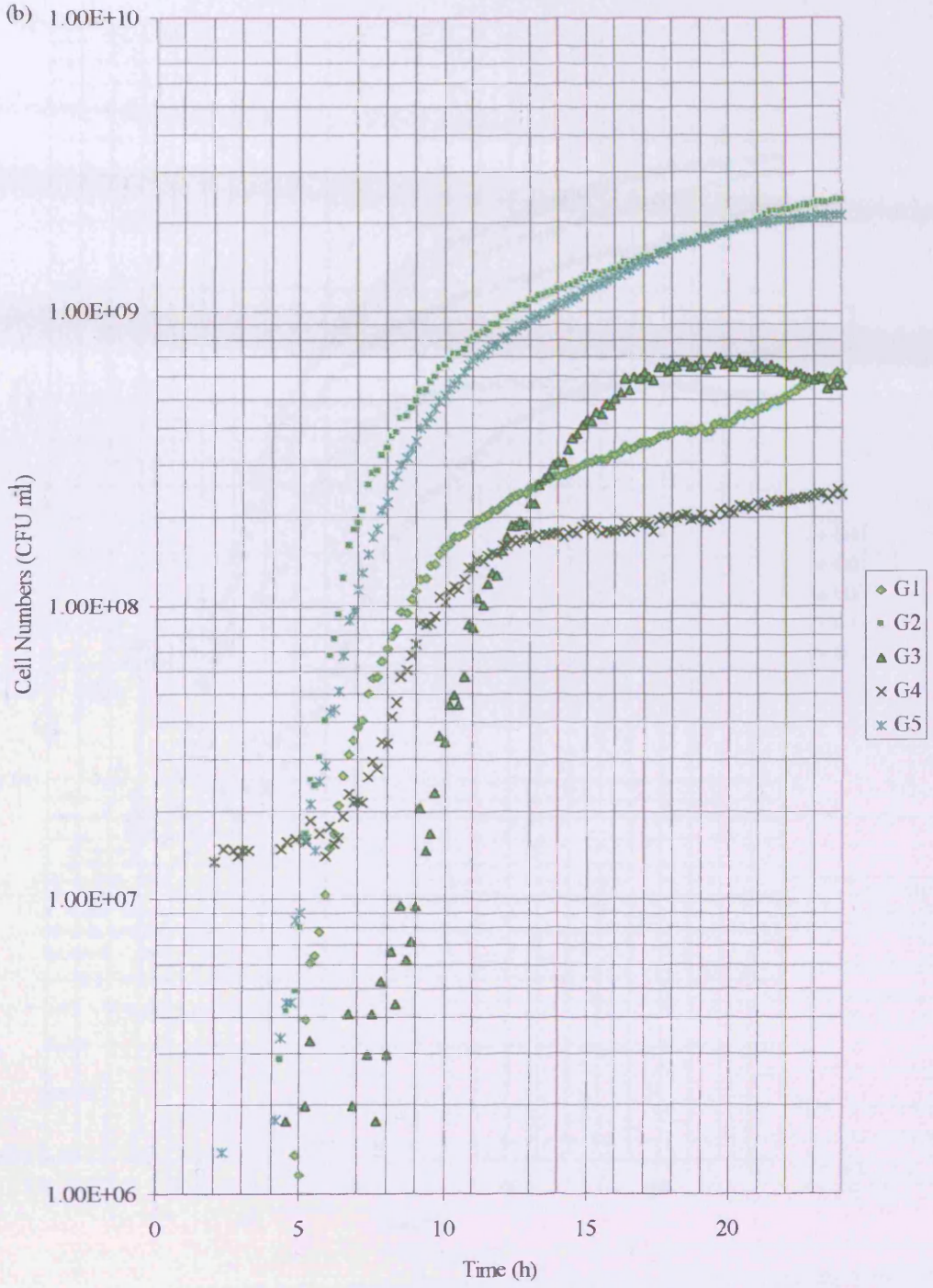
Appendix 2

2.1. Growth profiles for *Escherichia coli*

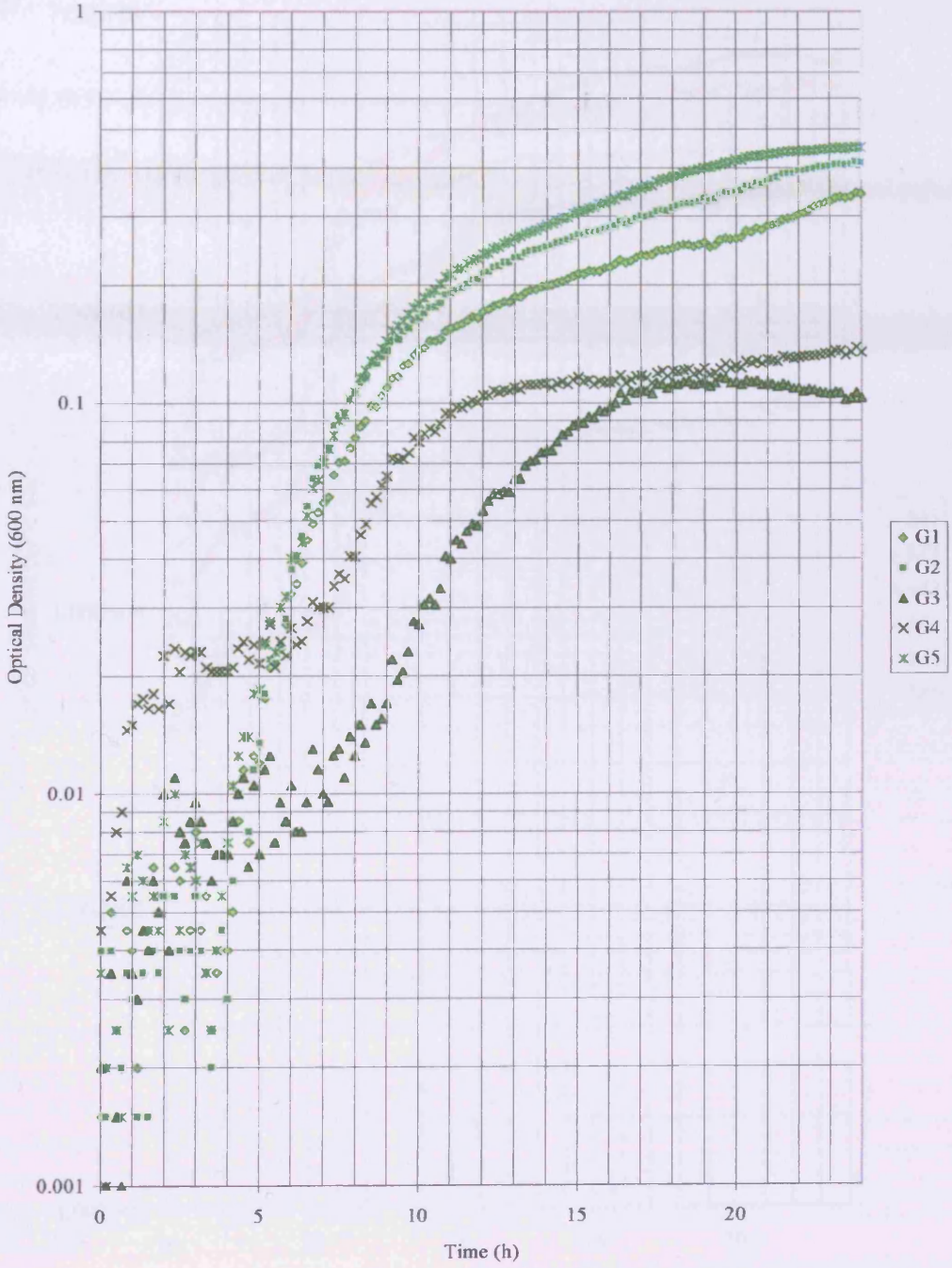
2.1.1. *Escherichia coli* growth in casein peptone-based BPW



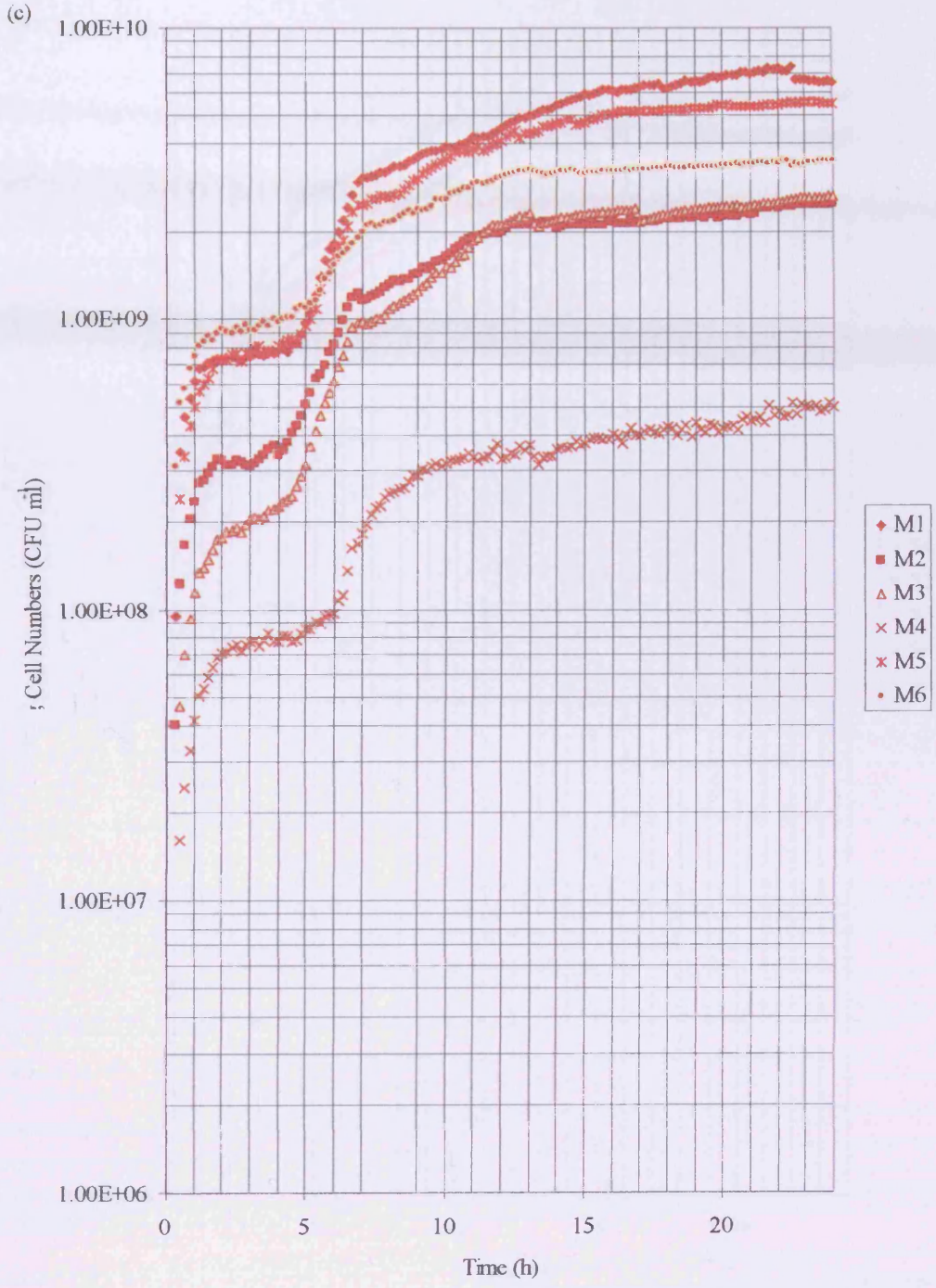
2.1.2.1. *Escherichia coli* growth in gelatin peptone-based BPW



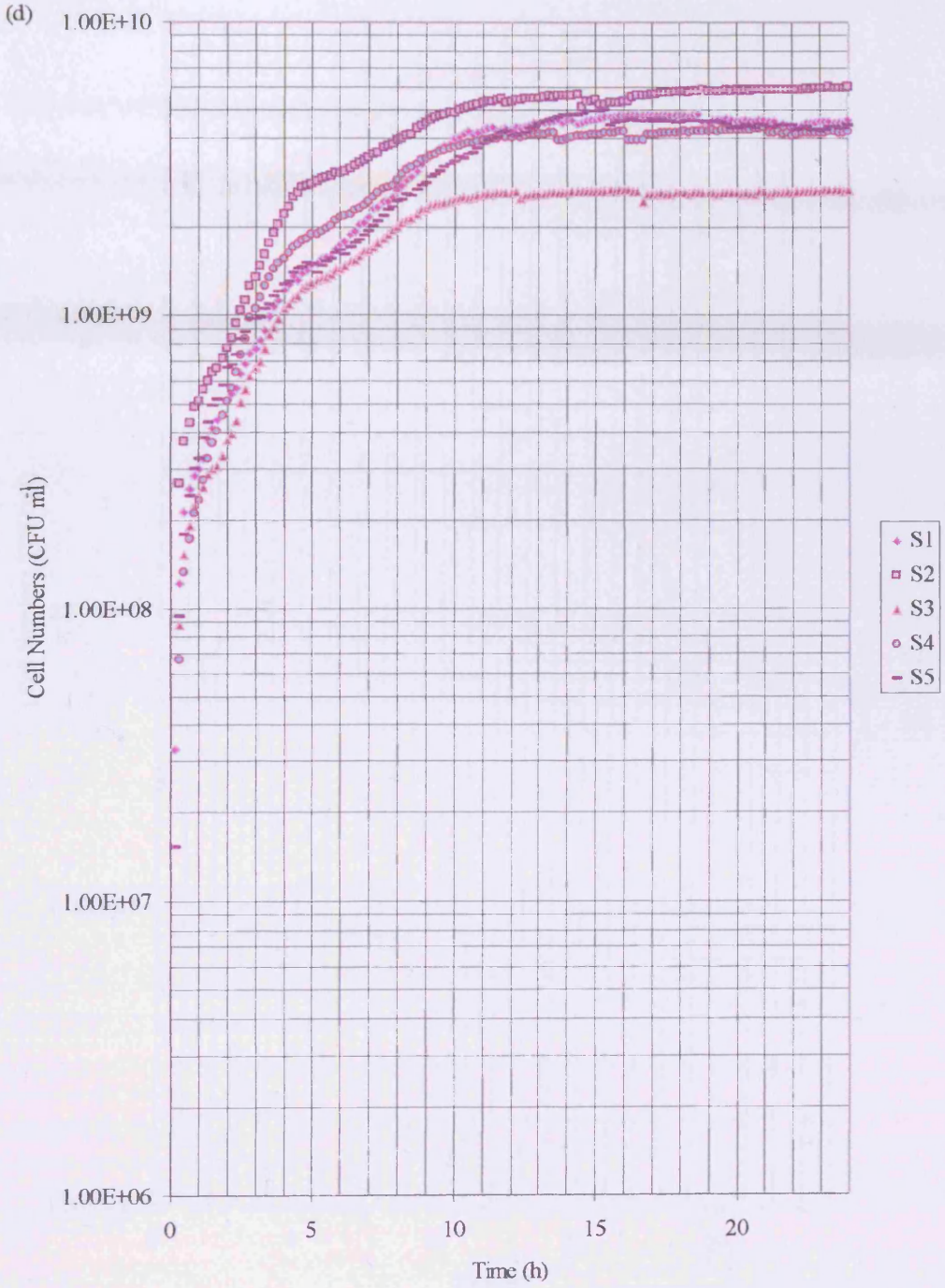
2.1.2.2. *Escherichia coli* growth in gelatin peptone-based BPW



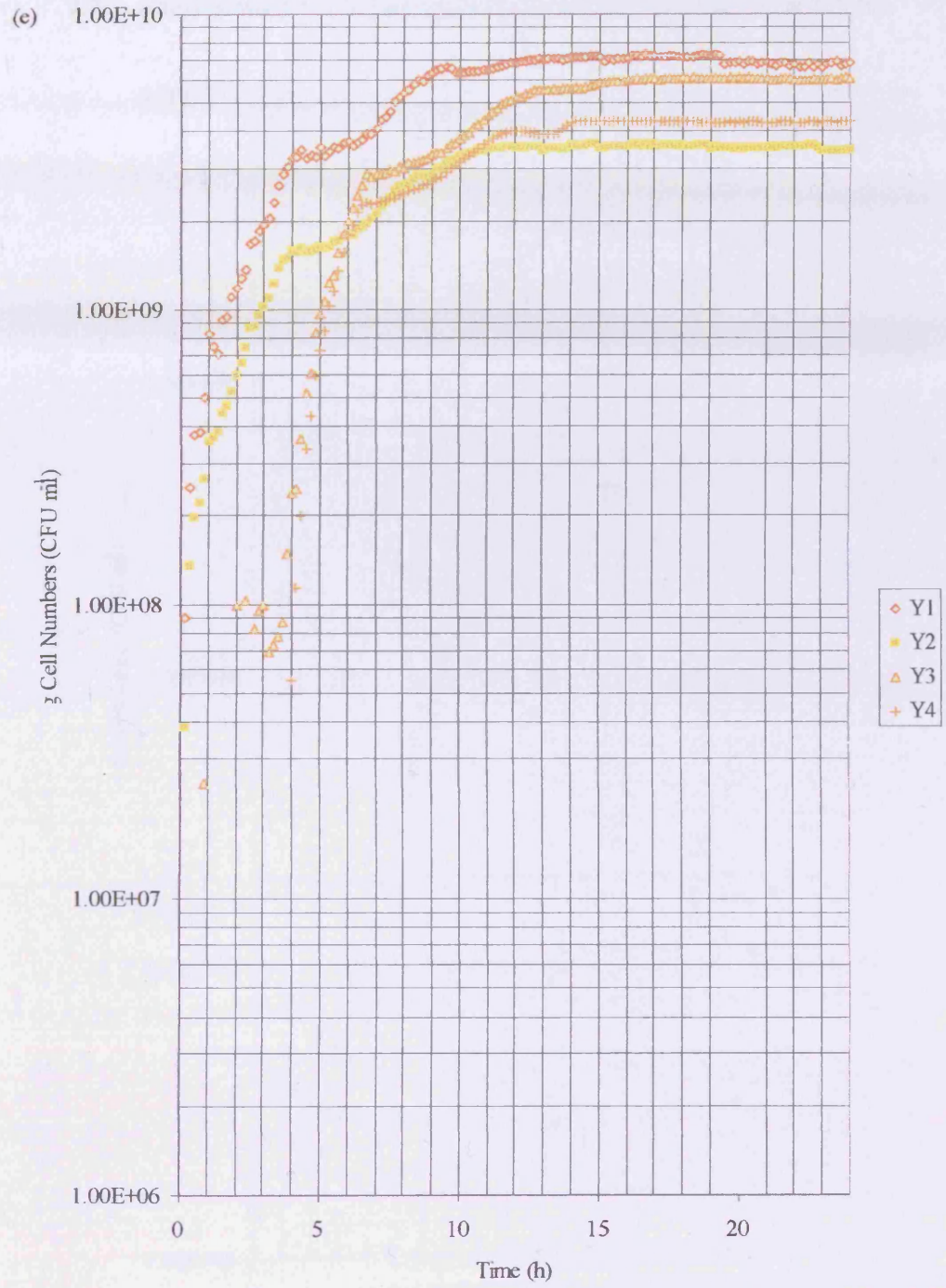
2.1.3. *Escherichia coli* growth in meat peptone-based BPW



2.1.4. *Escherichia coli* growth in soy peptone-based BPW

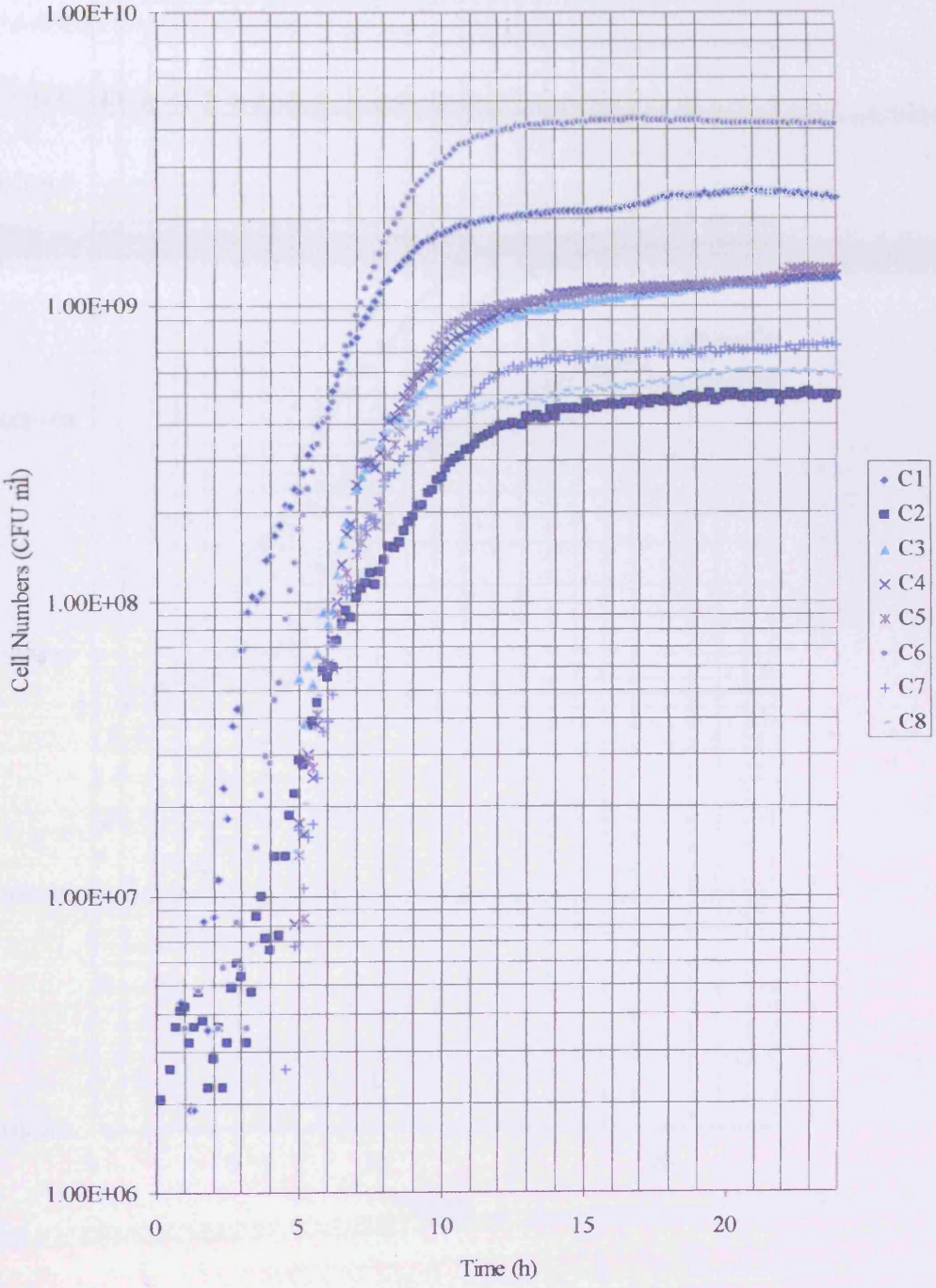


2.1.5. *Escherichia coli* growth in yeast peptone-based BPW

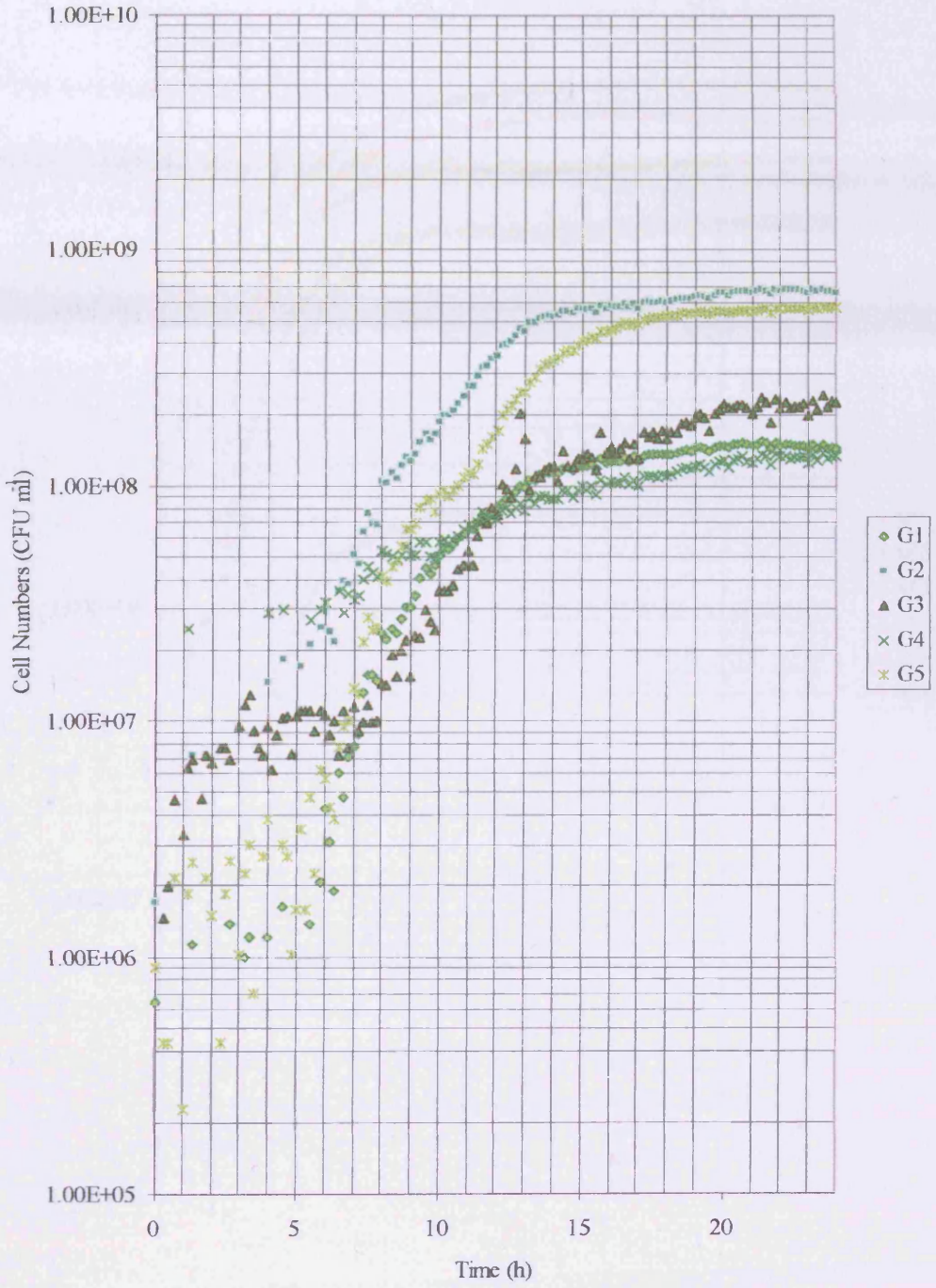


2.2. Growth profiles for *Salmonella* ser. Poona

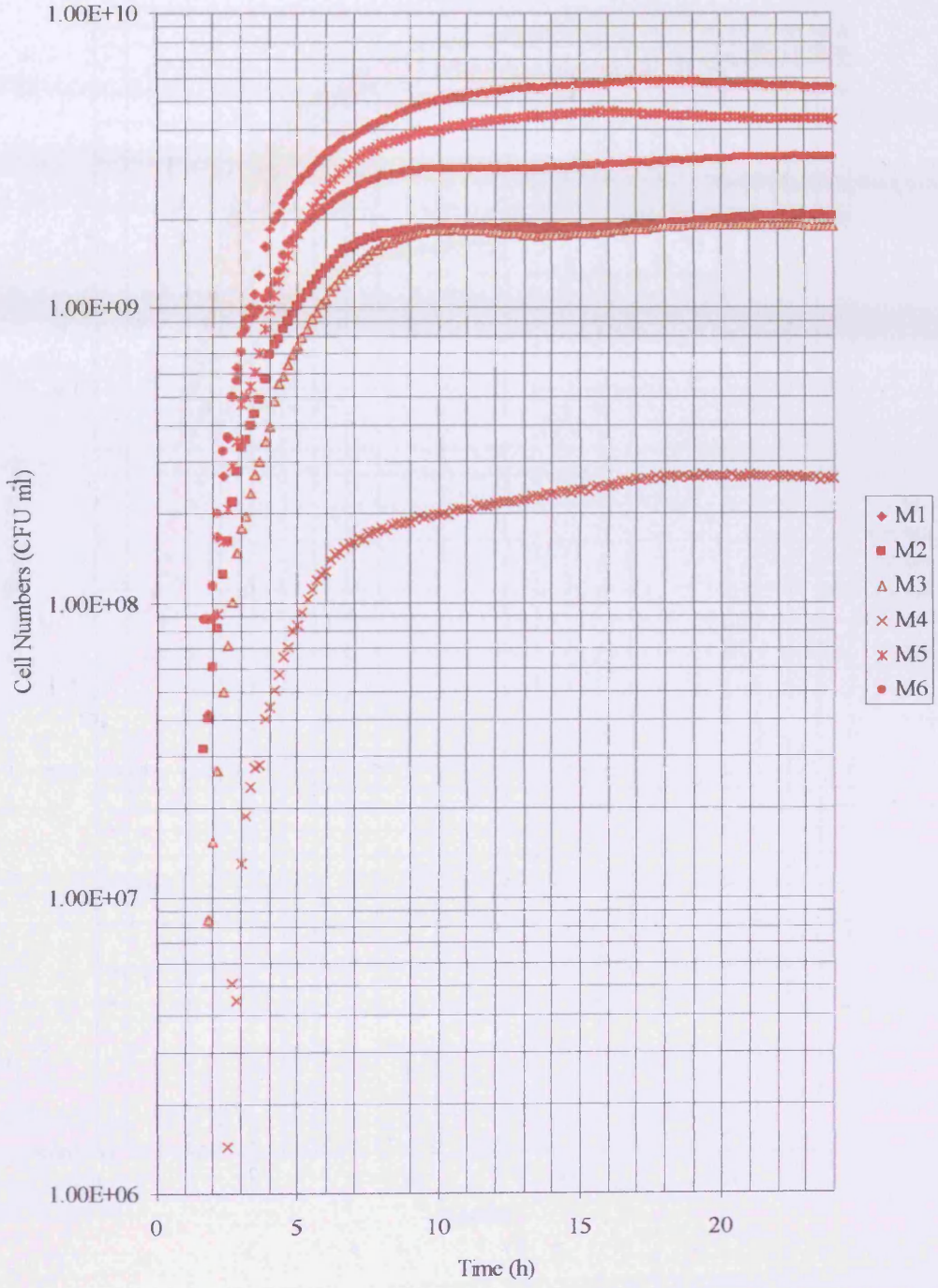
2.2.1. *Salmonella* ser. Poona growth in casein peptone-based BPW



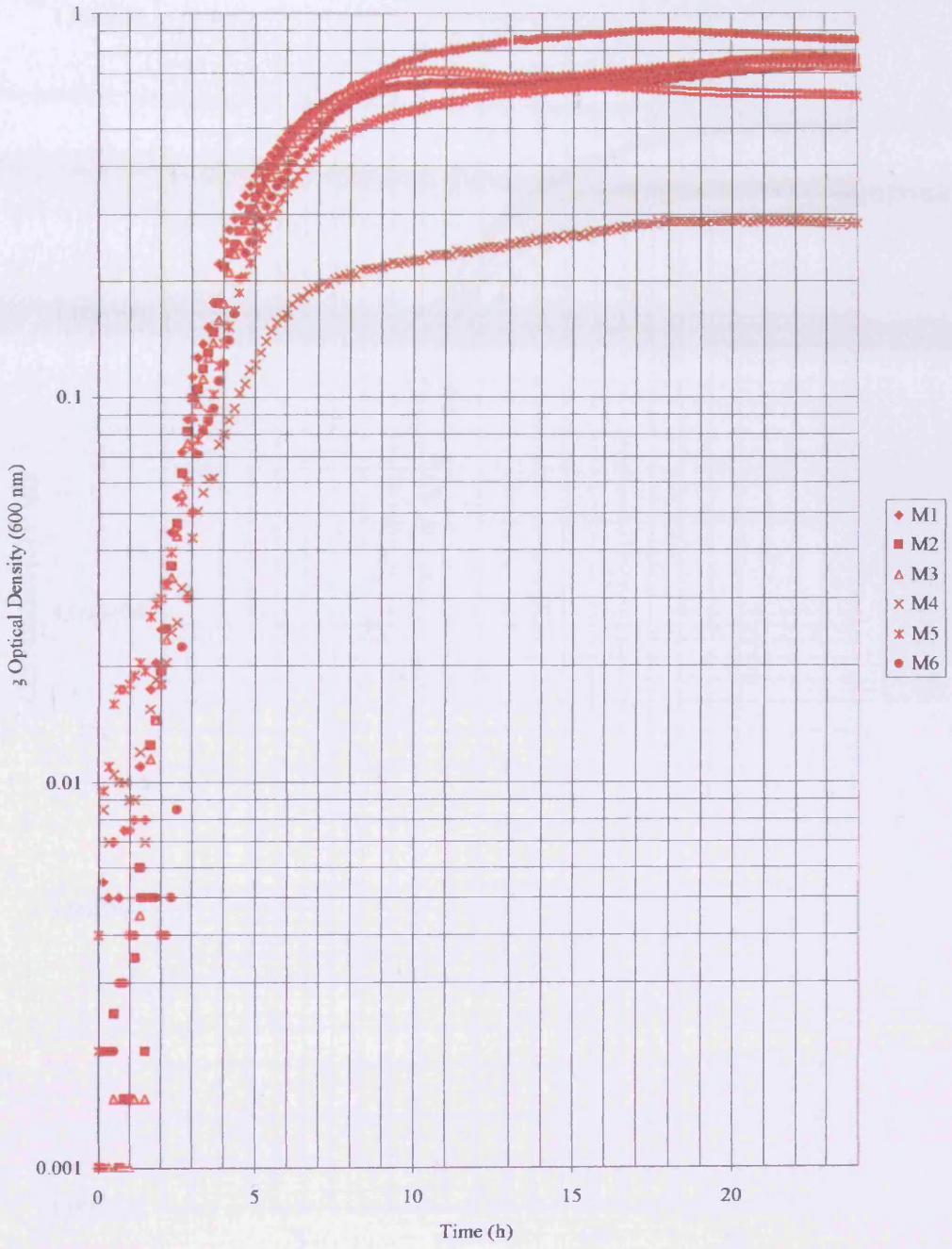
2.2.2. *Salmonella* ser. Poona growth in gelatin peptone-based BPW



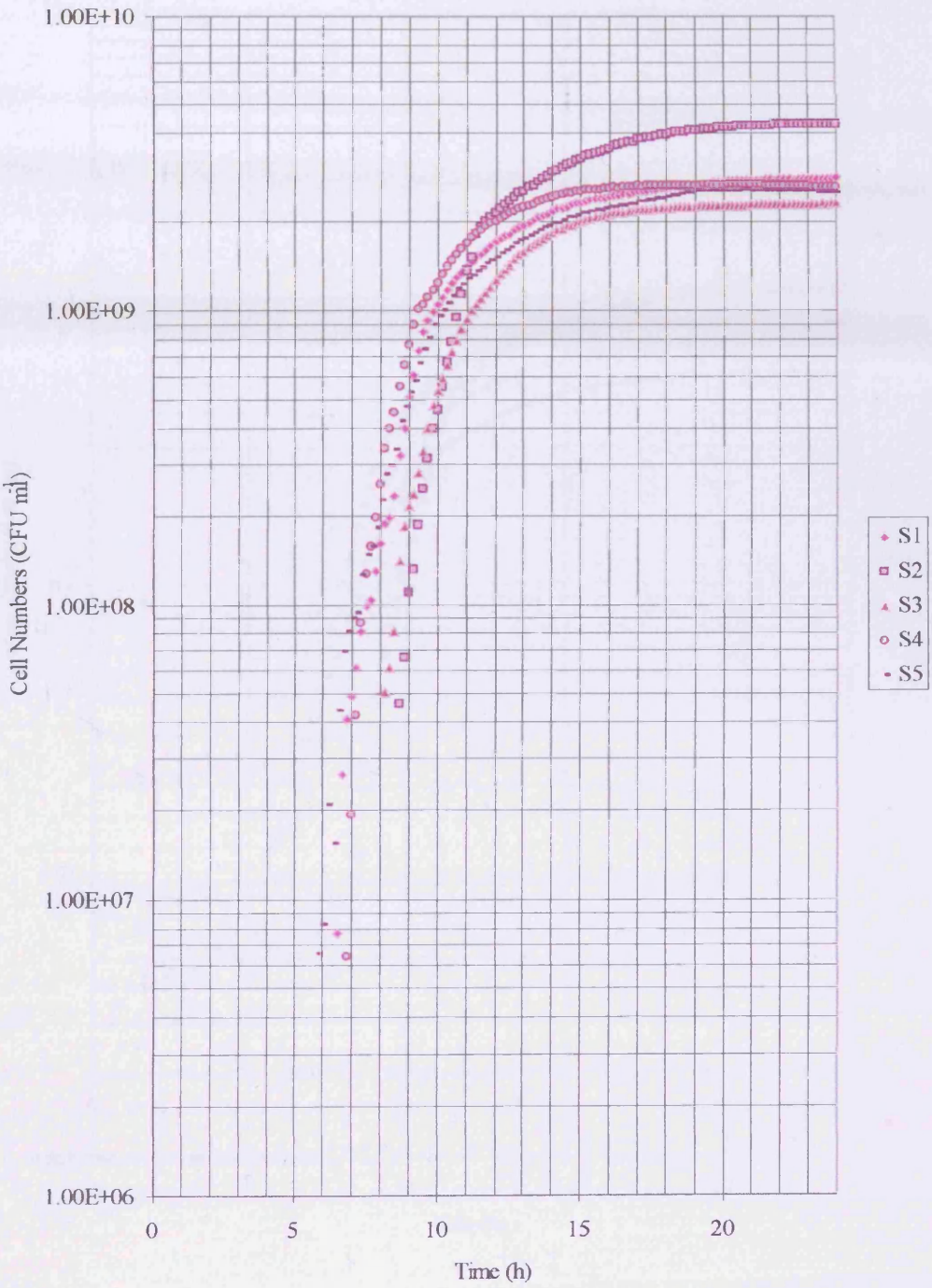
2.2.3.1. *Salmonella* ser. Poona growth in meat peptone-based BPW



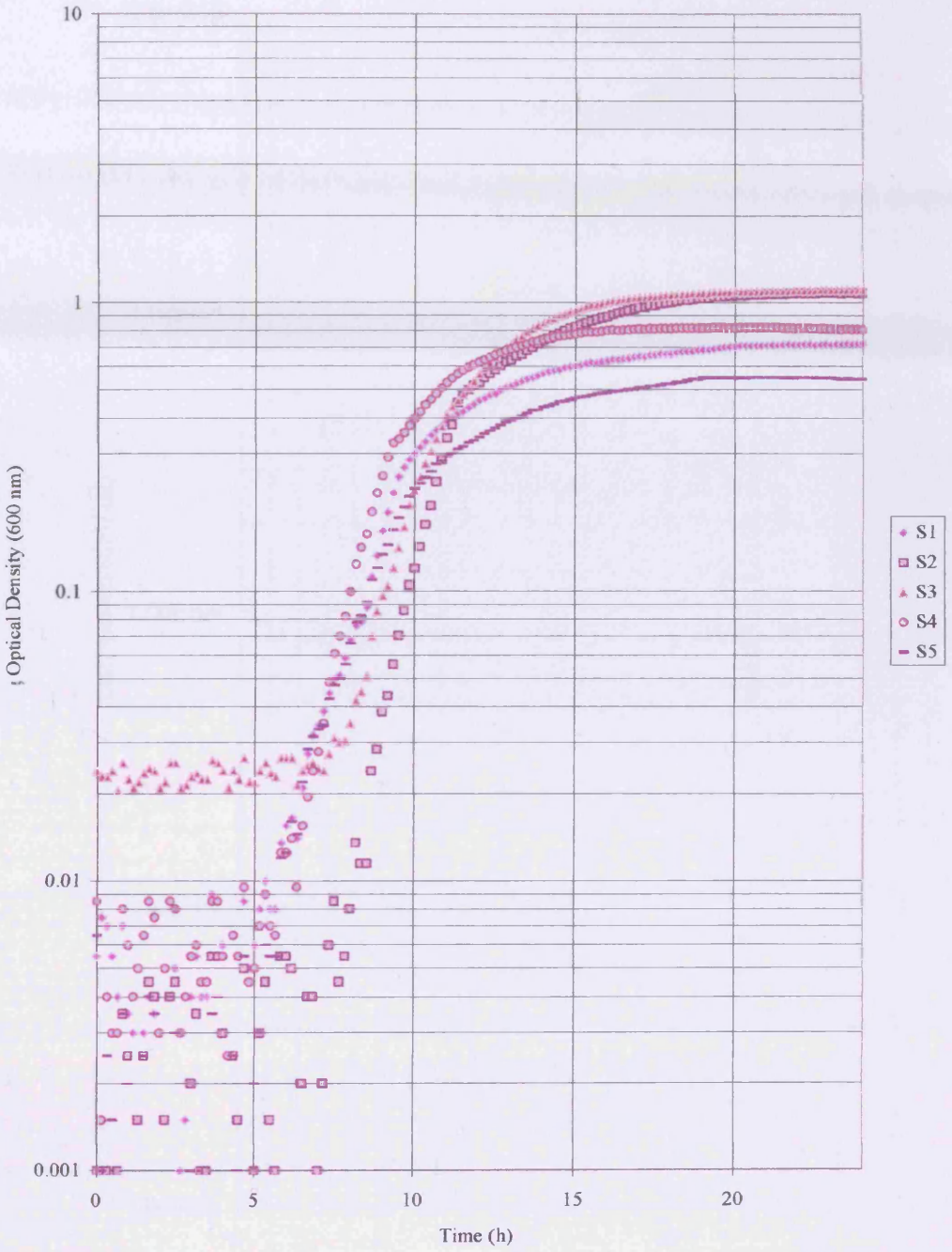
2.2.3.2. *Salmonella* ser. Poona growth in meat peptone-based BPW



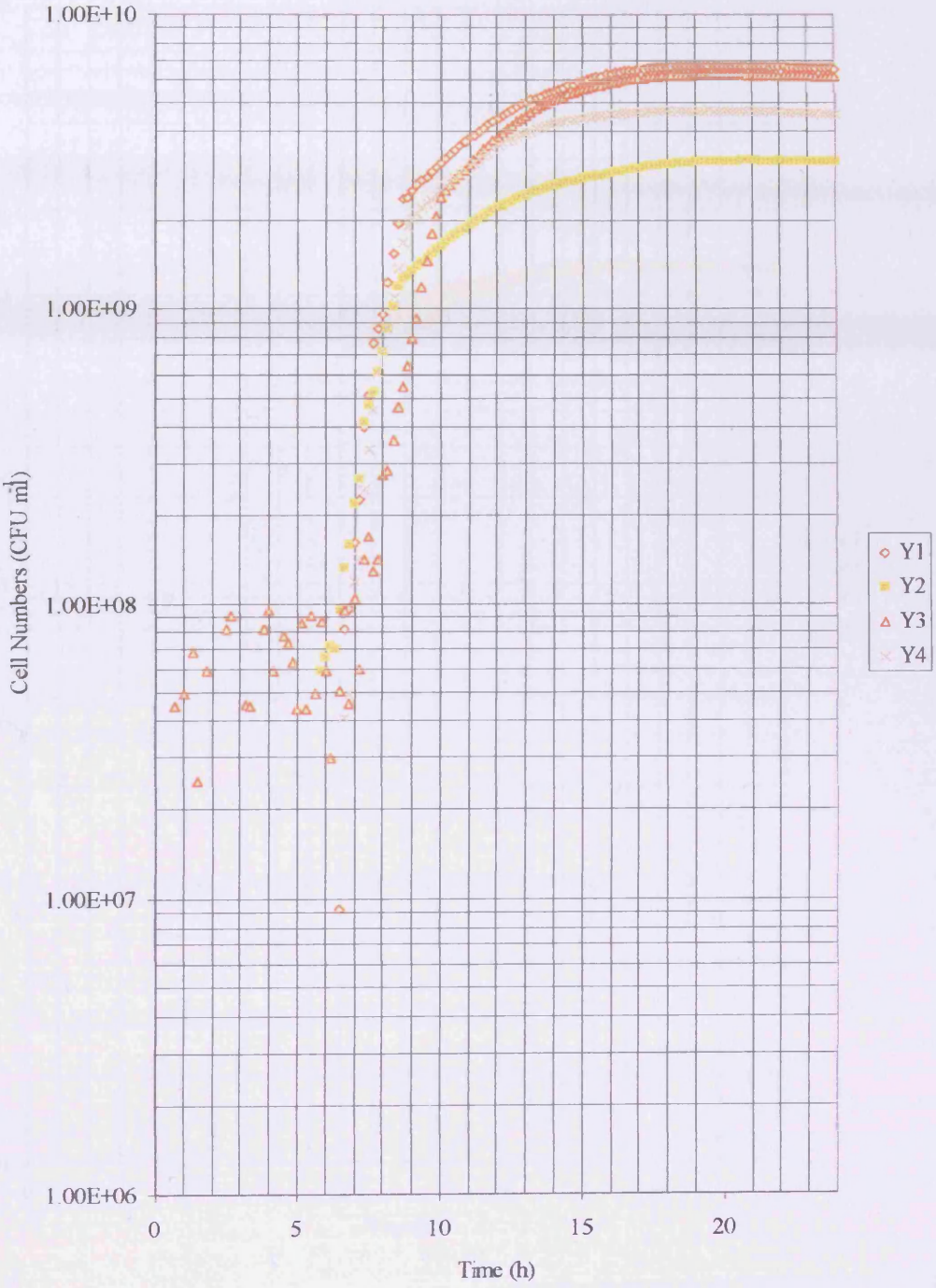
2.2.4.1. *Salmonella* ser. Poona growth in soy peptone-based BPW



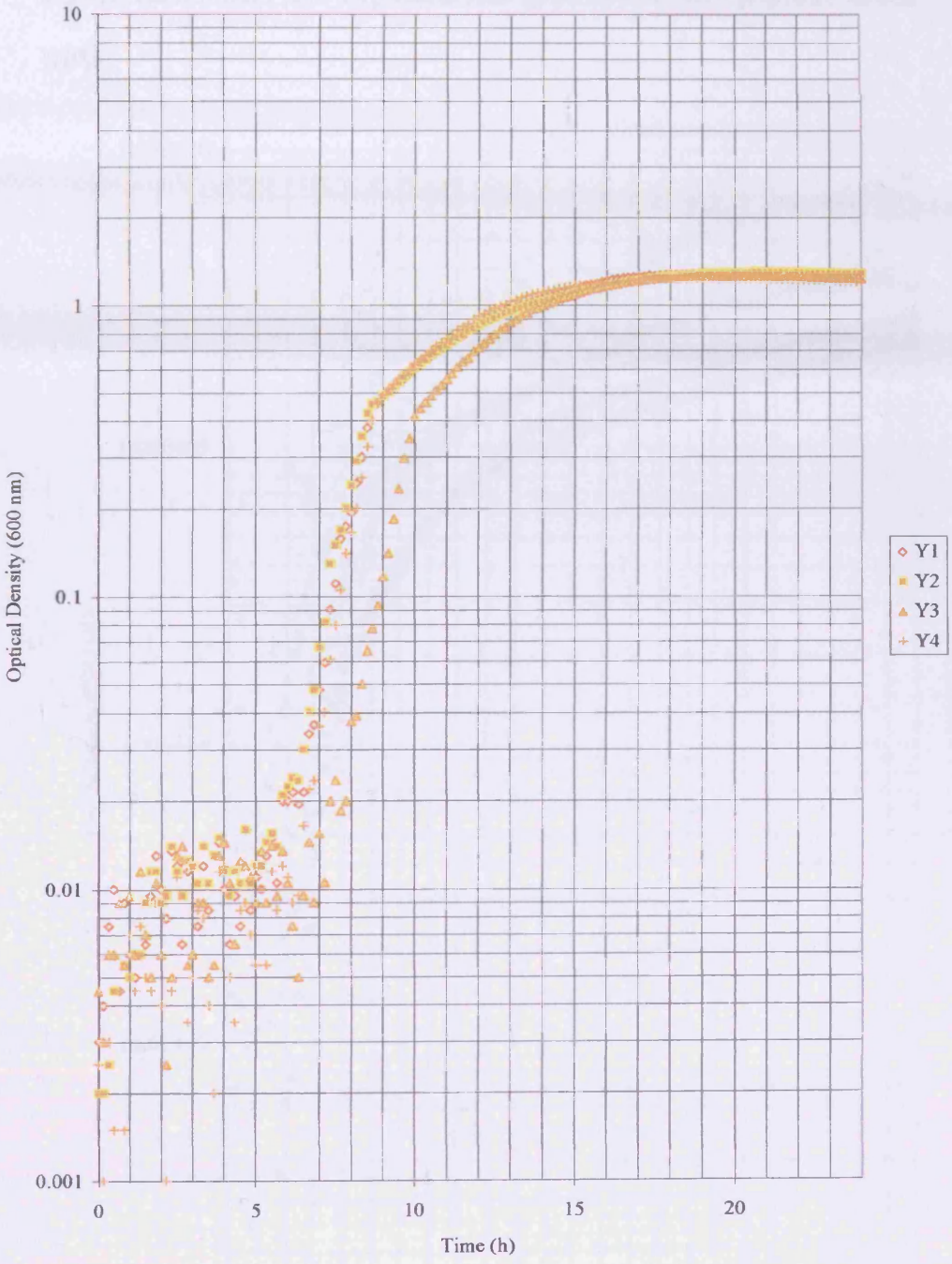
2.2.4.2. *Salmonella* ser. Poona growth in soy peptone-based BPW



2.2.5.1. *Salmonella* ser. Poona growth in yeast peptone-based BPW

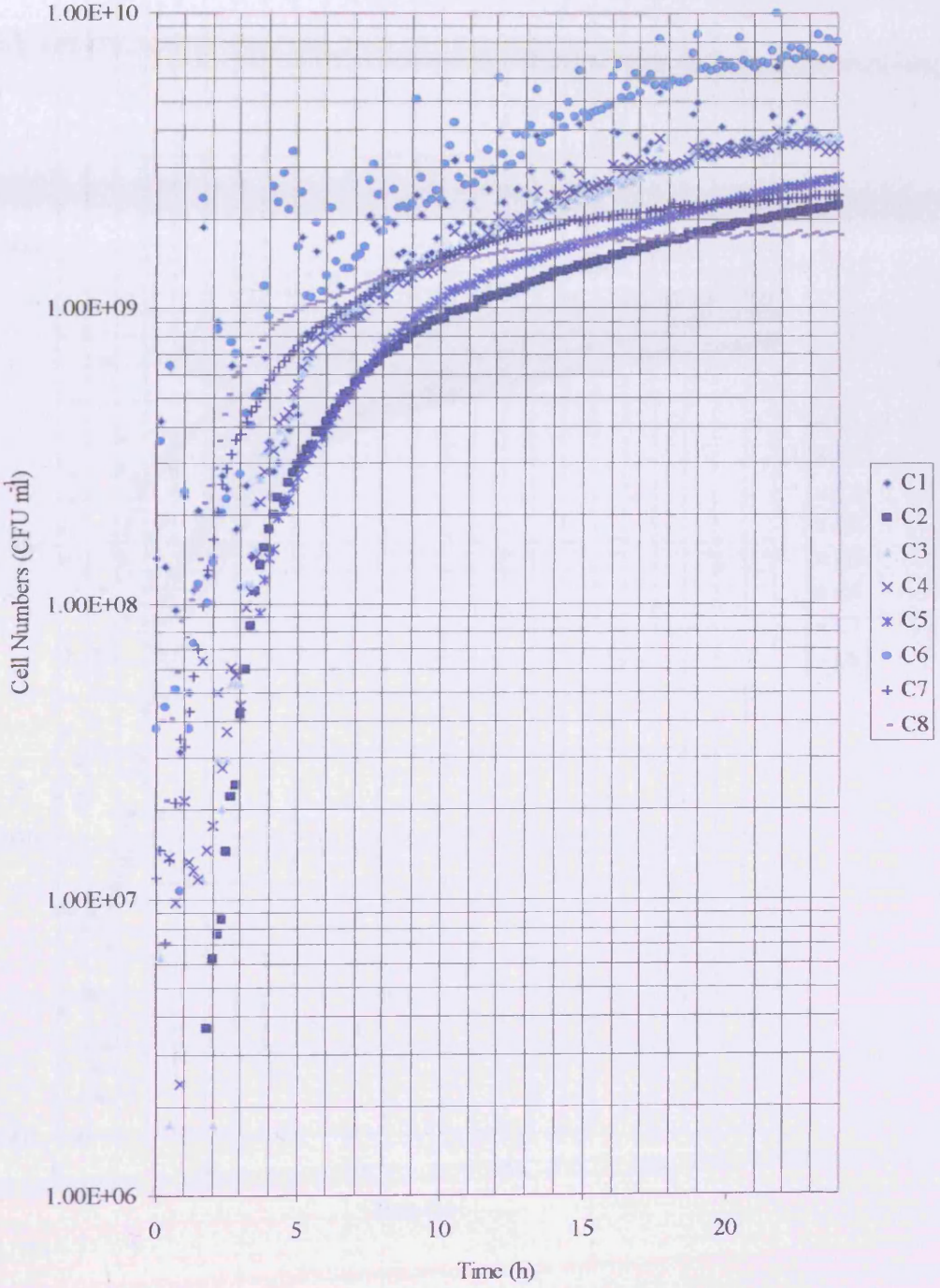


2.2.5.2. *Salmonella* ser. Poona growth in yeast peptone-based BPW

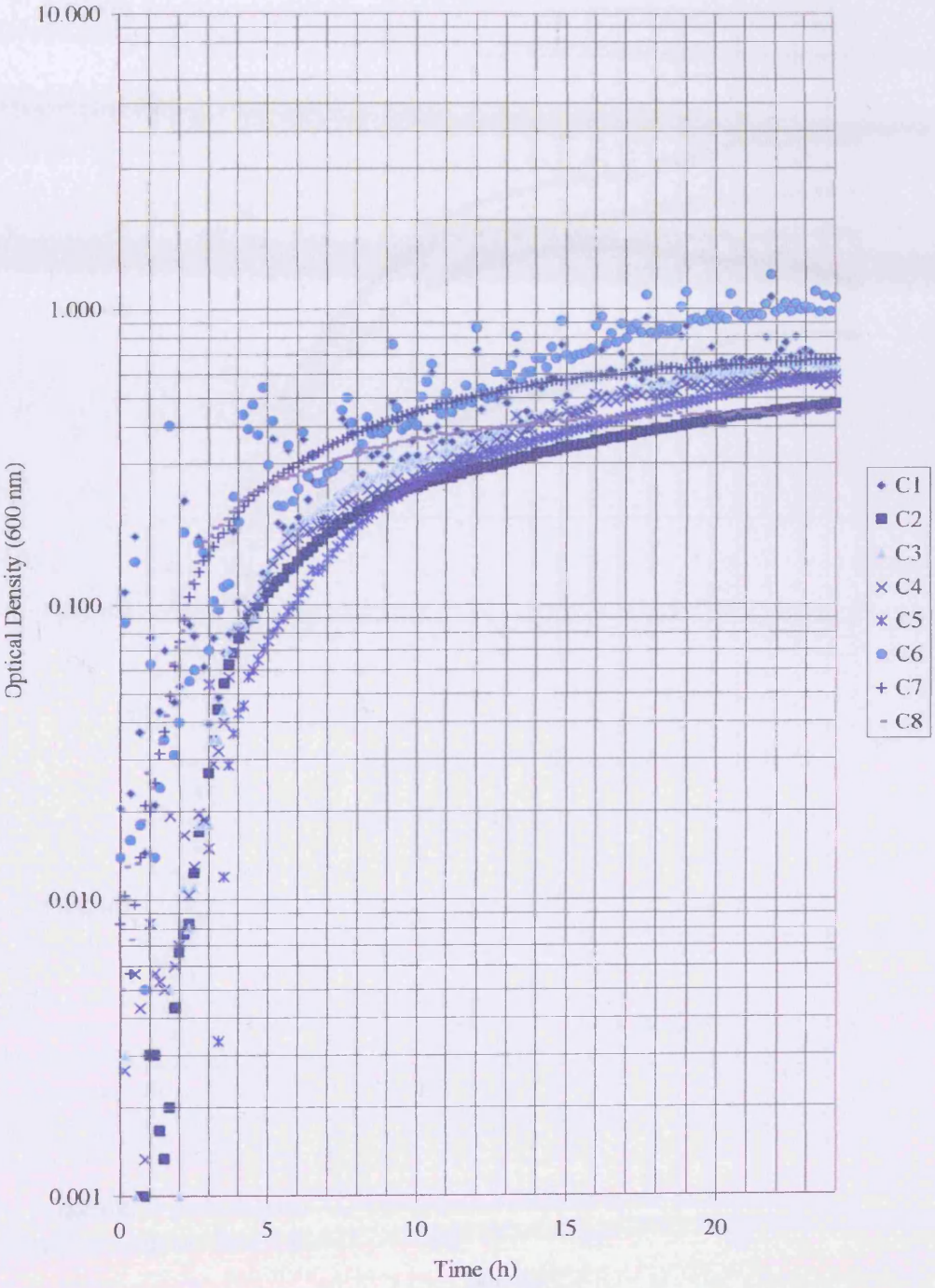


2.3. Growth profiles for *Salmonella* ser. Typhimurium

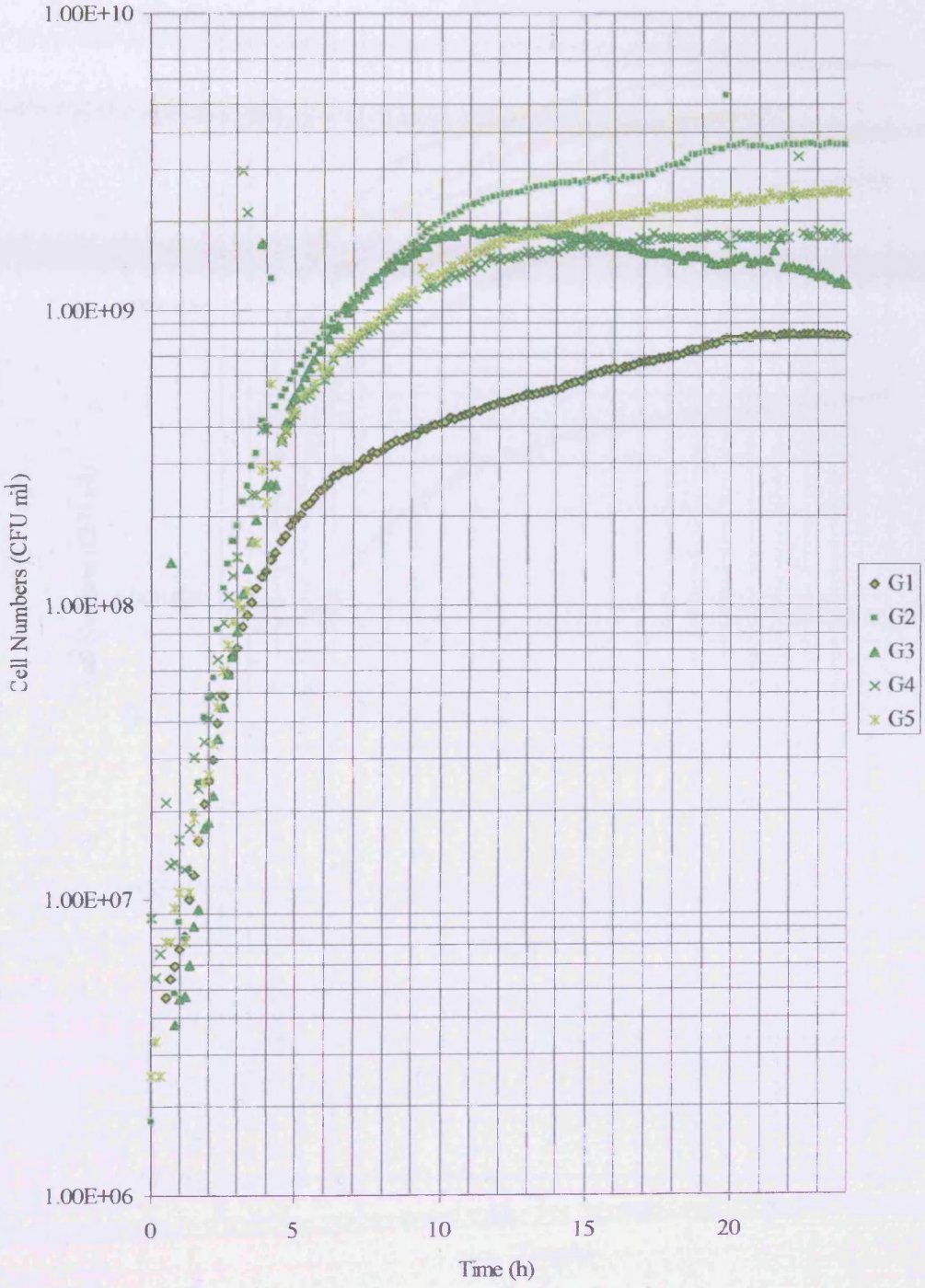
2.3.1.1. *Salmonella* ser. Typhimurium growth in casein peptone-based BPW



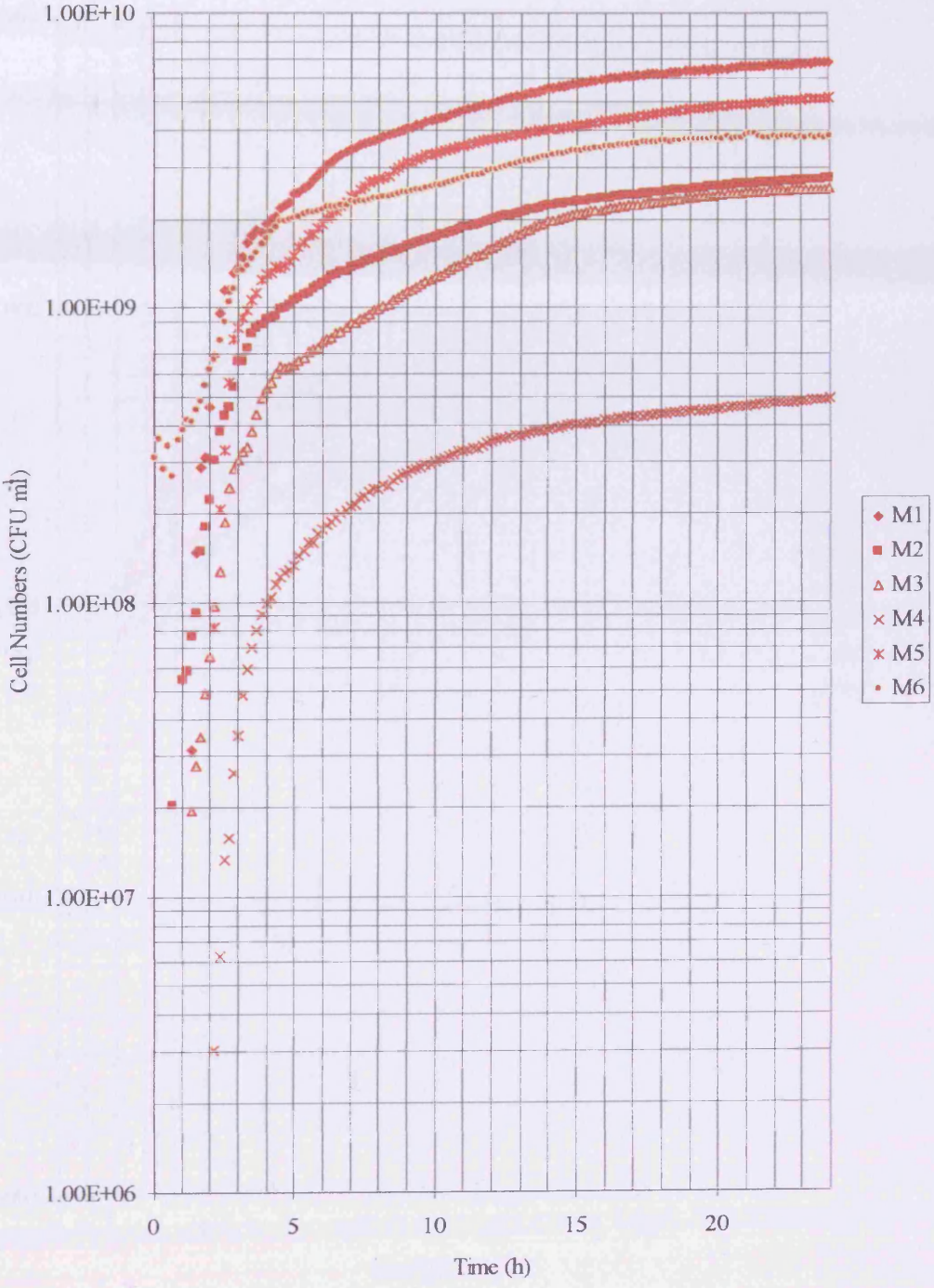
2.3.1.2. *Salmonella* ser. Typhimurium growth in casein peptone-based BPW



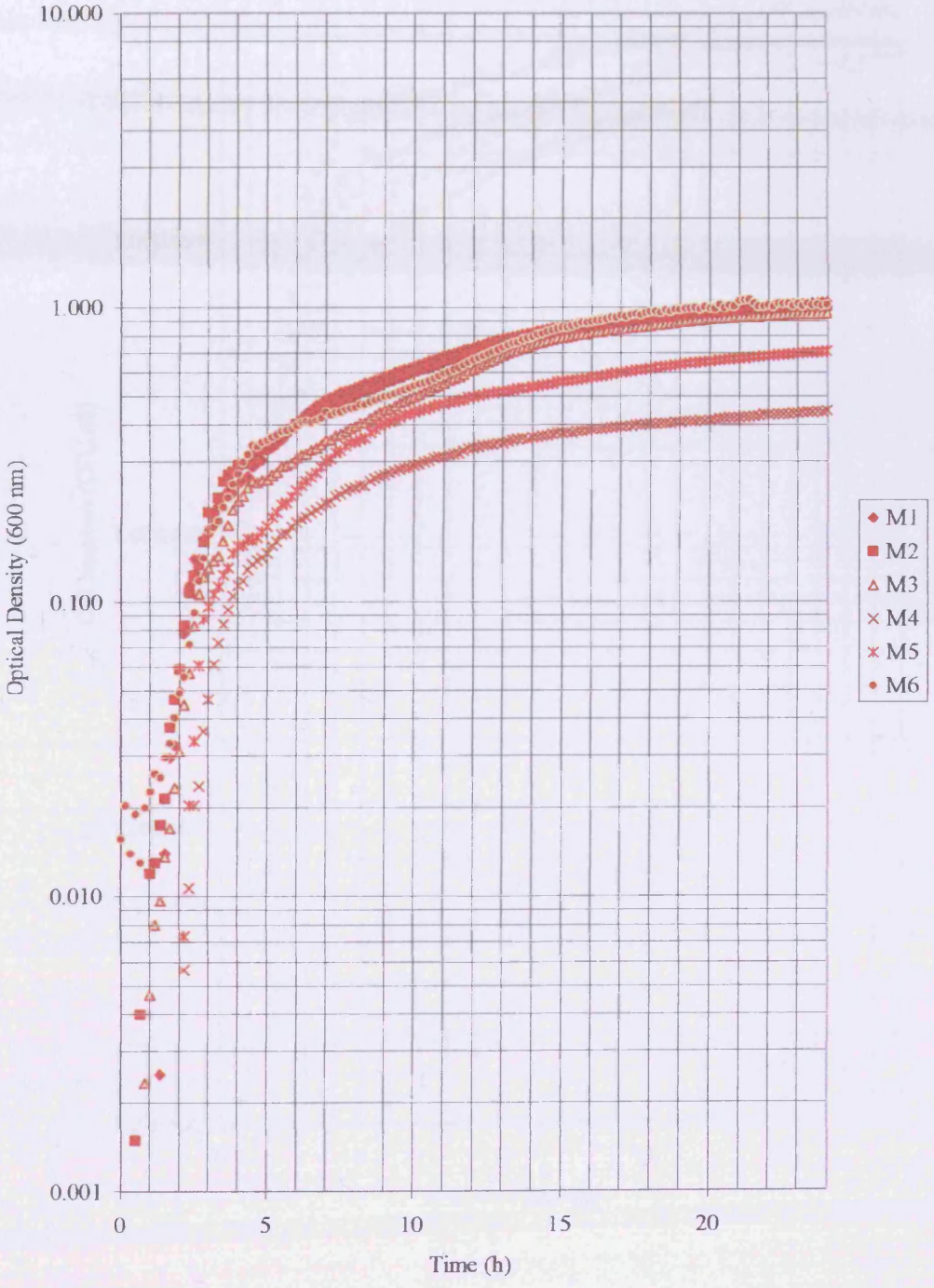
2.3.2. *Salmonella* ser. Typhimurium growth in gelatin peptone-based BPW



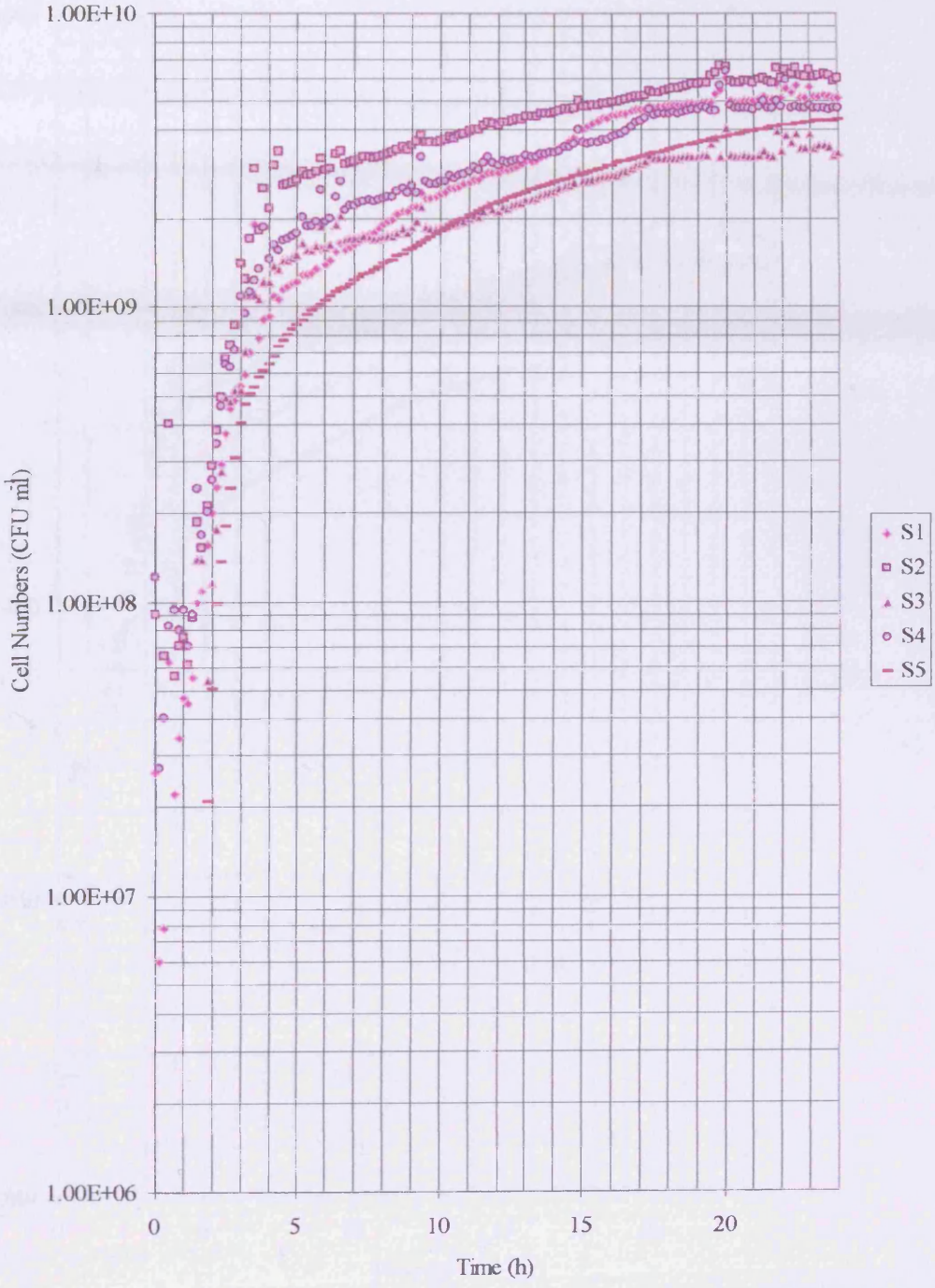
2.3.3.1. *Salmonella* ser. Typhimurium growth in meat peptone-based BPW



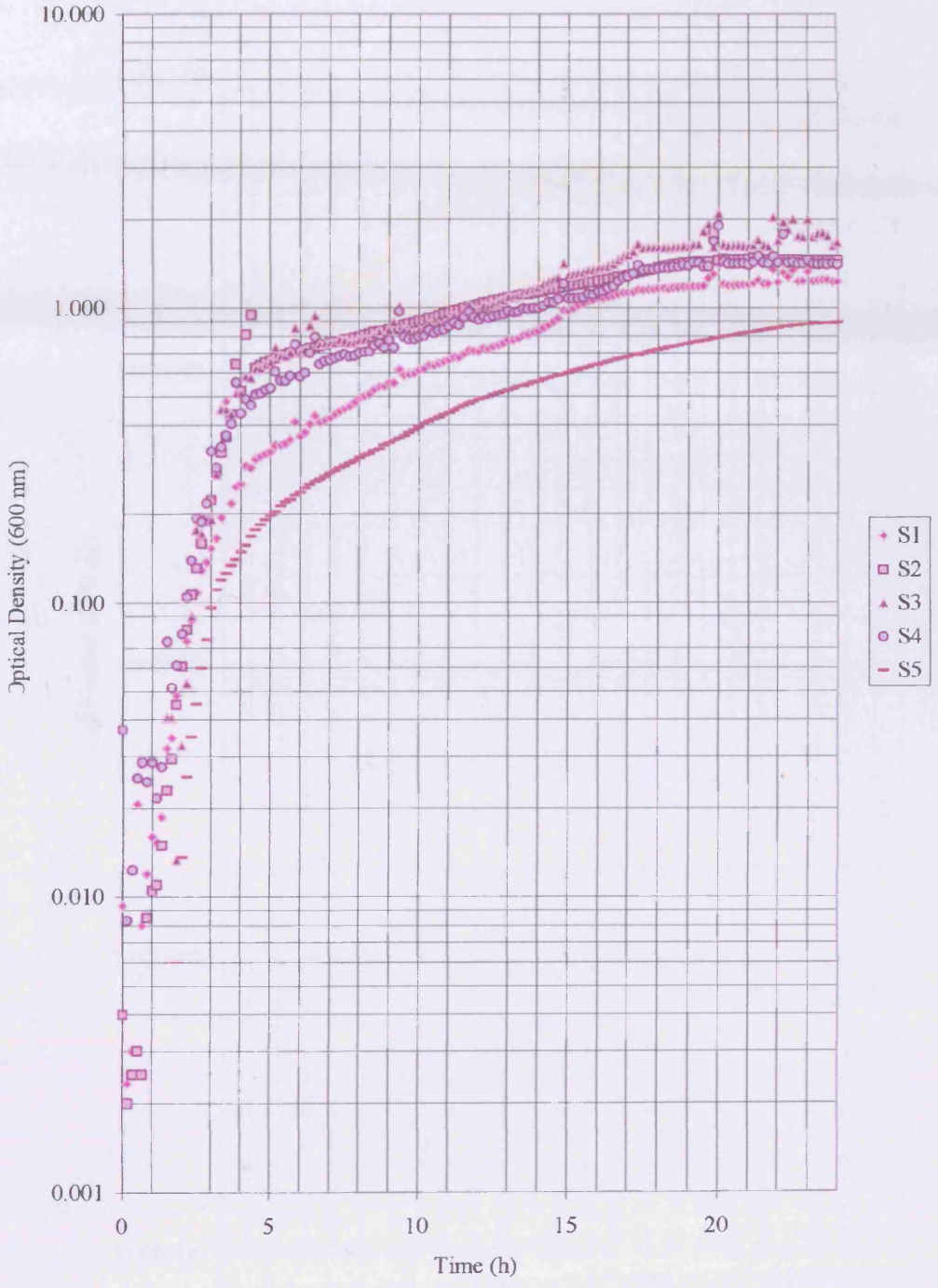
2.3.3.2. *Salmonella* ser. Typhimurium growth in meat peptone-based BPW



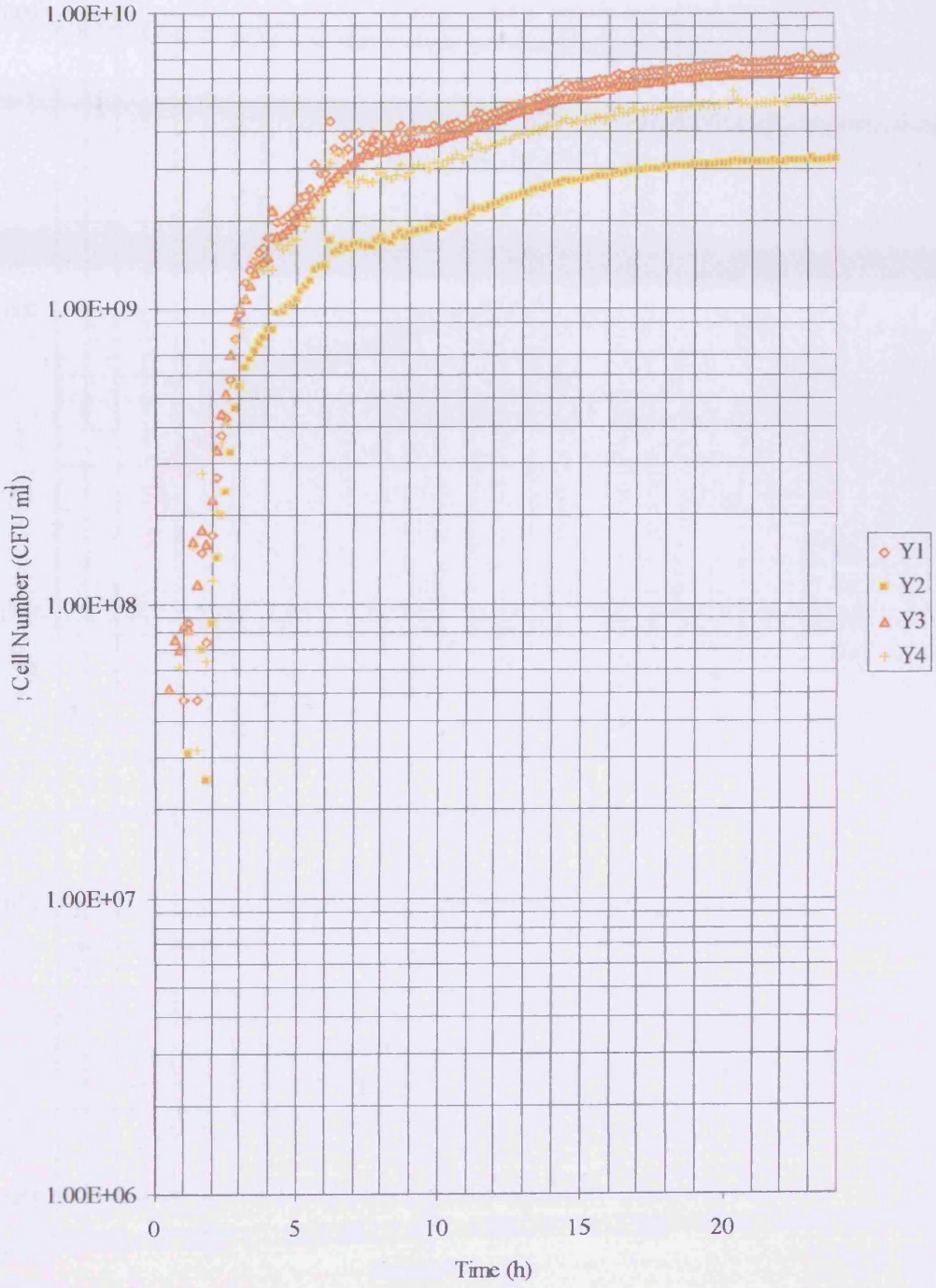
2.3.4.1. *Salmonella* ser. Typhimurium growth in soy peptone-based BPW



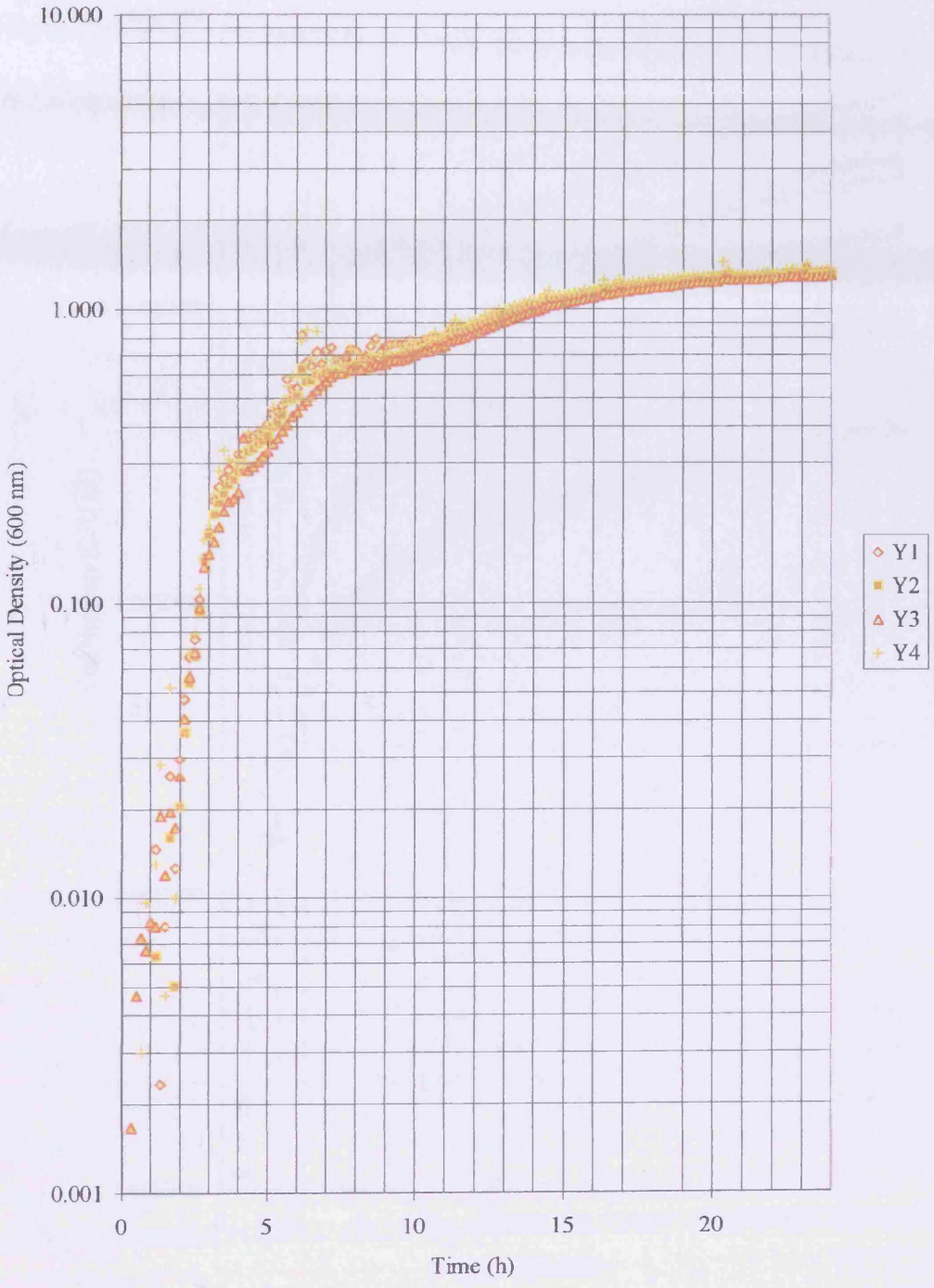
2.3.4.2. *Salmonella* ser. Typhimurium growth in soy peptone-based BPW



2.3.5.1. *Salmonella* ser. Typhimurium growth in yeast peptone-based BPW

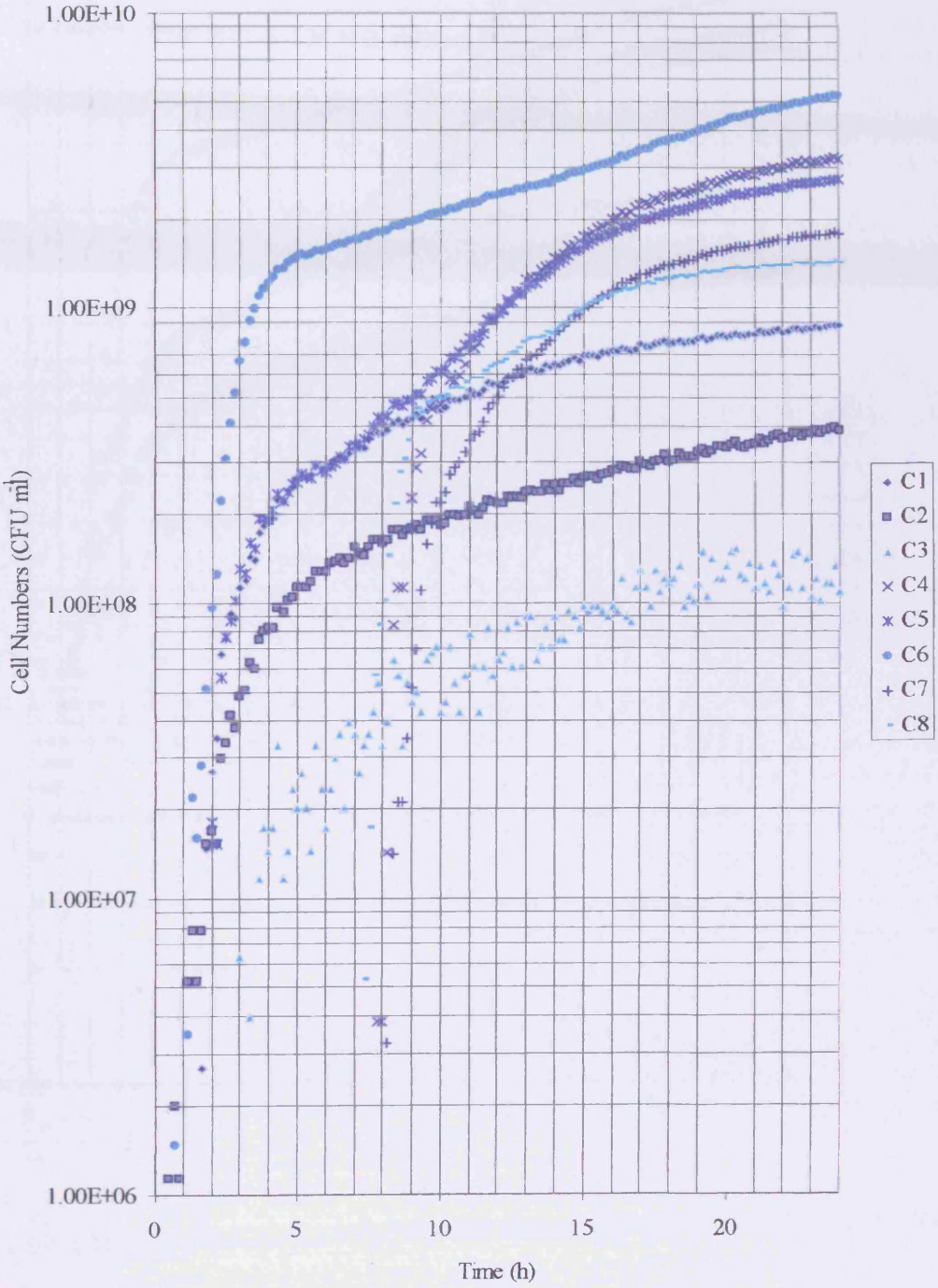


2.3.5.2. *Salmonella* ser. Typhimurium growth in yeast peptone-based BPW

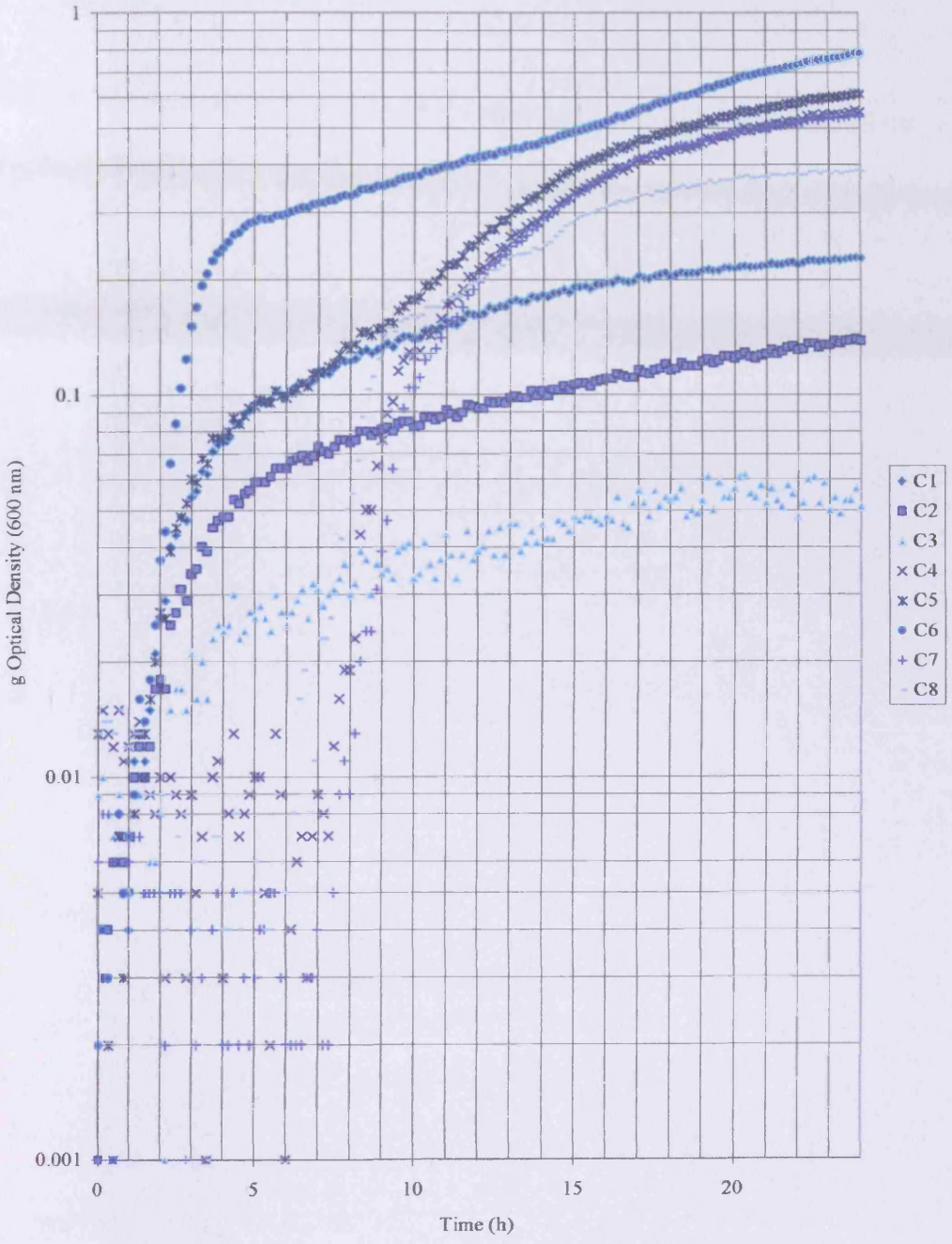


2.4. Growth profiles for *Staphylococcus aureus*

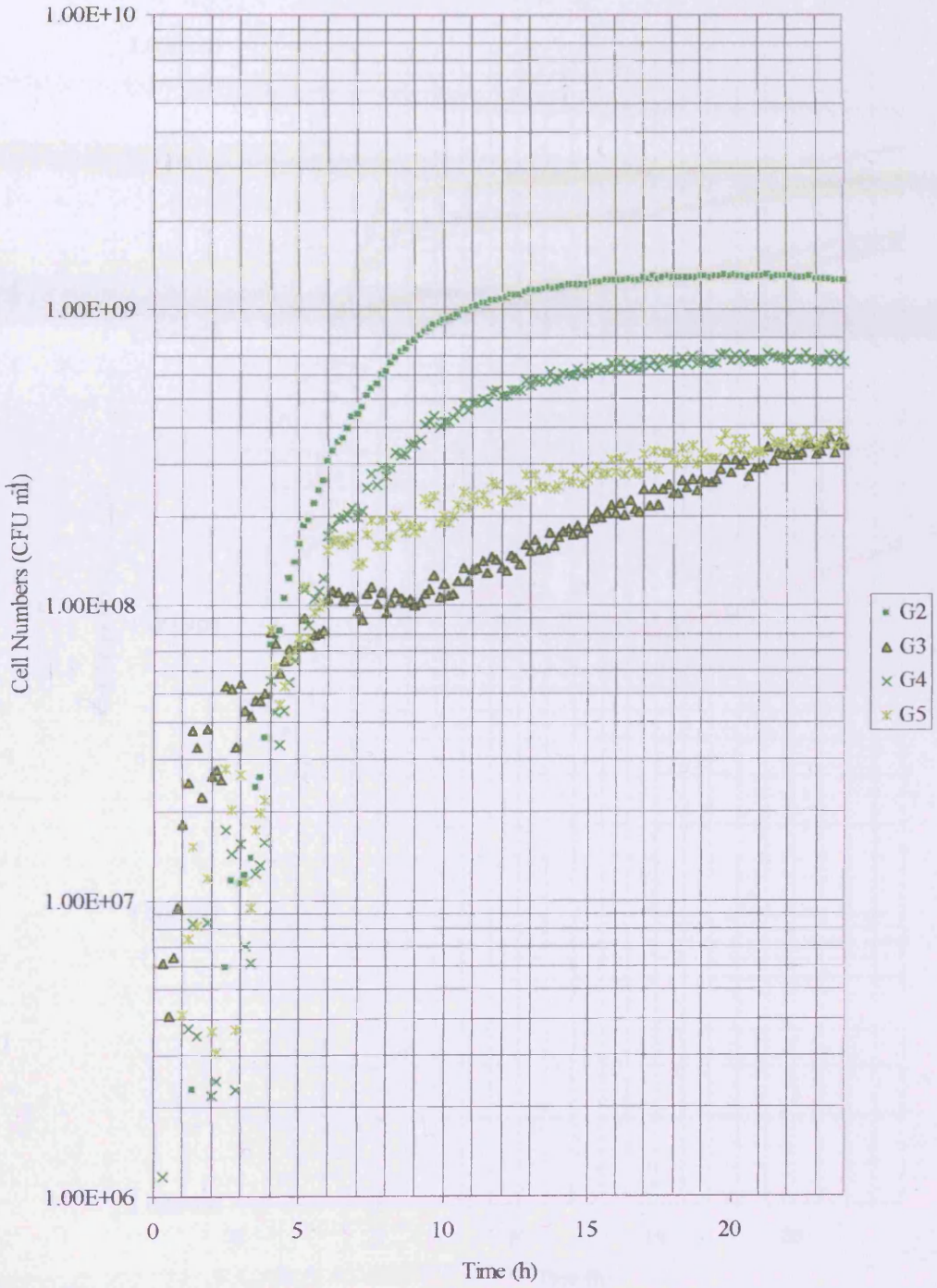
2.4.1.1. *Staphylococcus aureus* growth in casein peptone-based BPW



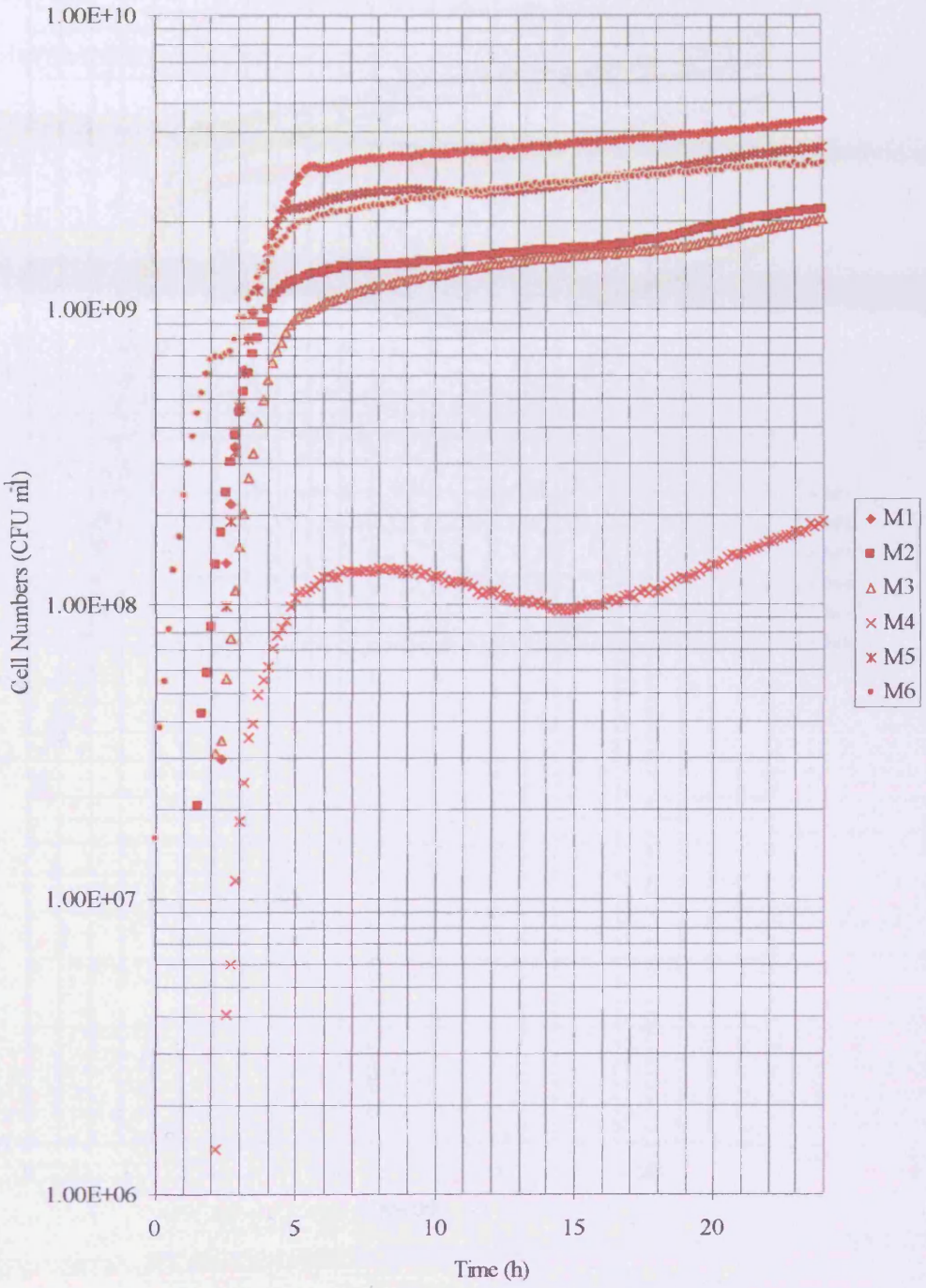
2.4.1.2. *Staphylococcus aureus* growth in casein peptone-based BPW



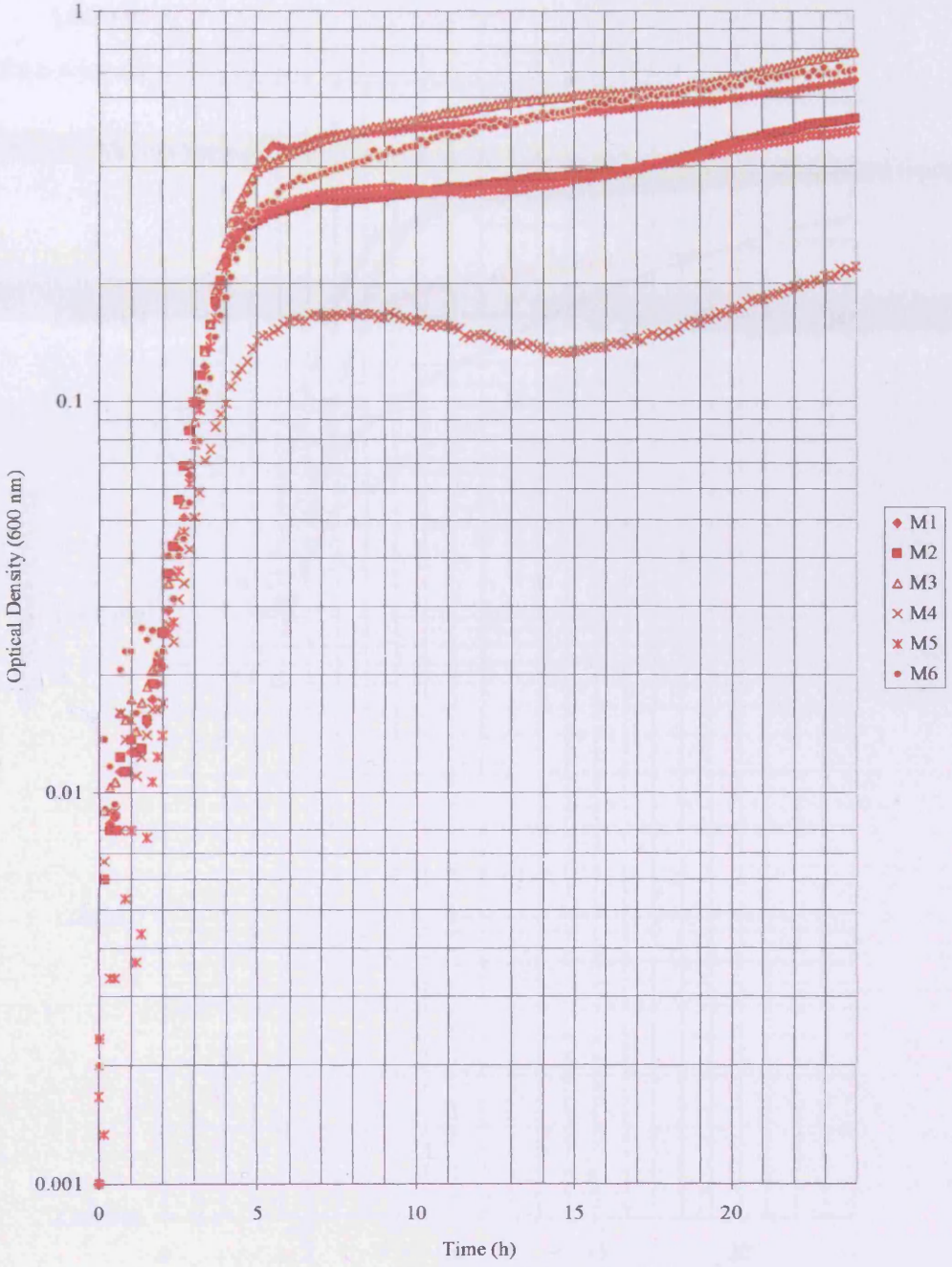
2.4.2. *Staphylococcus aureus* growth in gelatin peptone-based BPW



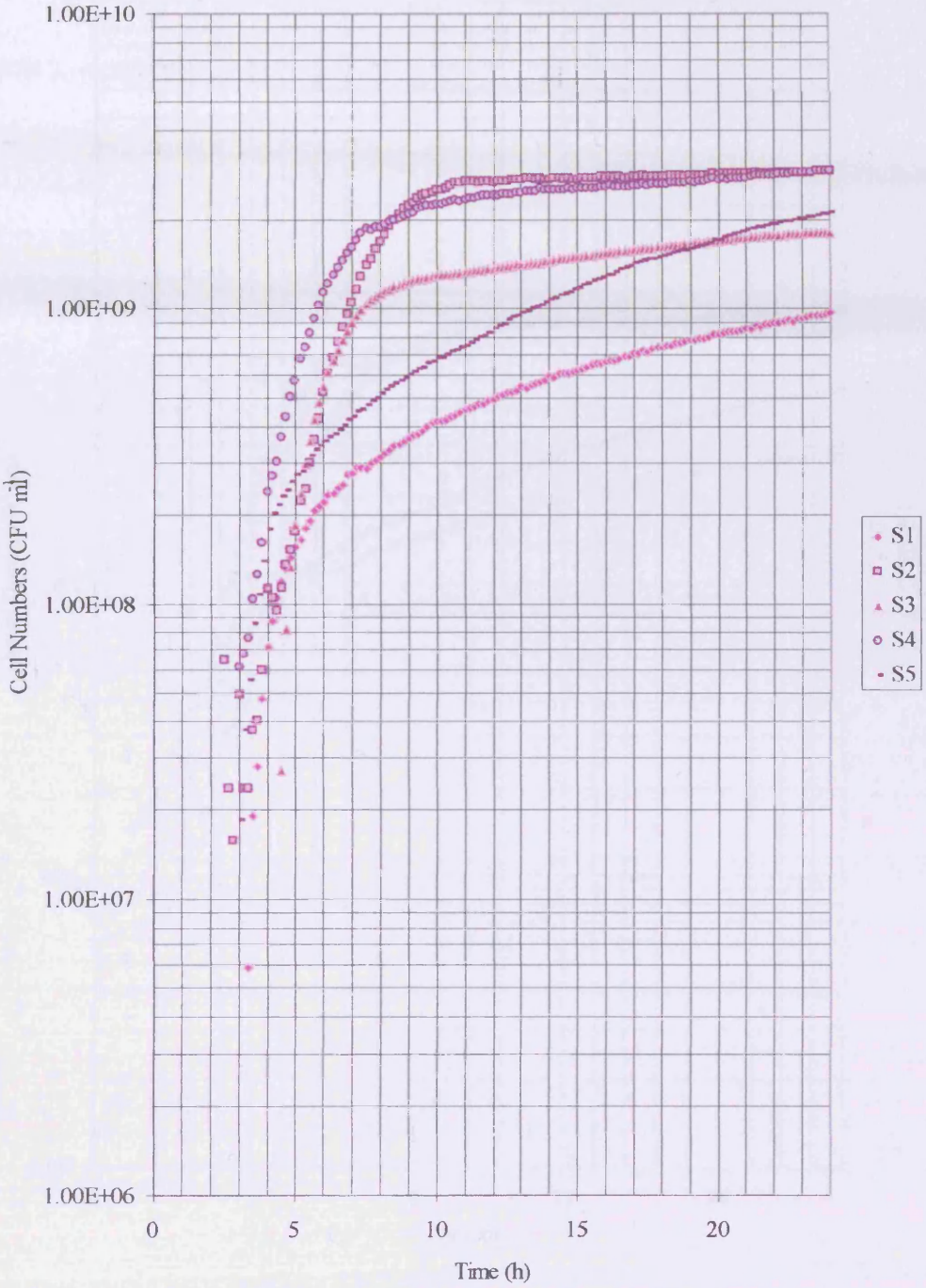
2.4.3.1. *Staphylococcus aureus* growth in meat peptone-based BPW



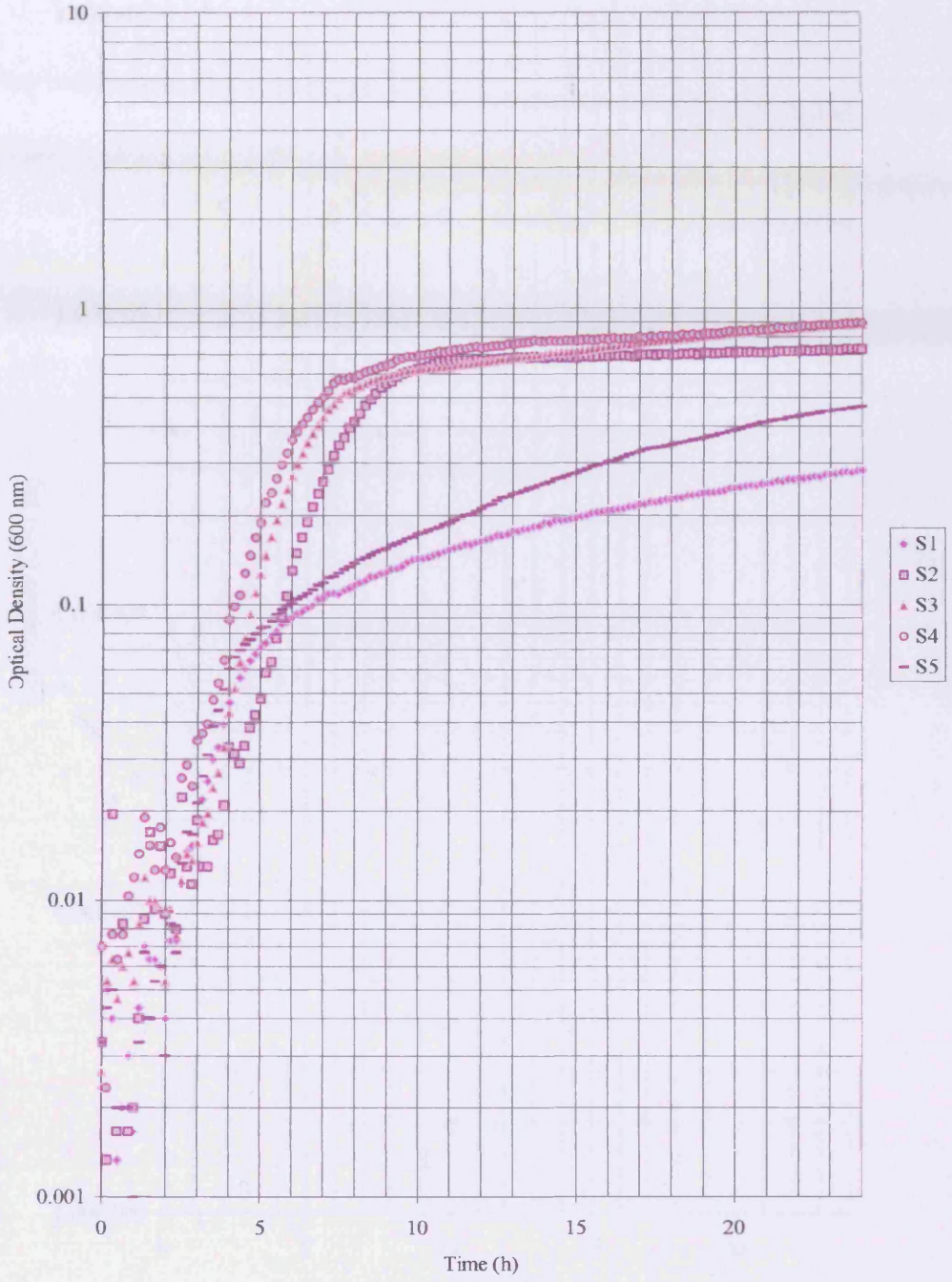
2.4.3.2. *Staphylococcus aureus* growth in meat peptone-based BPW



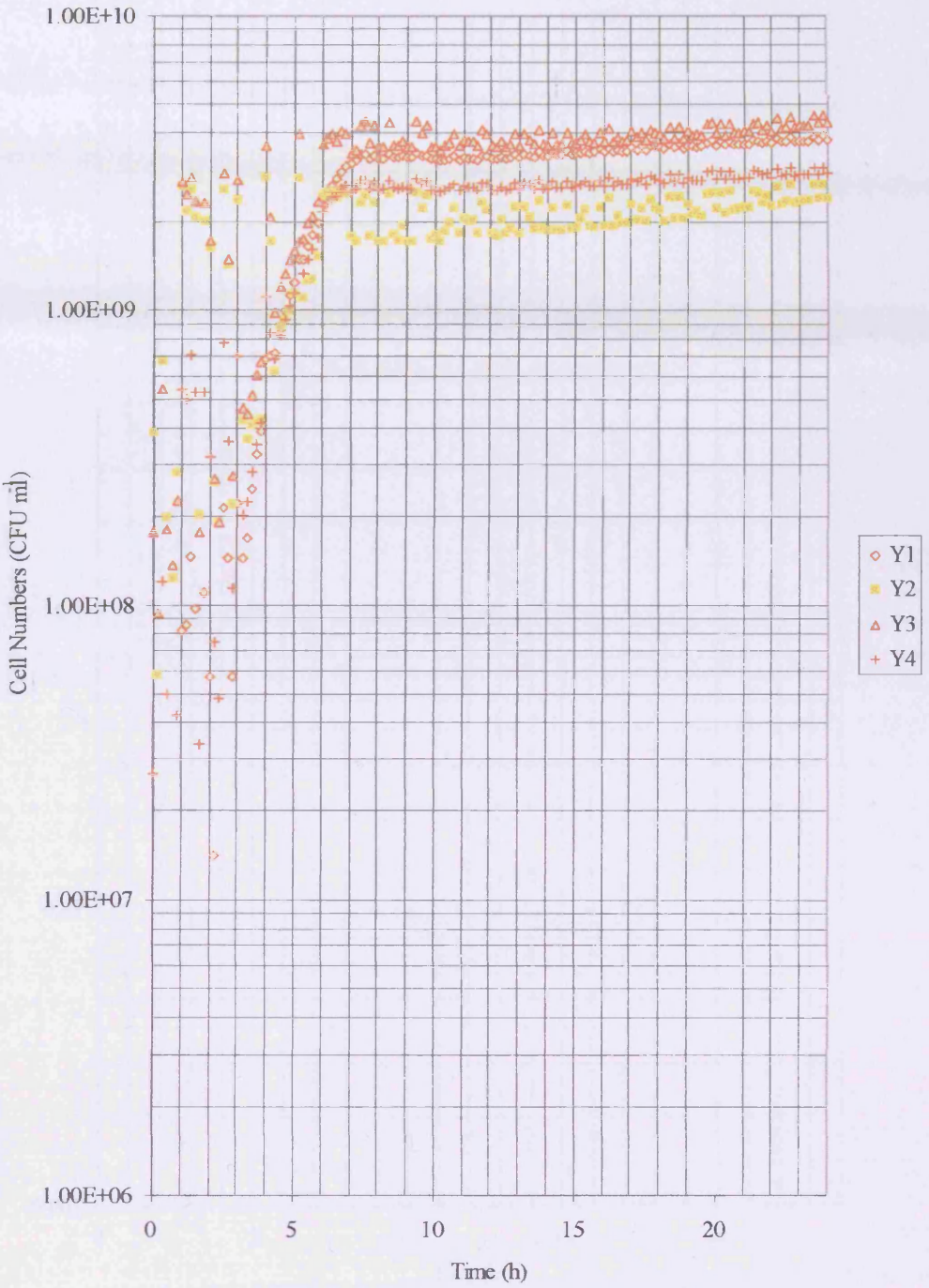
2.4.4.1. *Staphylococcus aureus* growth in soy peptone-based BPW



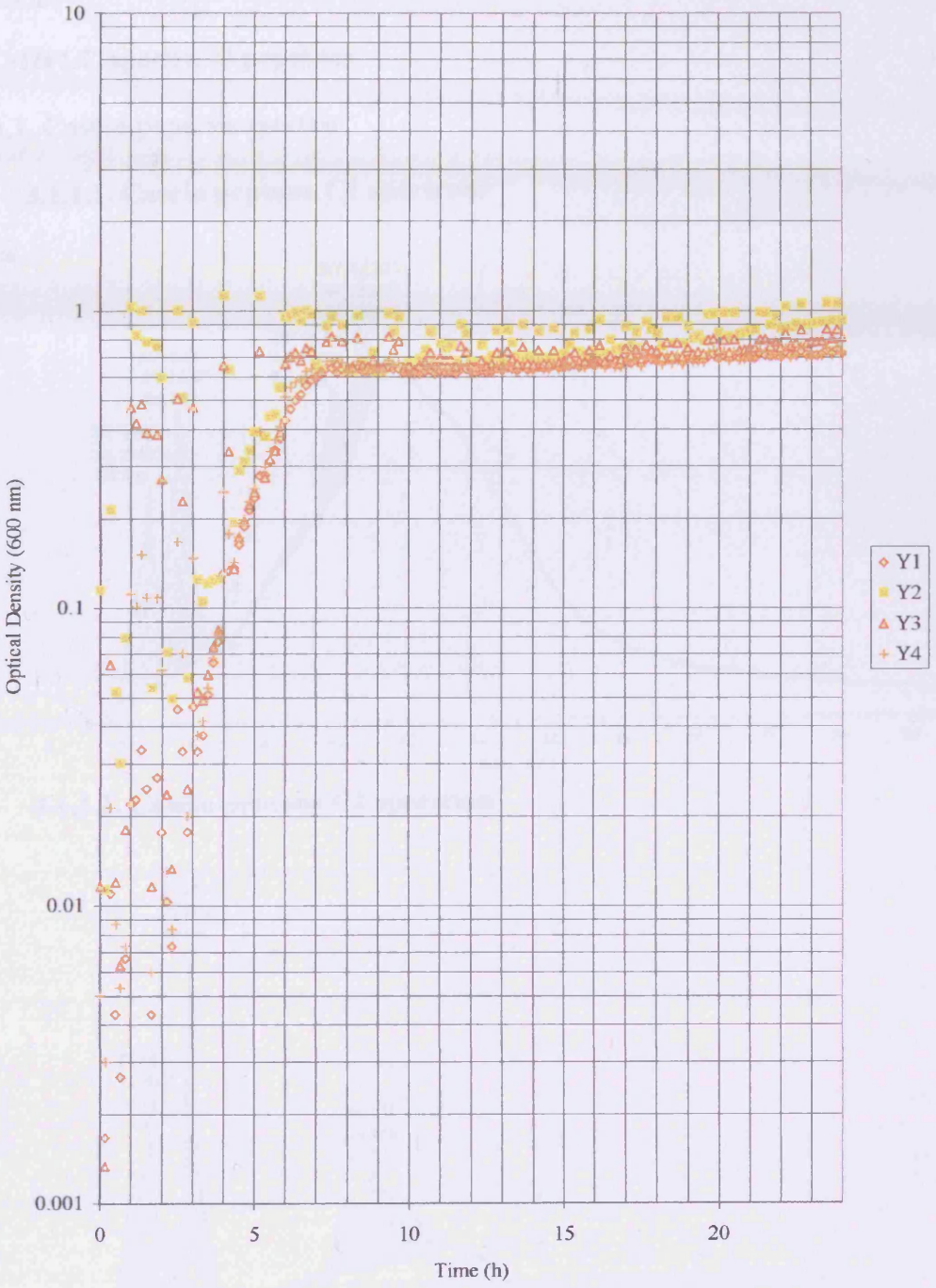
2.4.4.2. *Staphylococcus aureus* growth in soy peptone-based BPW



2.4.5.1. *Staphylococcus aureus* growth in yeast peptone-based BPW



2.4.5.2. *Staphylococcus aureus* growth in yeast peptone-based BPW

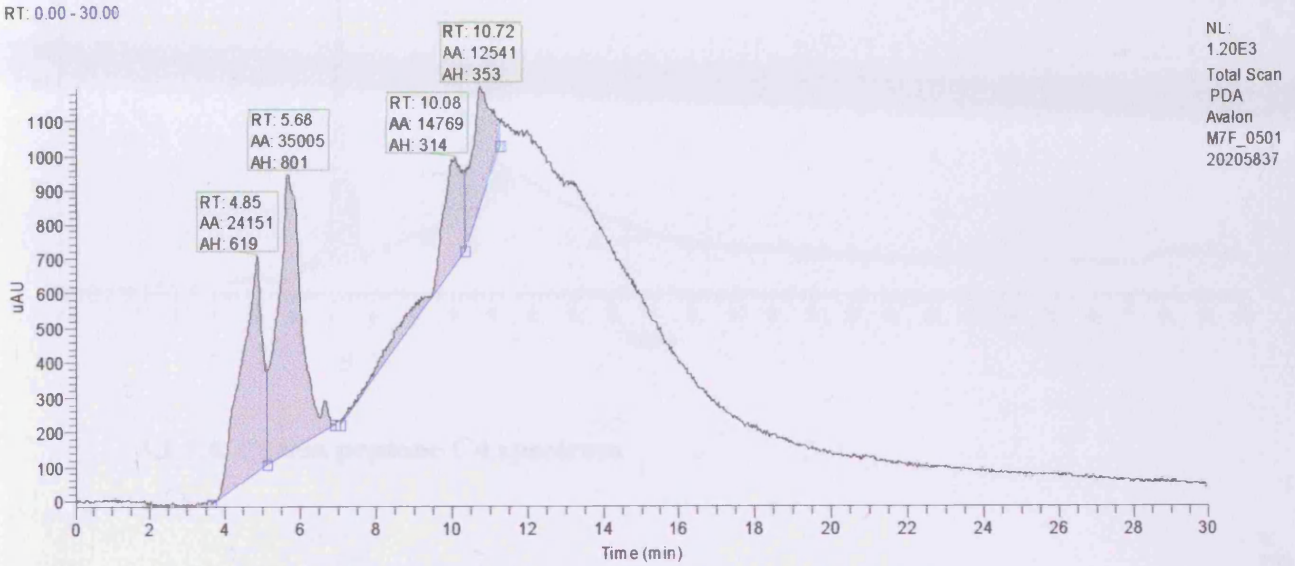


Appendix 3

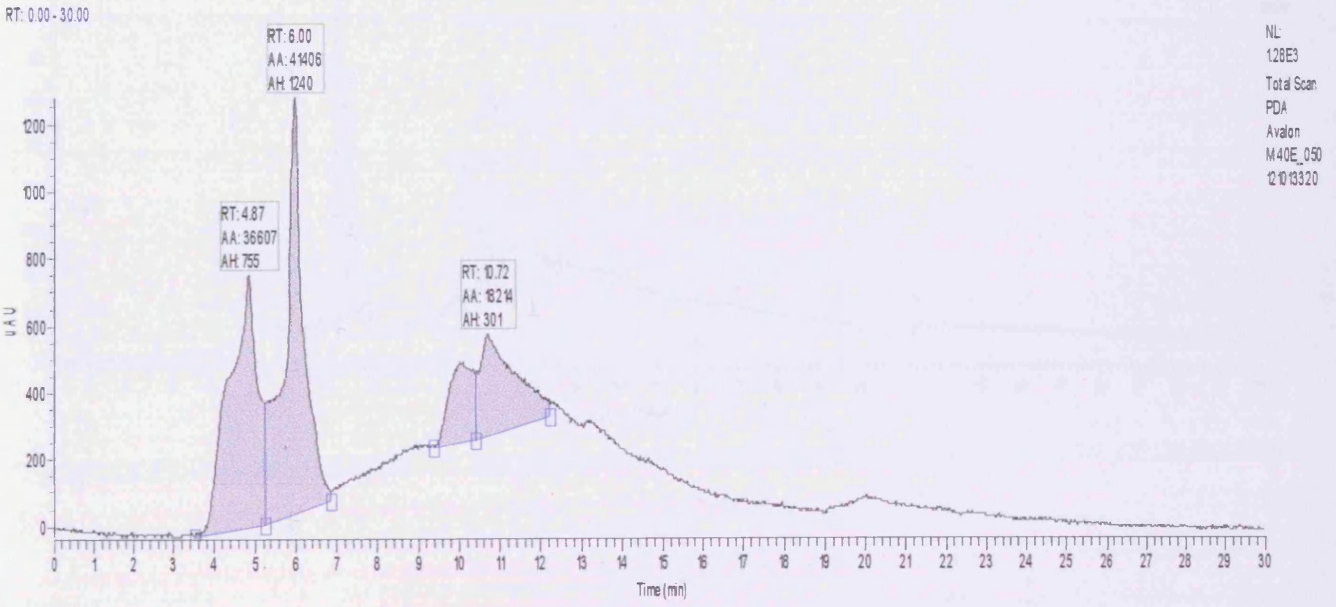
3.1. SEC-HPLC spectra of peptones

3.1.1. Casein peptone spectra

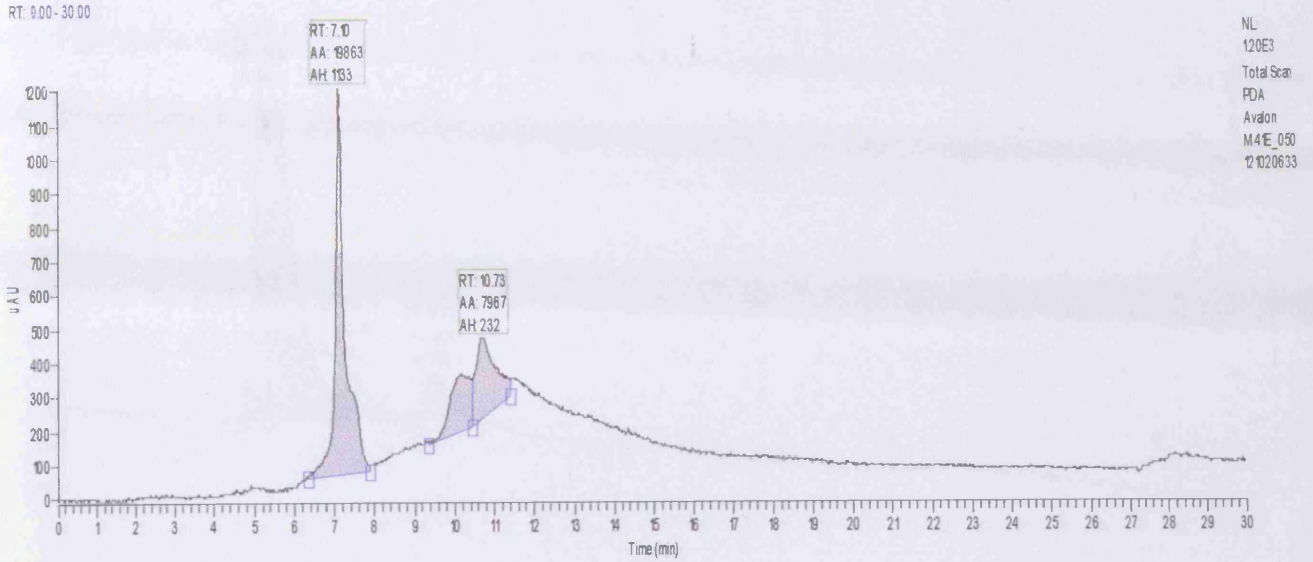
3.1.1.1. Casein peptone C1 spectrum



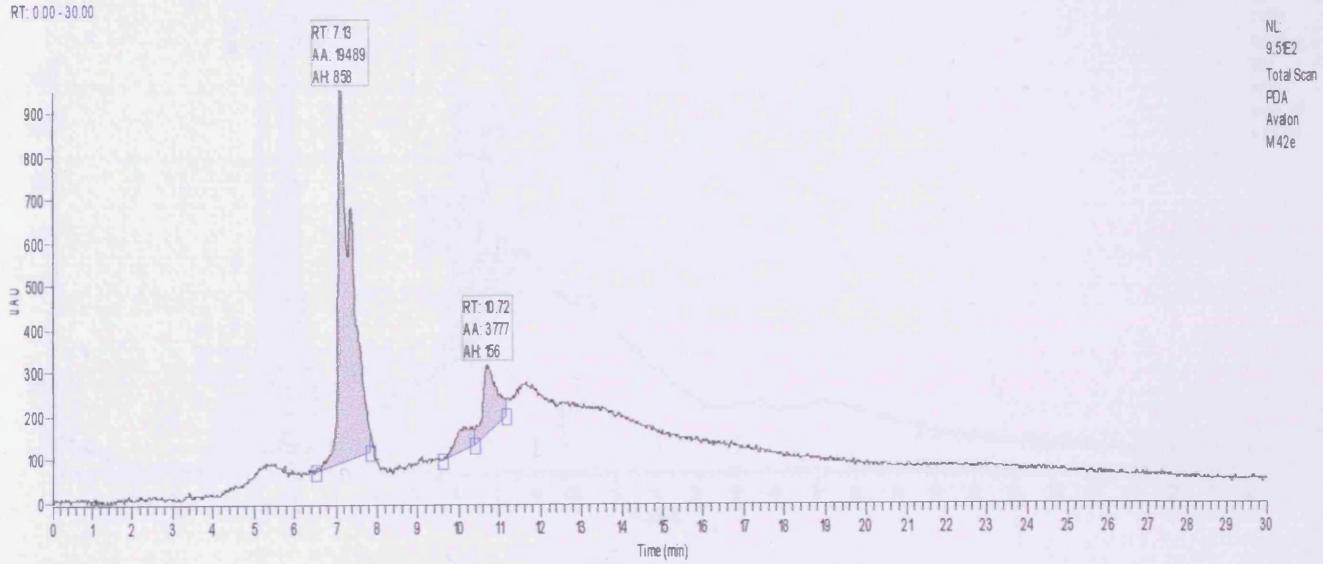
3.1.1.2. Casein peptone C2 spectrum



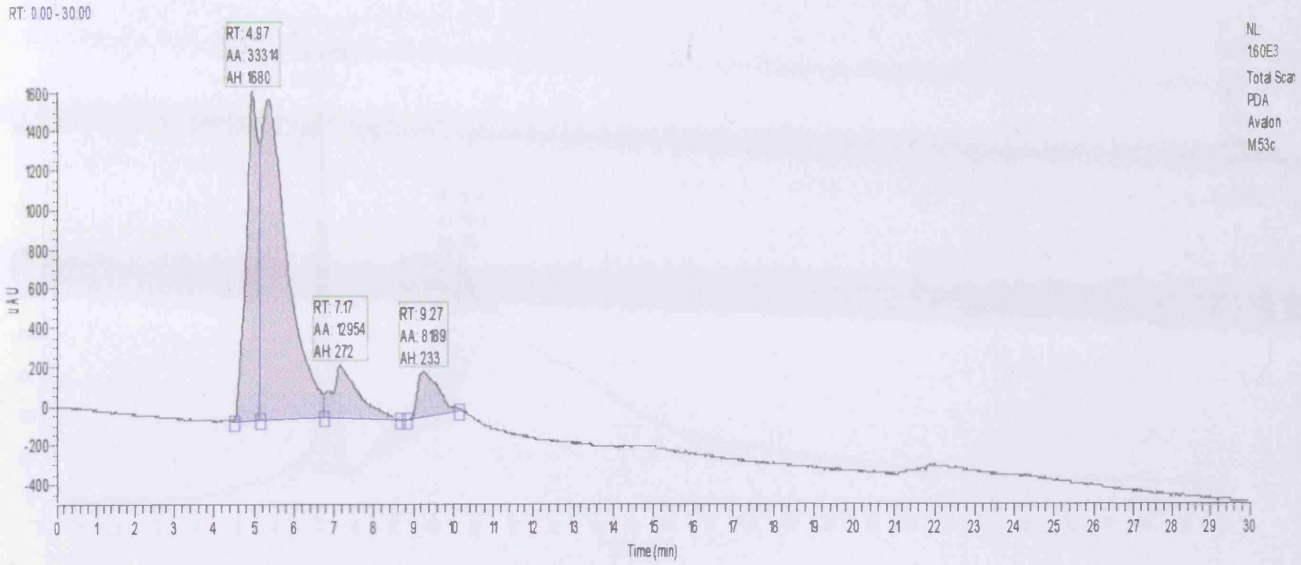
3.1.1.3. Casein peptone C3 spectrum



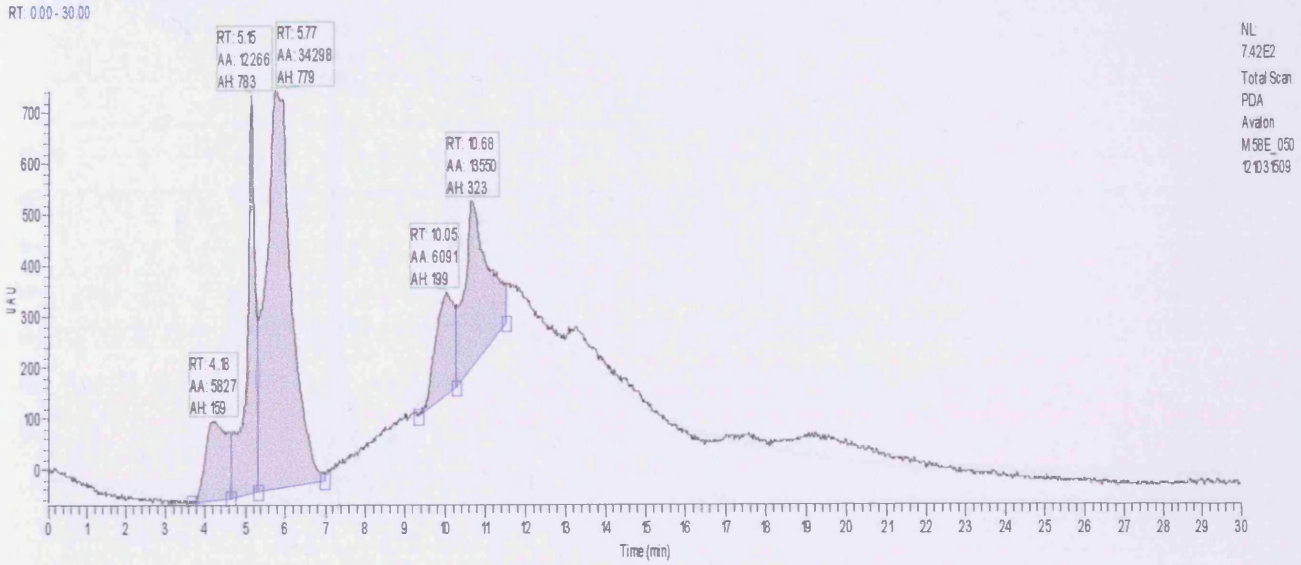
3.1.1.4. Casein peptone C4 spectrum



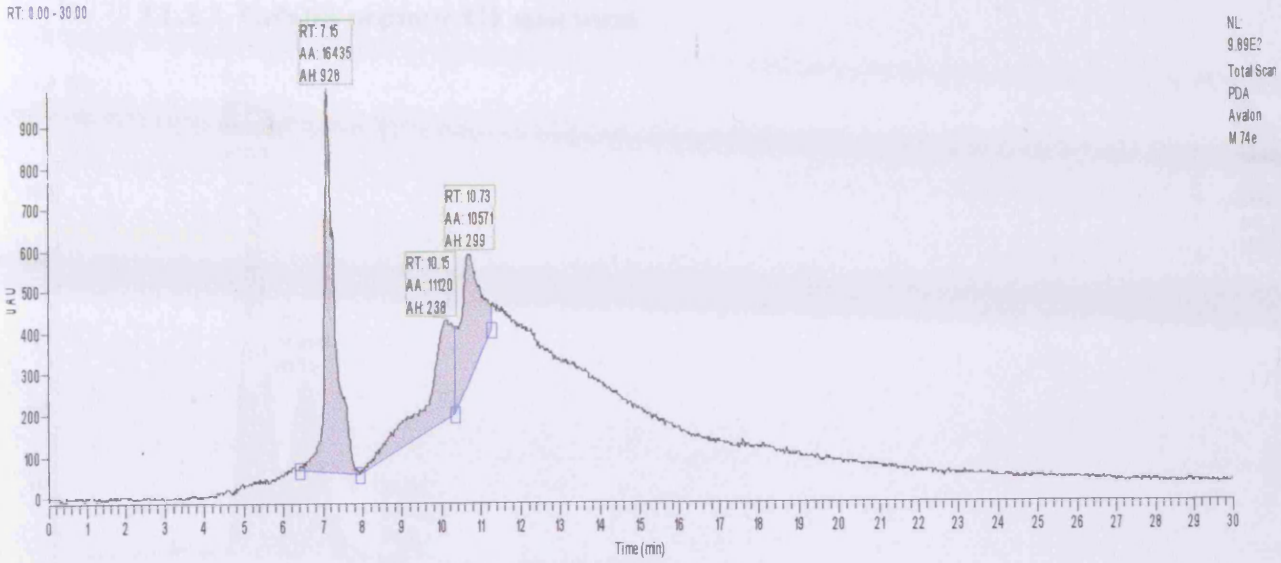
3.1.1.5. Casein peptone C5 spectrum



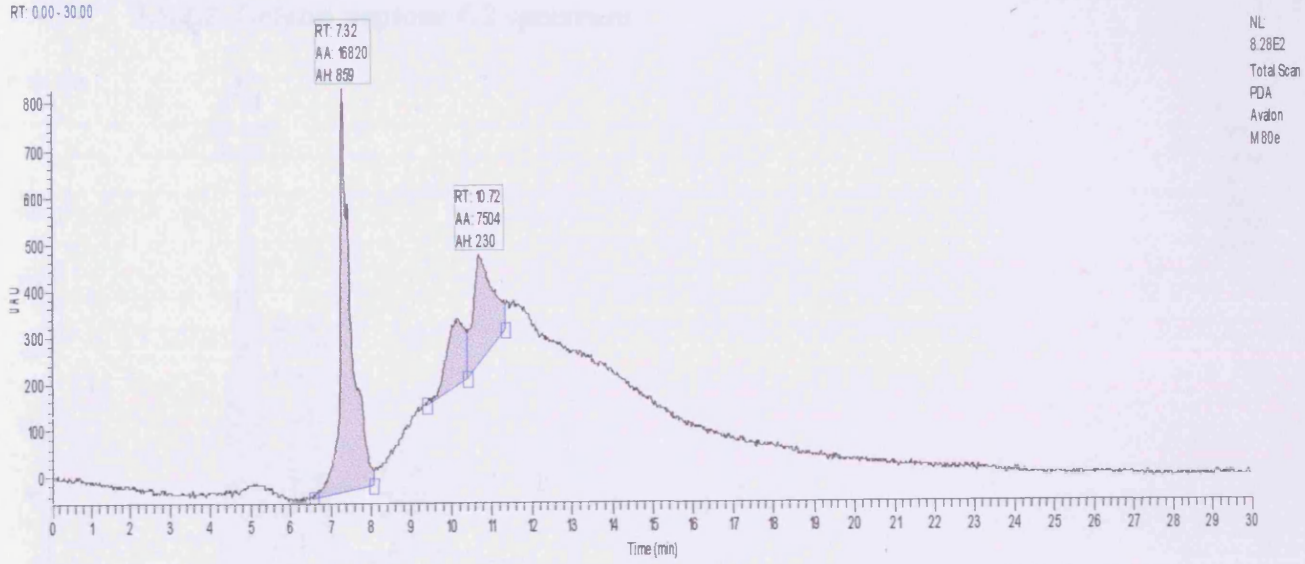
3.1.1.6. Casein peptone C6 spectrum



3.1.1.7. Casein peptone C7 spectrum

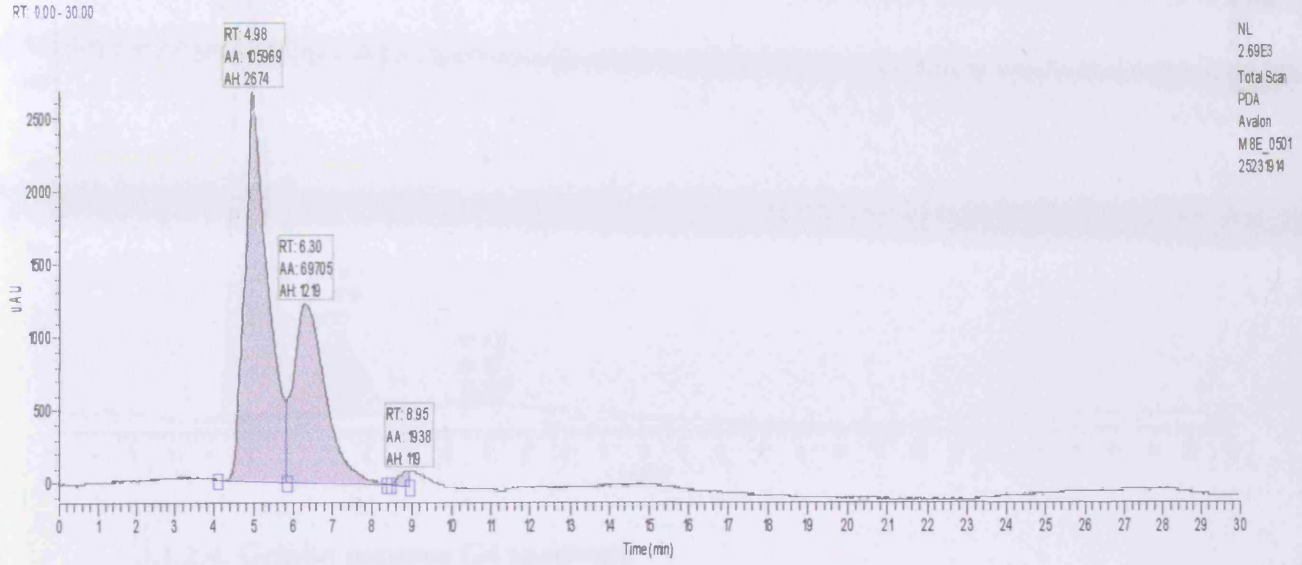


3.1.1.8. Casein peptone C8 spectrum

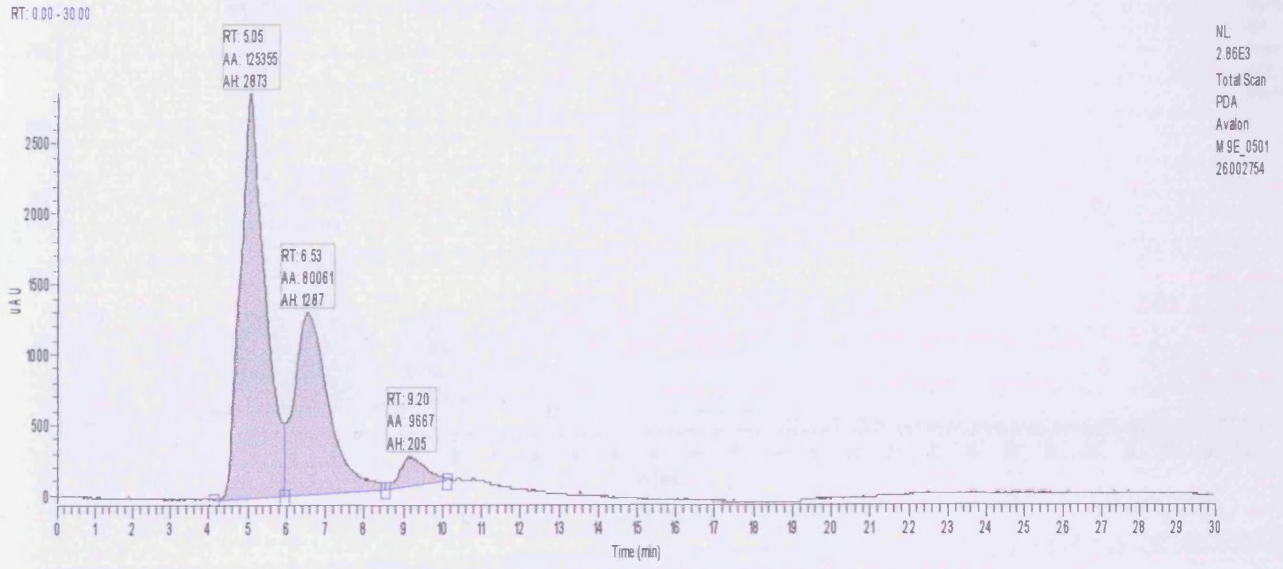


3.1.2. Gelatin peptone spectra

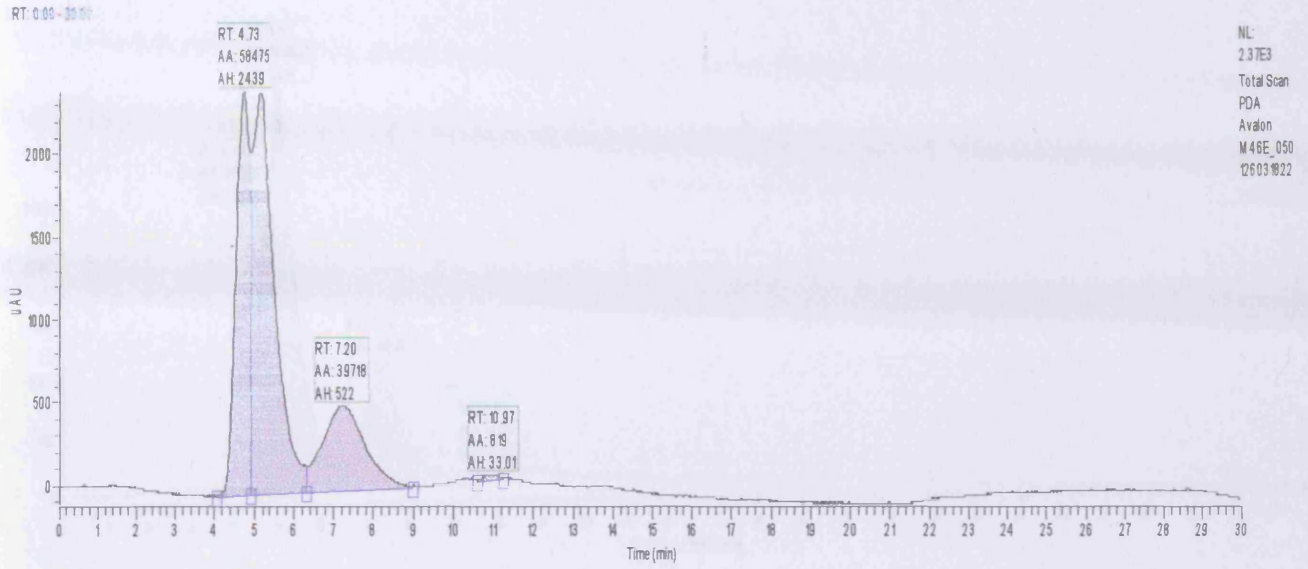
3.1.2.1. Gelatin peptone G1 spectrum



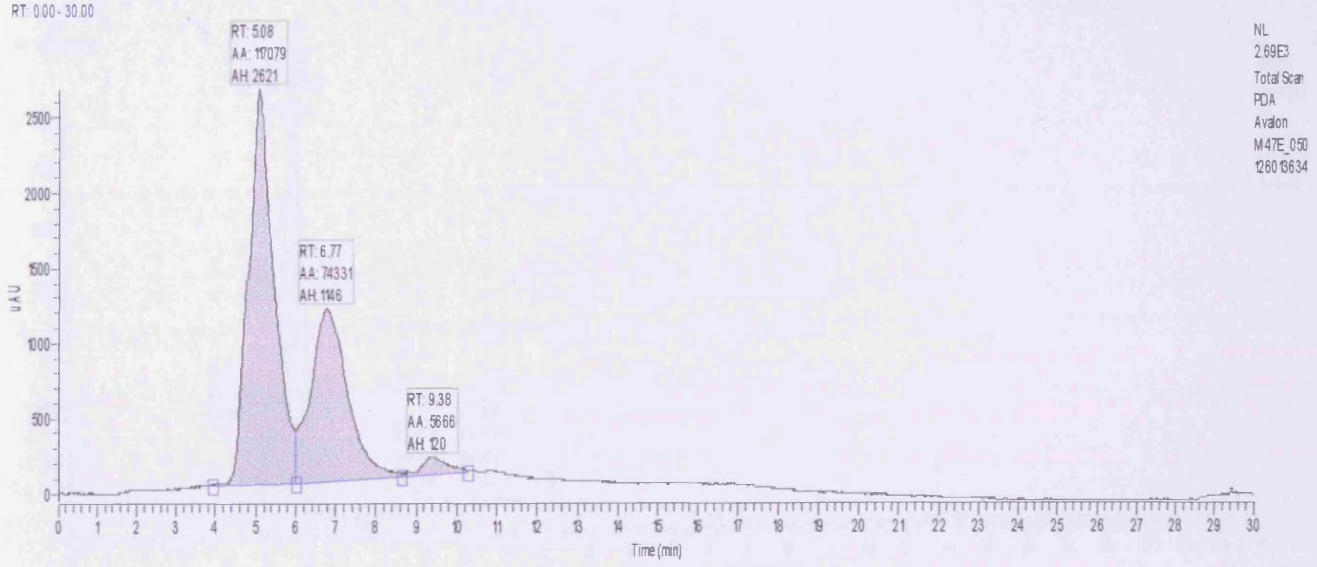
3.1.2.2. Gelatin peptone G2 spectrum



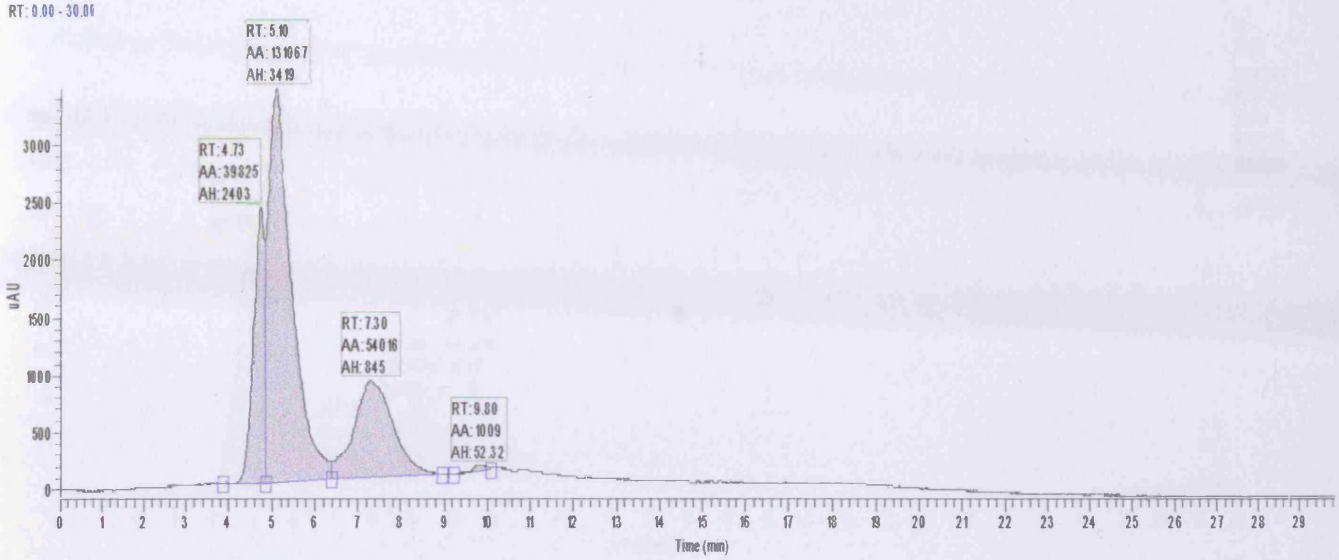
3.1.2.3. Gelatin peptone G3 spectrum



3.1.2.4. Gelatin peptone G4 spectrum

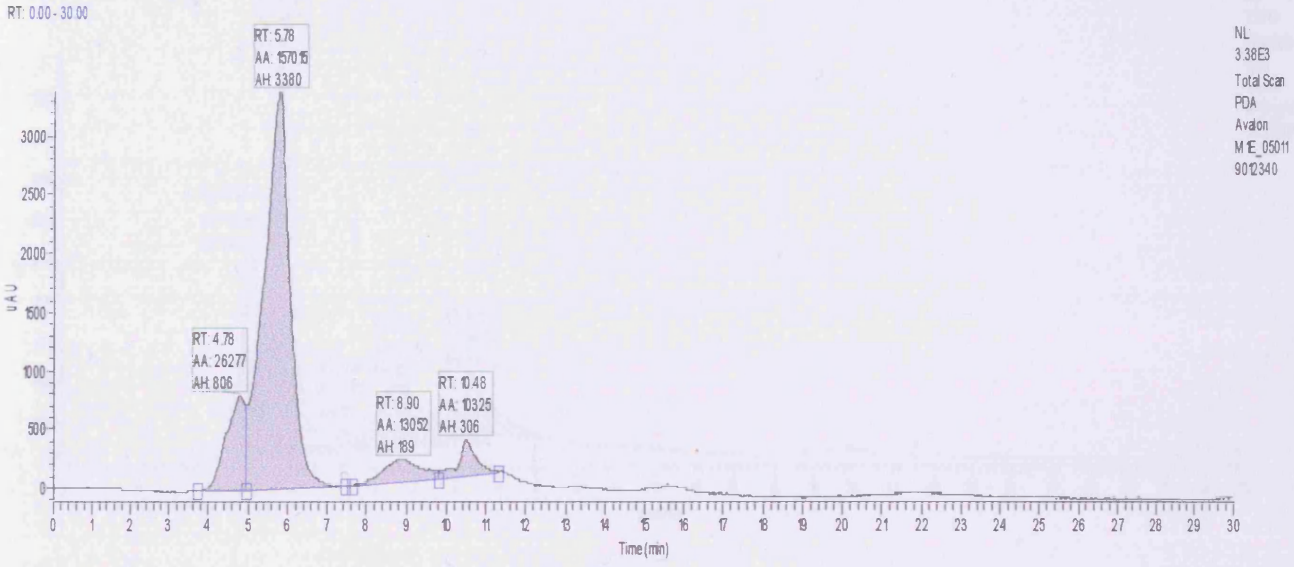


3.1.2.5. Gelatin peptone G5 spectrum



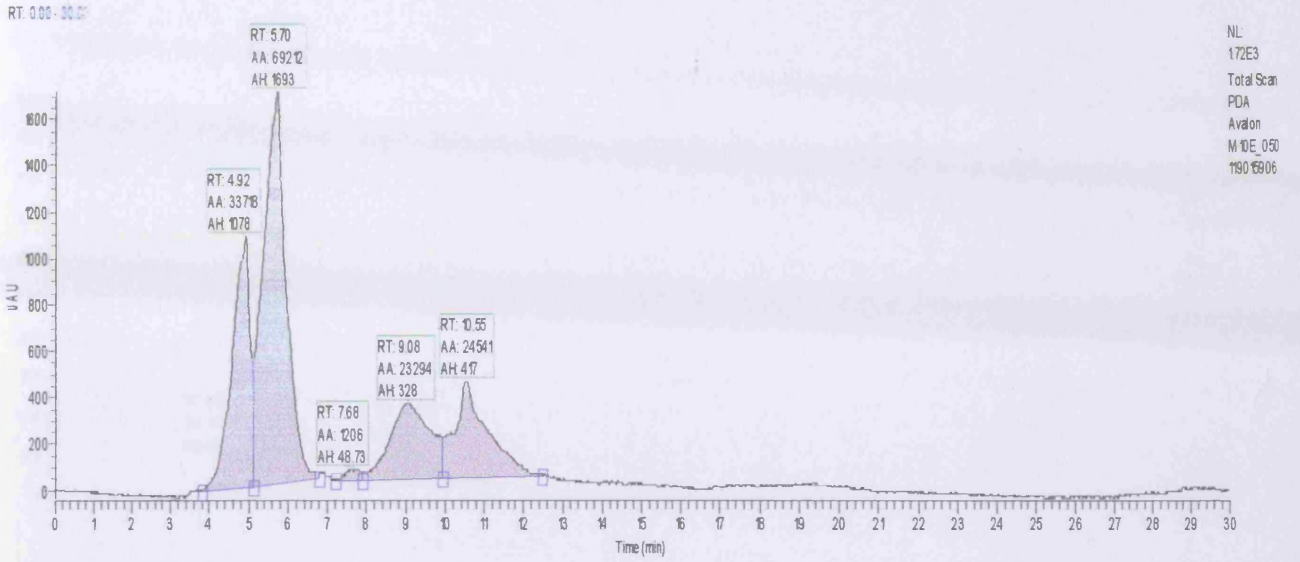
3.1.3. Meat peptone spectra

3.1.3.1. Meat peptone M1 spectrum

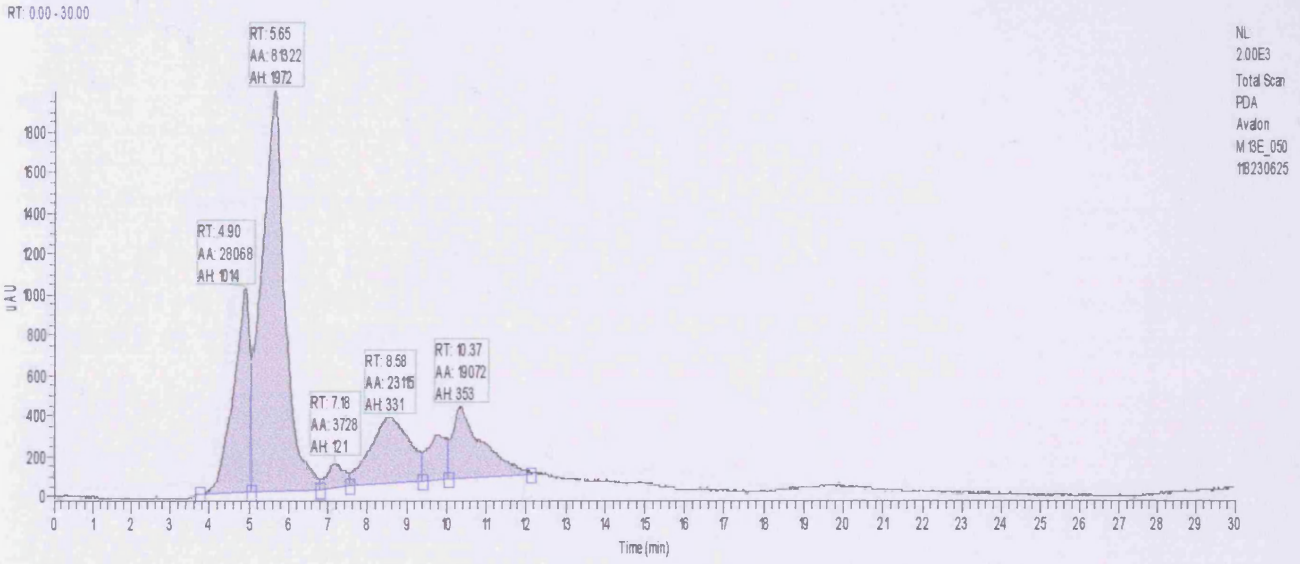


Appendix 3

3.1.3.2. Meat peptone M2 spectrum

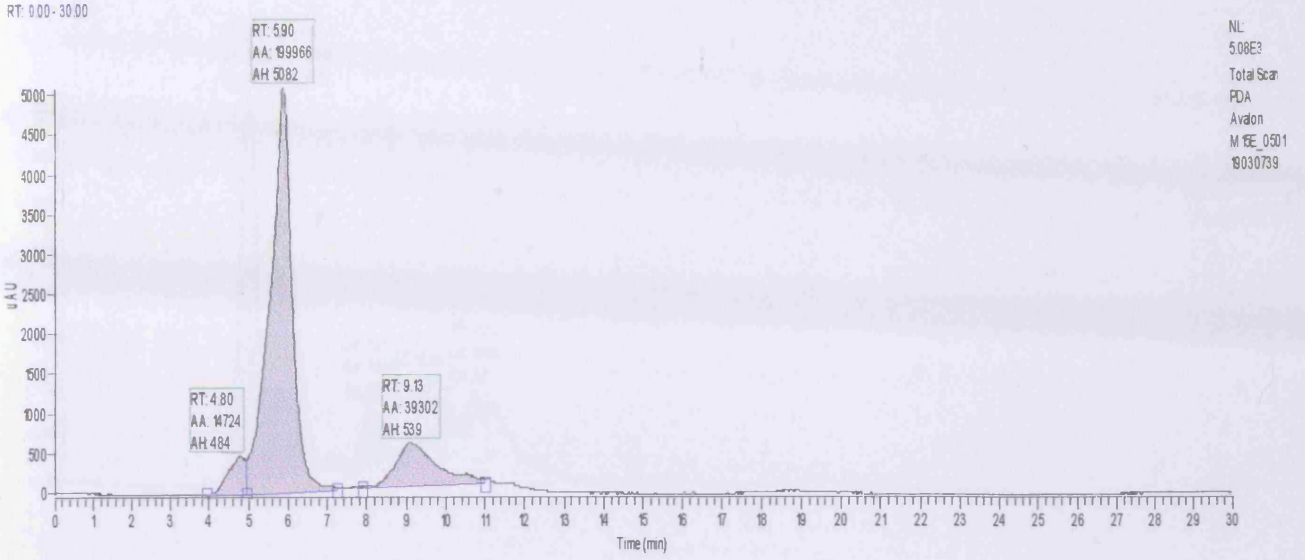


3.1.3.3. Meat peptone M3 spectrum

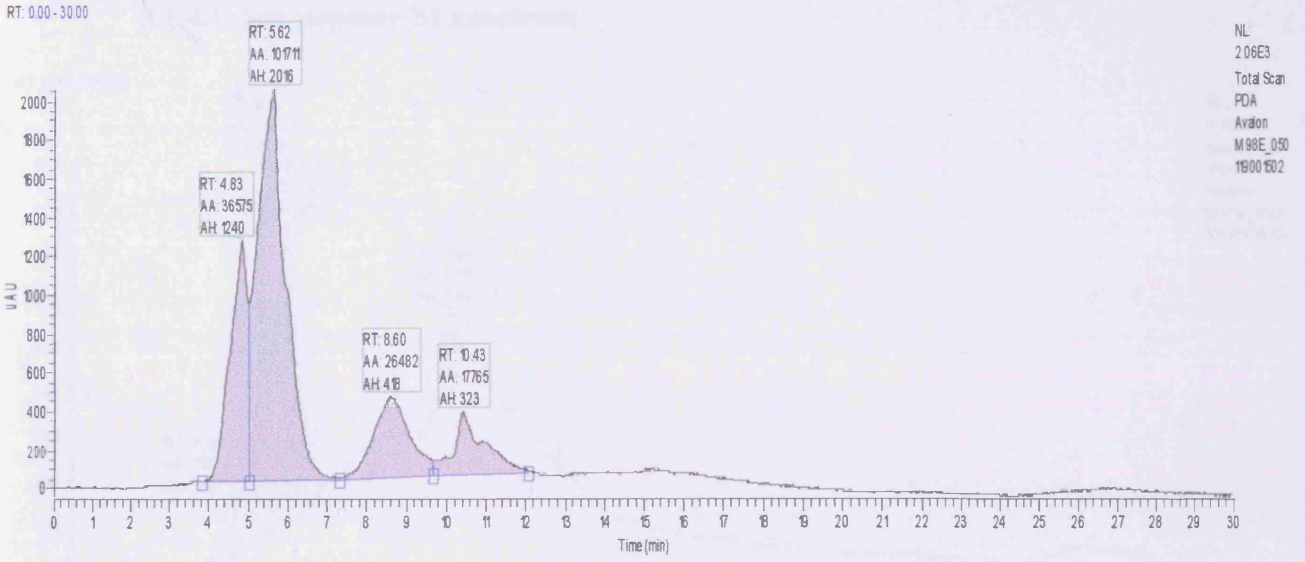


Appendix 3

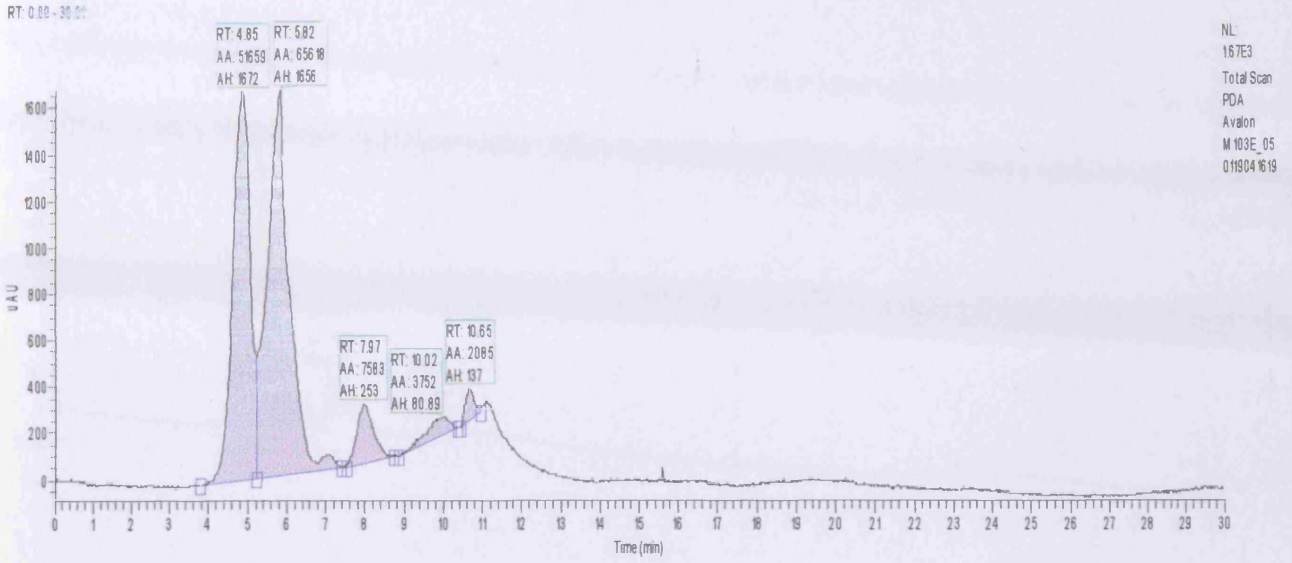
3.1.3.4. Meat peptone M4 spectrum



3.1.3.5. Meat peptone M5 spectrum

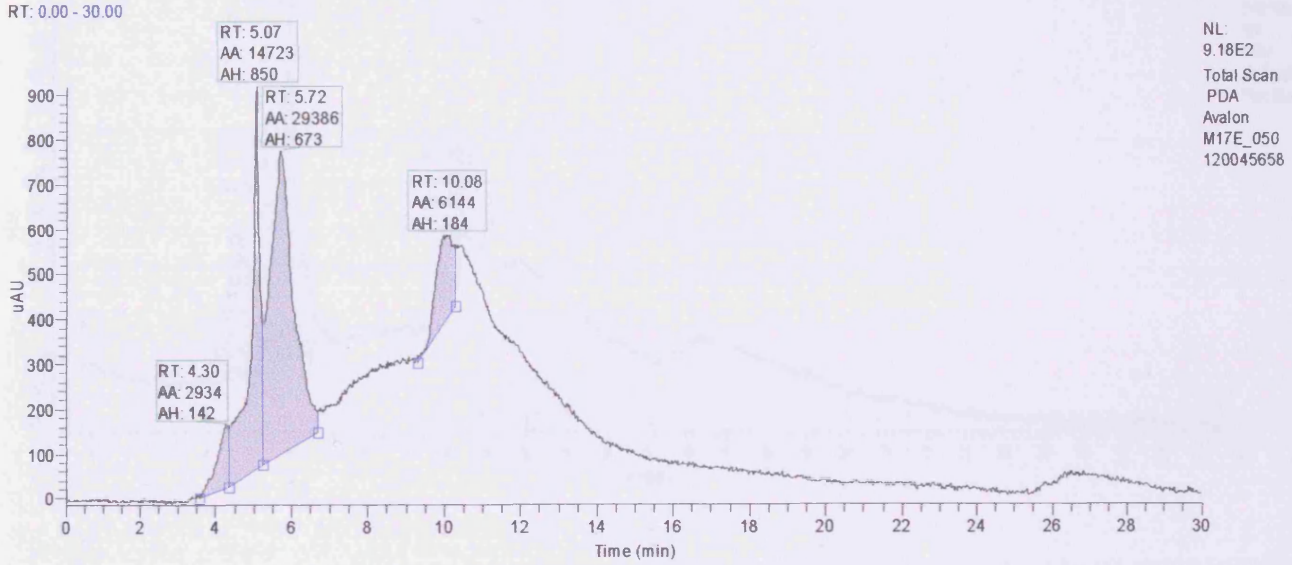


3.1.3.6. Meat peptone M6 spectrum

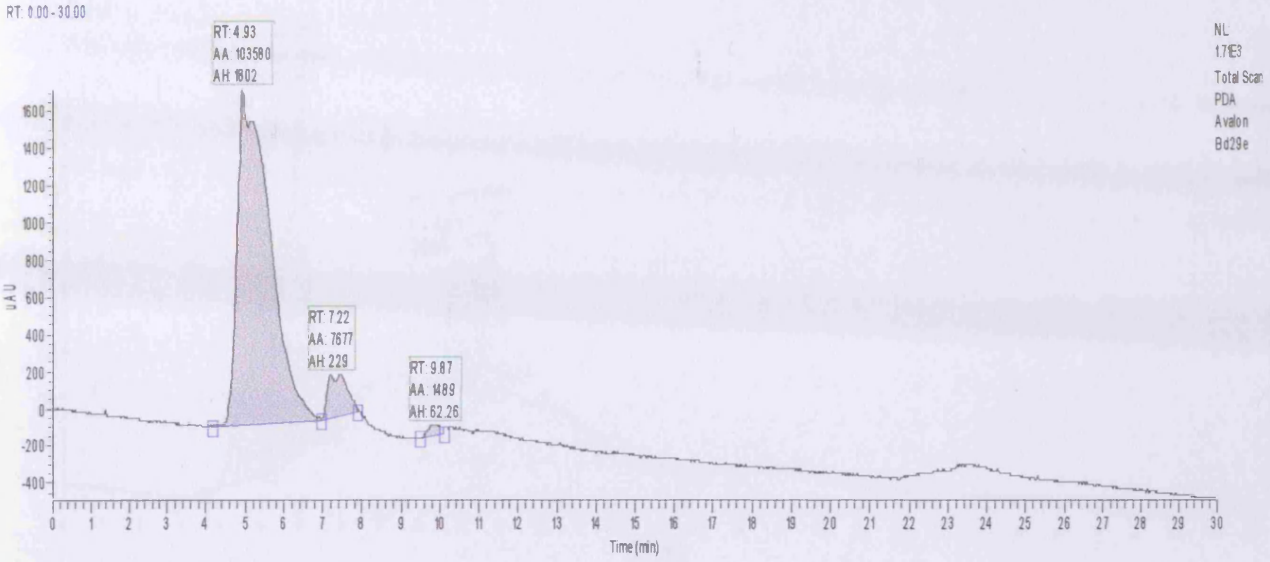


3.1.4. Soy peptone spectra

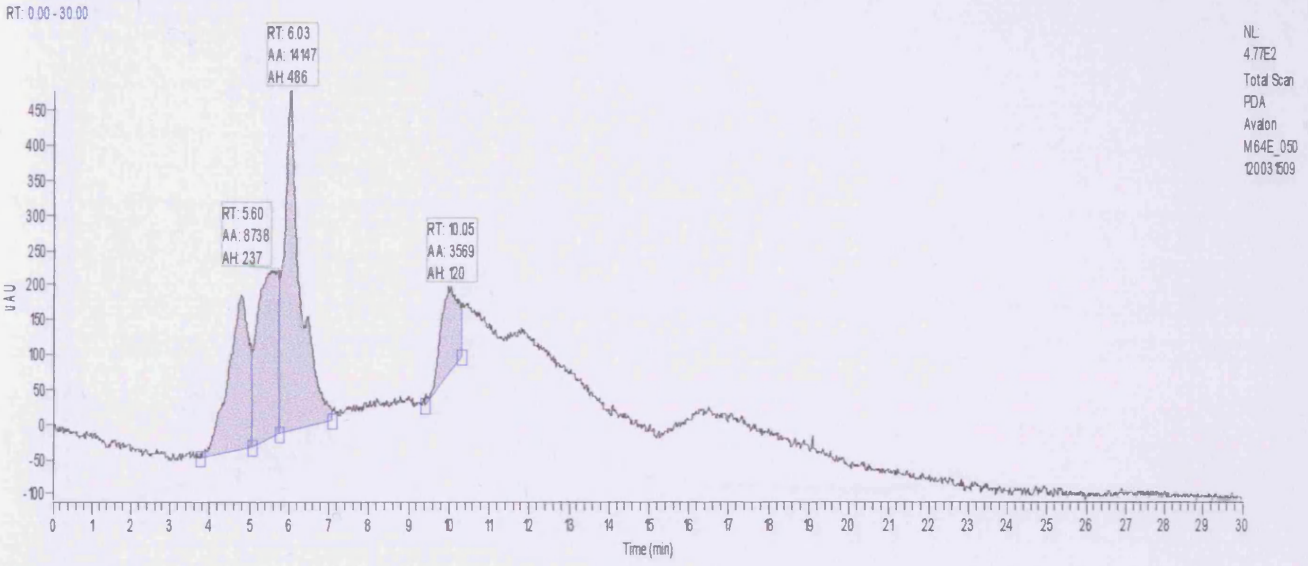
3.1.4.1. Soy peptone S1 spectrum



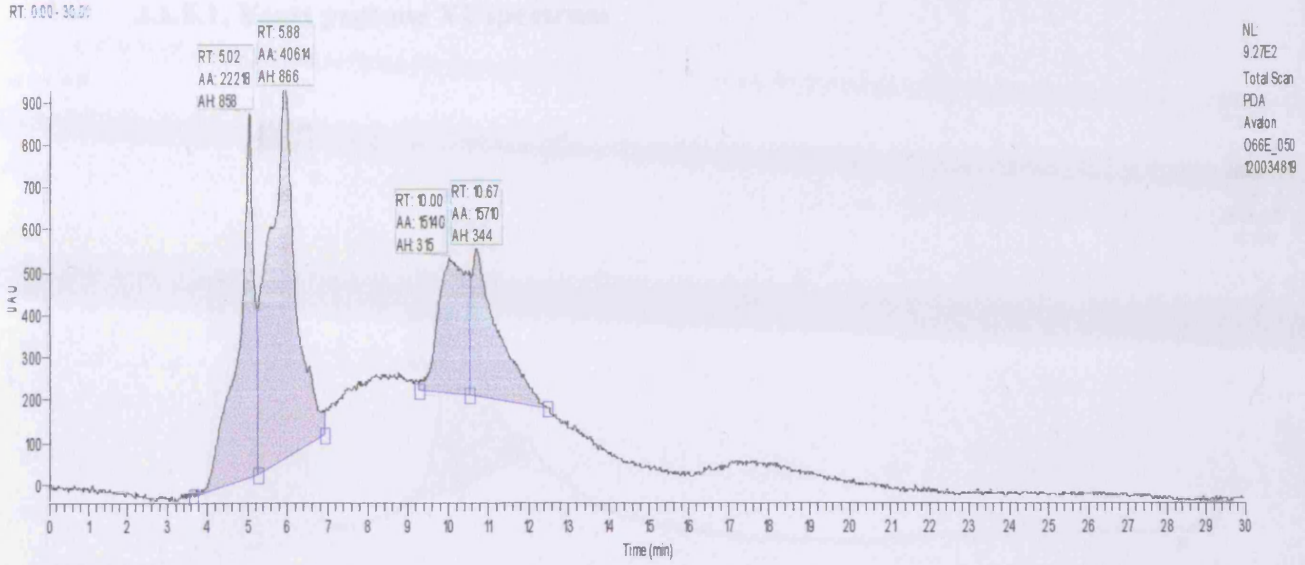
3.1.4.2. Soy peptone S2 spectrum



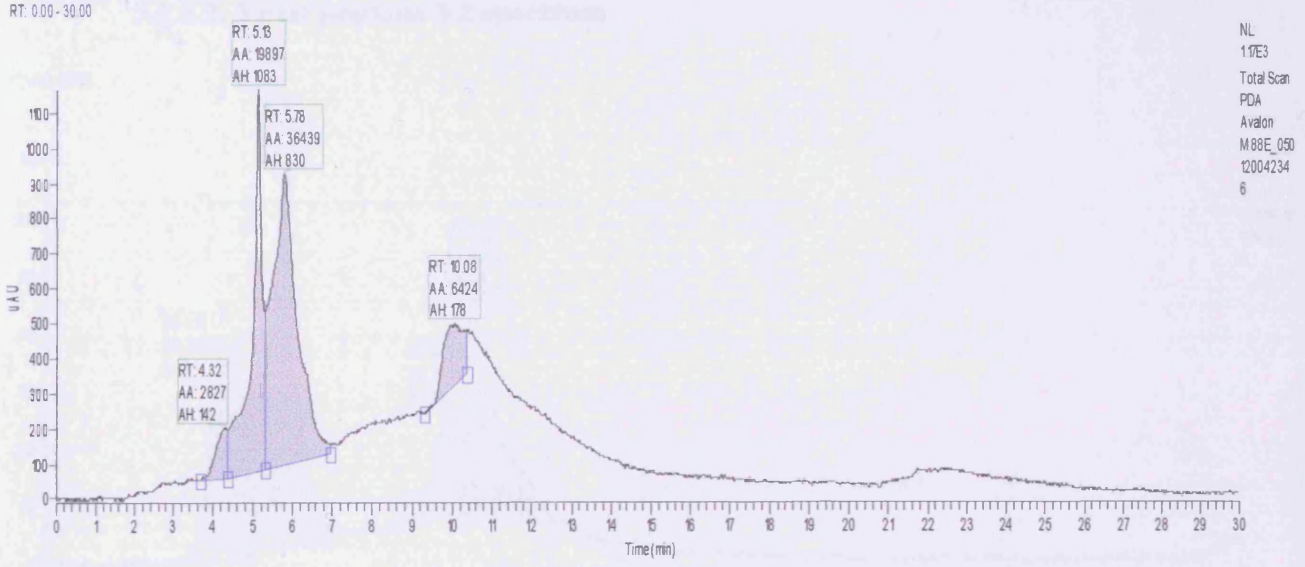
3.1.4.3. Soy peptone S3 spectrum



3.1.4.4. Soy peptone S4 spectrum

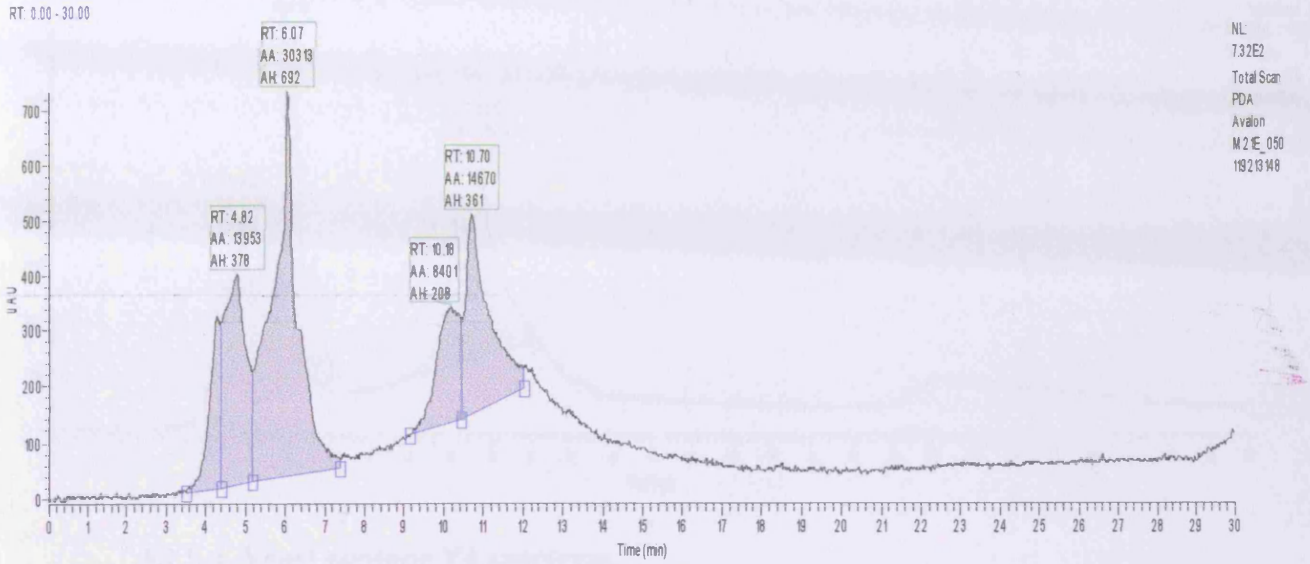


3.1.4.5. Soy peptone S5 spectrum

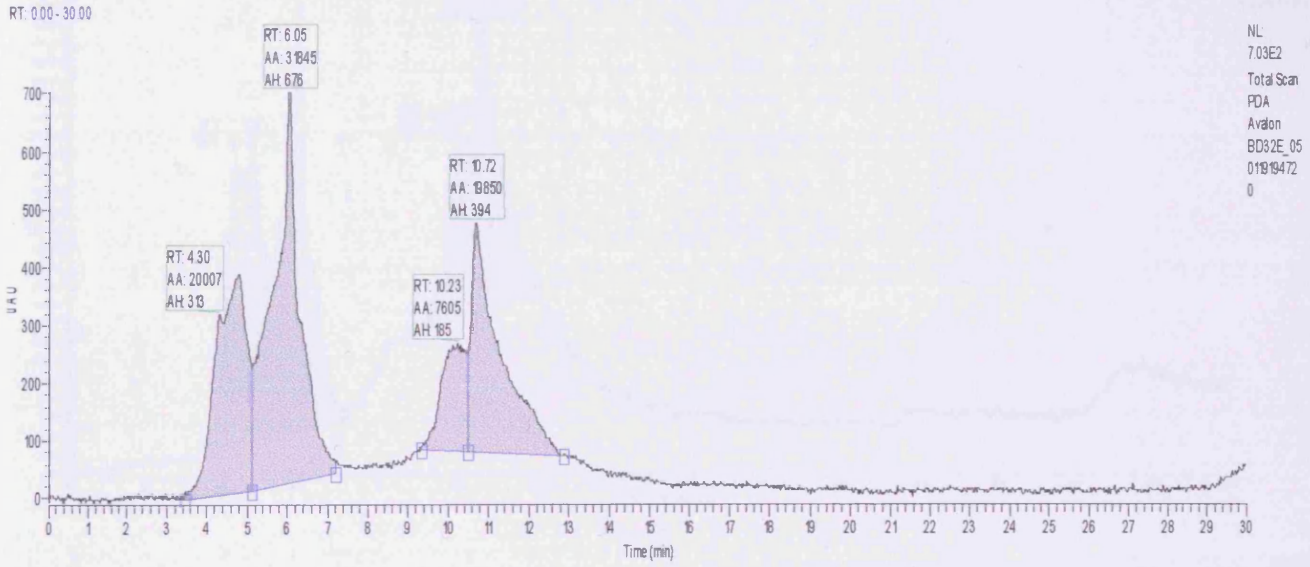


3.1.5. Yeast peptone spectra

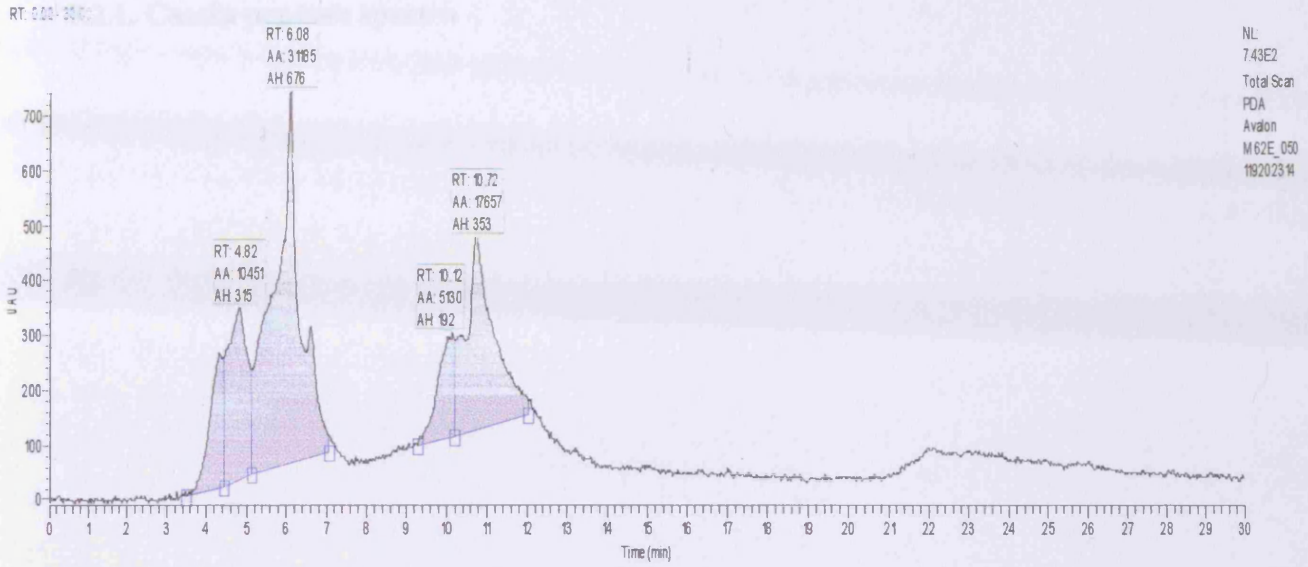
3.1.5.1. Yeast peptone Y1 spectrum



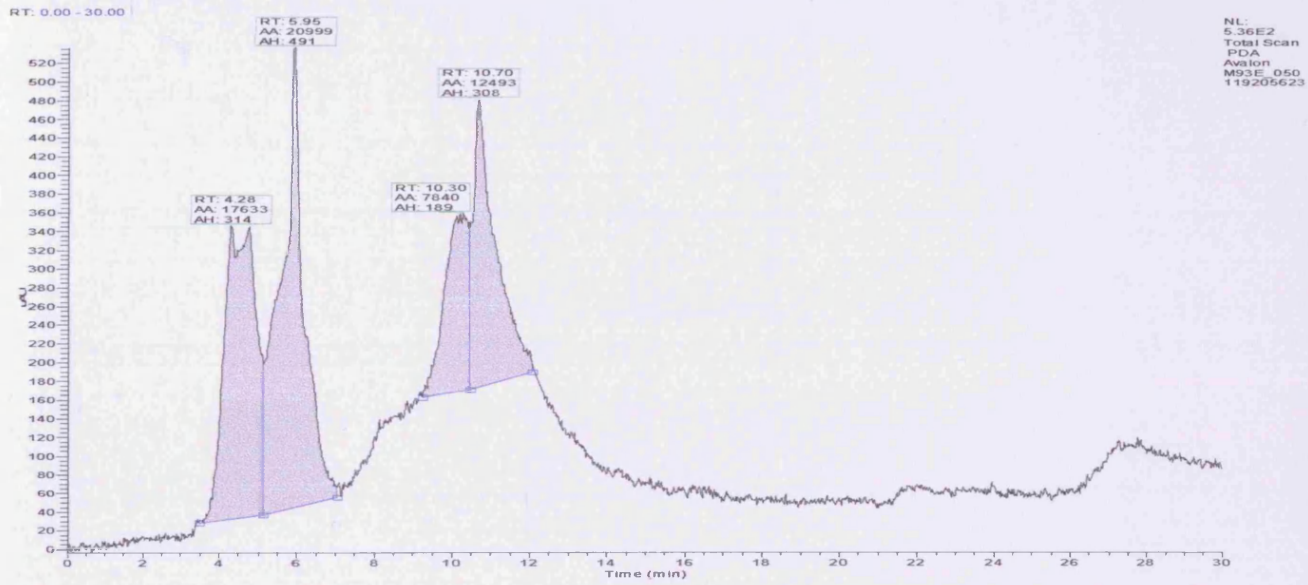
3.1.5.2. Yeast peptone Y2 spectrum



3.1.5.3. Yeast peptone Y3 spectrum



3.1.5.4. Yeast peptone Y4 spectrum

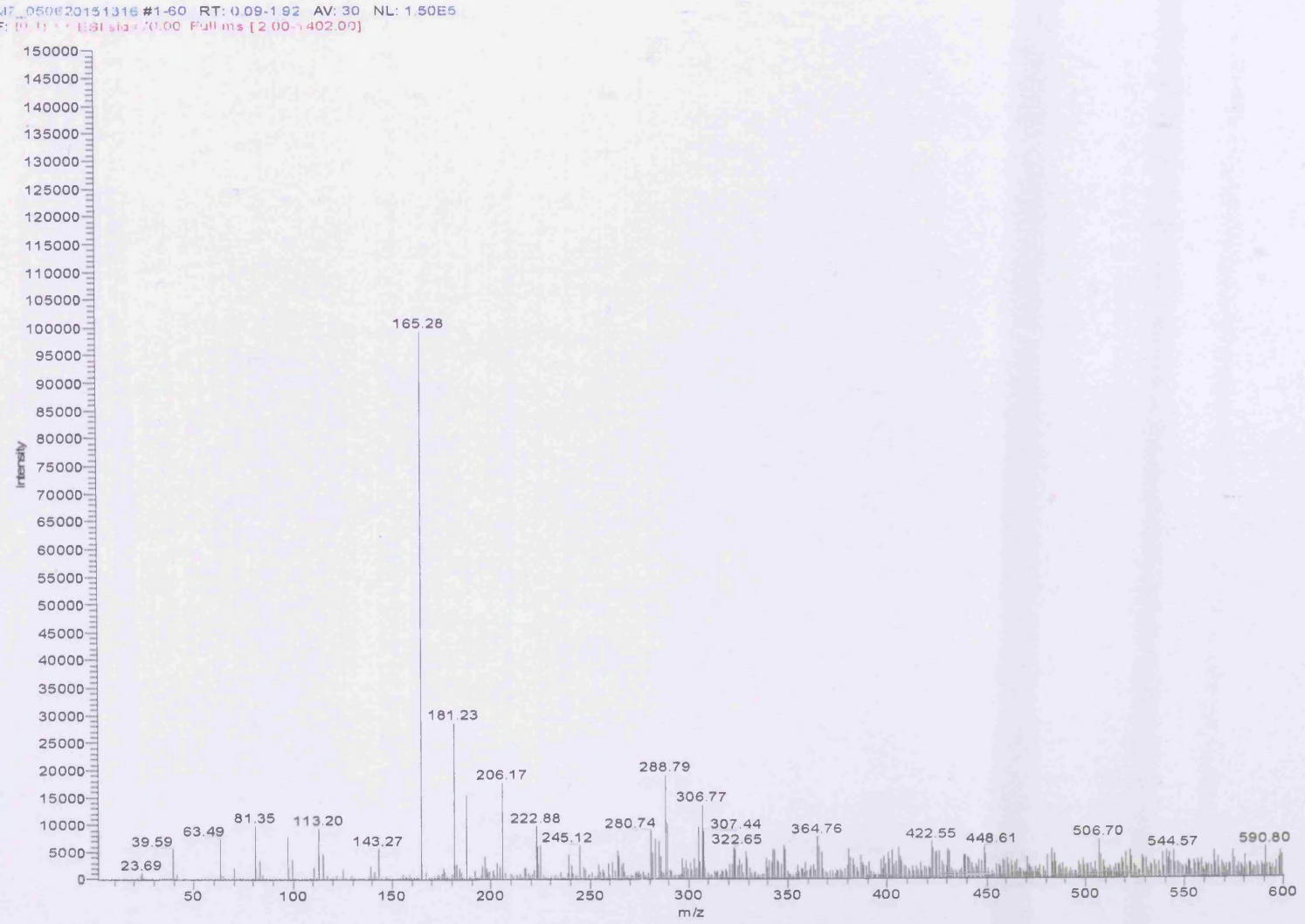


Appendix 3

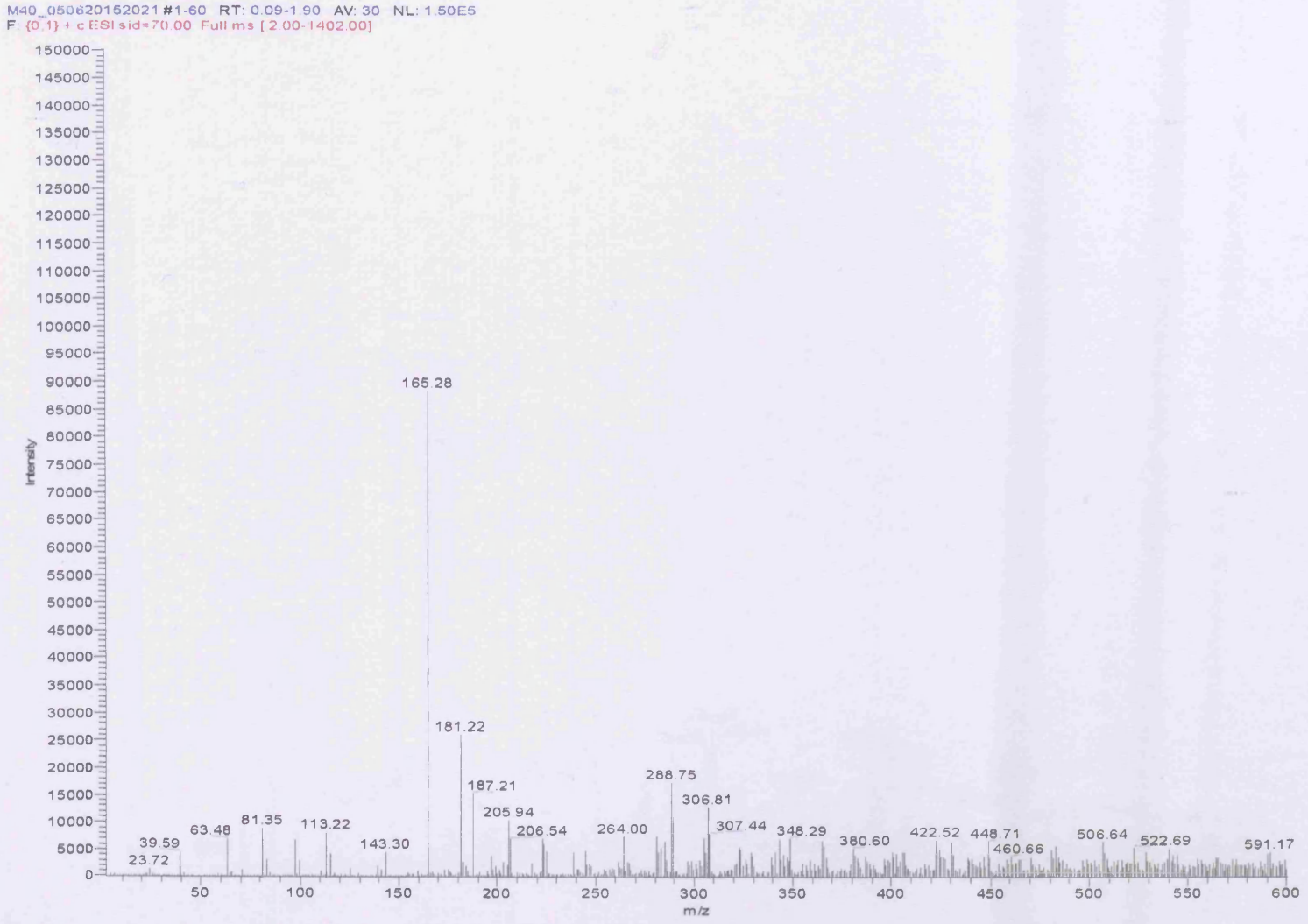
3.2. ESI-MS spectra of peptones

3.2.1. Casein peptone spectra

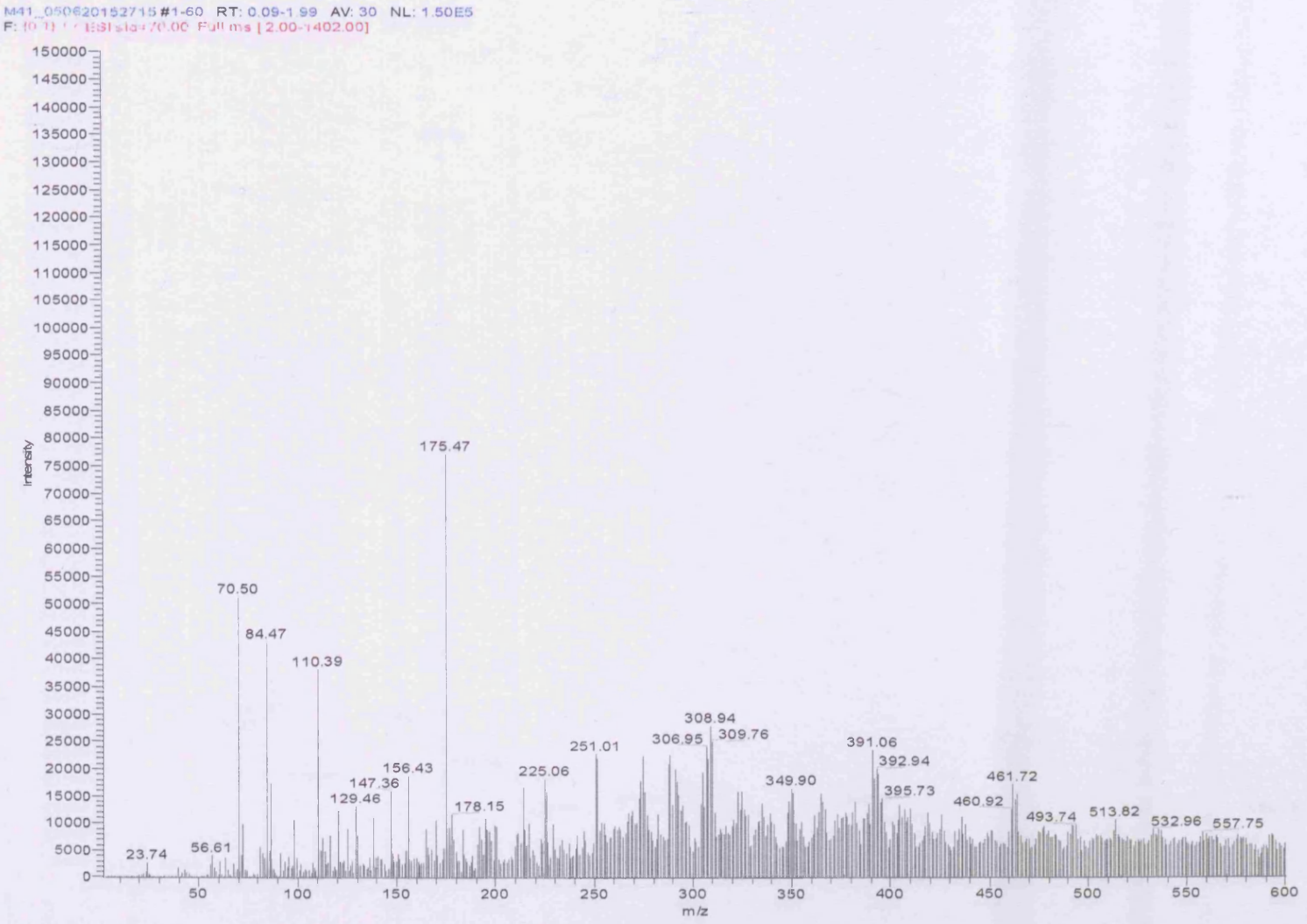
3.2.1.1. Casein peptone C1 spectrum



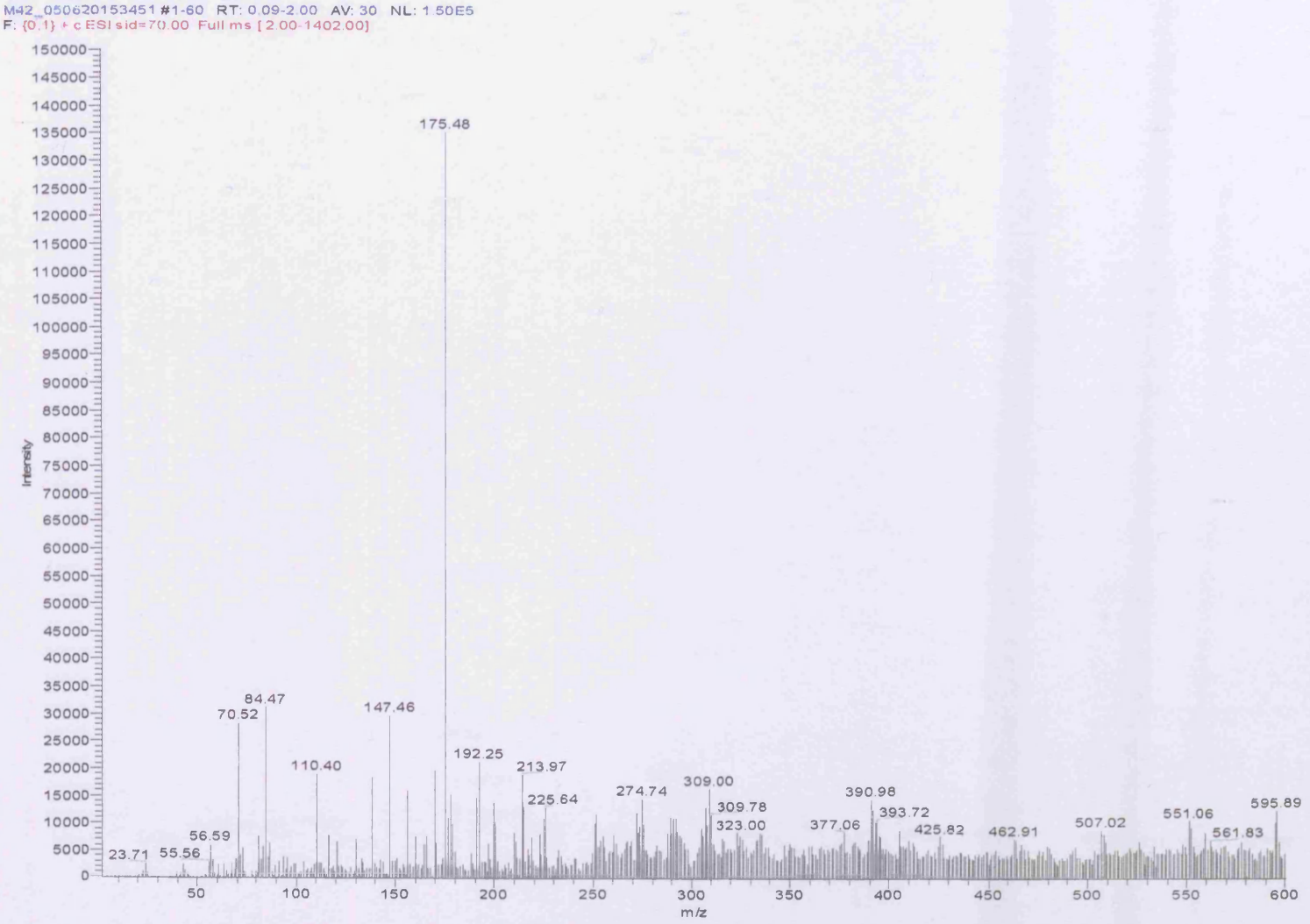
3.2.1.2. Casein peptone C2 spectrum



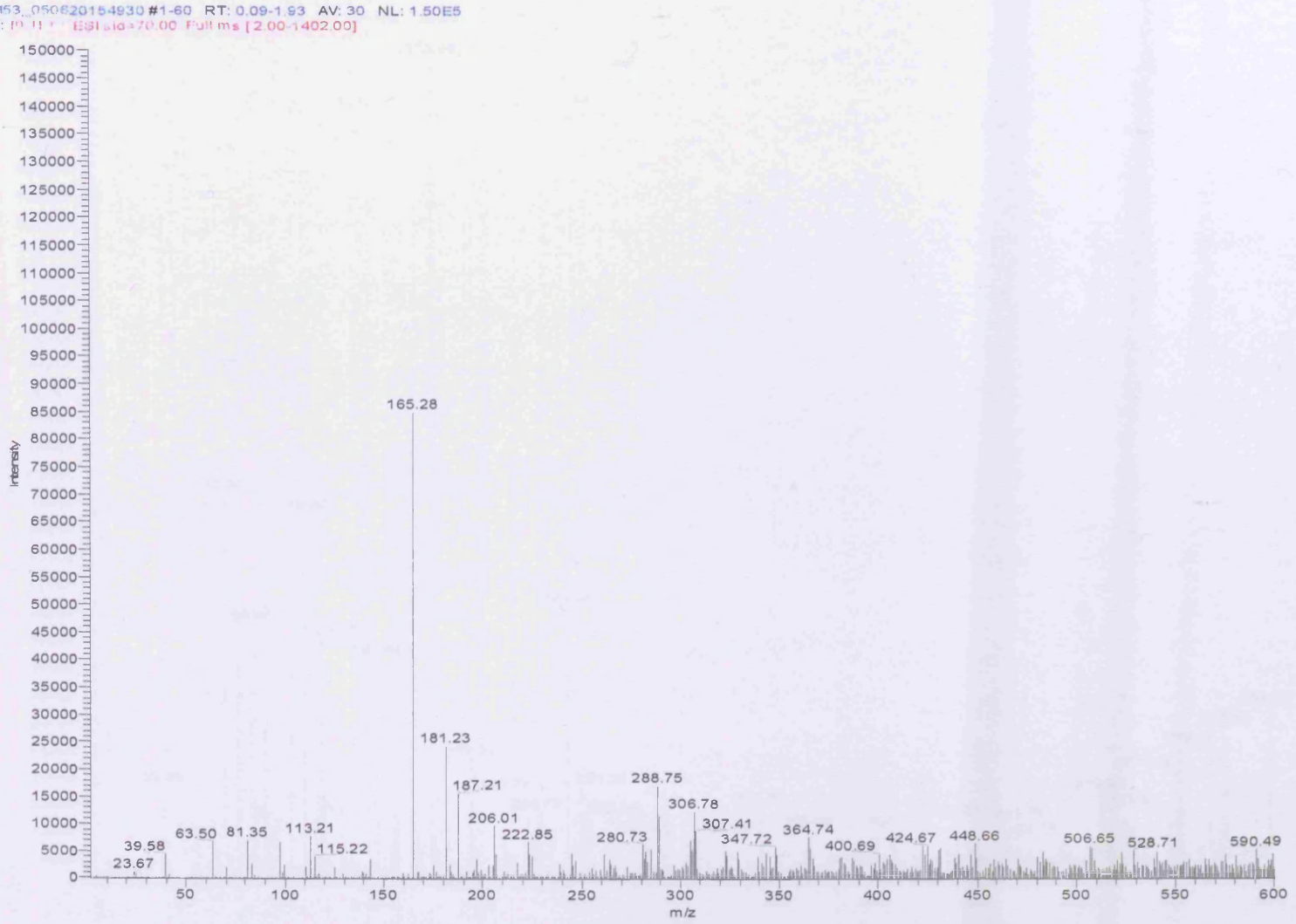
3.2.1.3. Casein peptone C3 spectrum



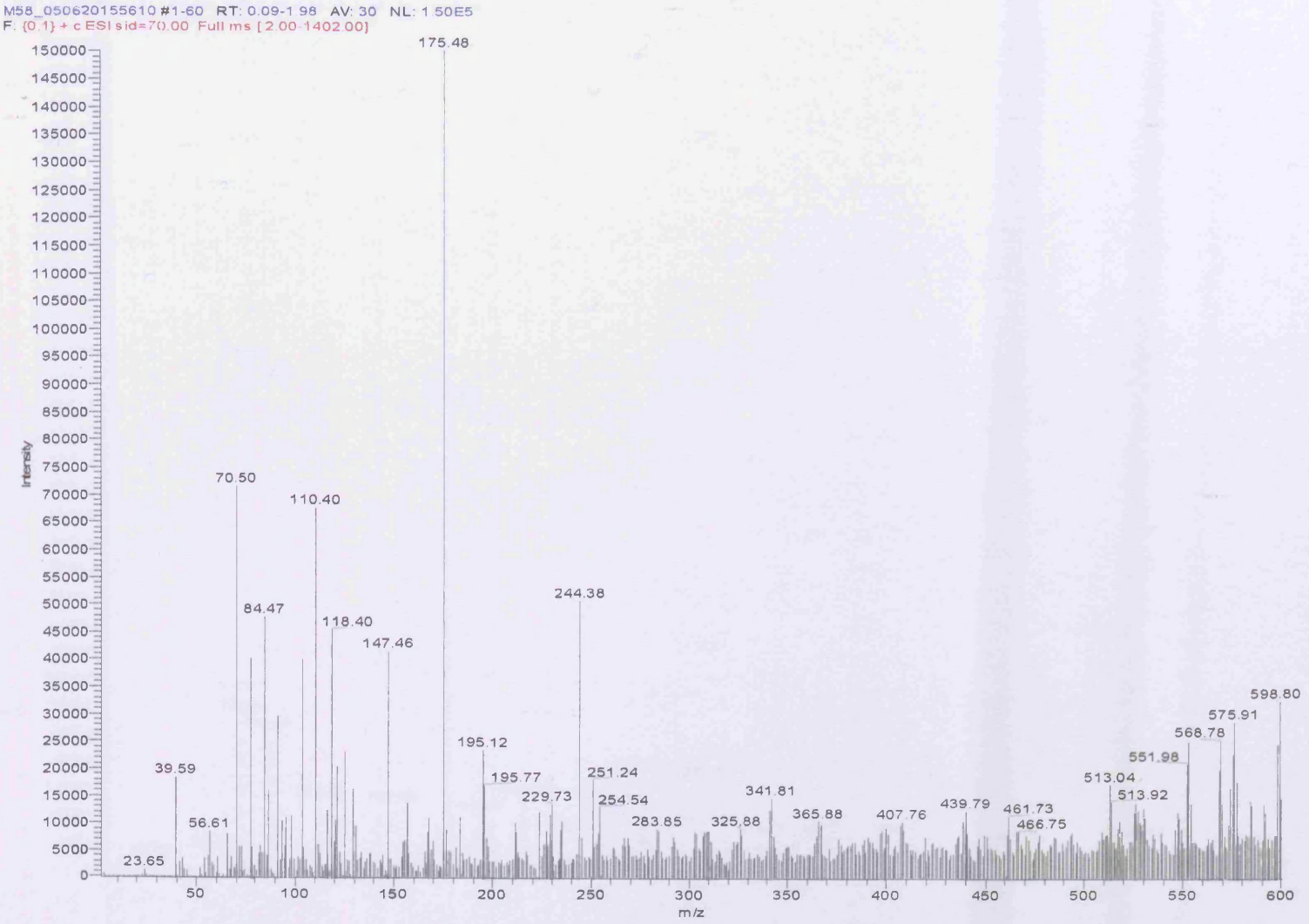
3.2.1.4. Casein peptone C4 spectrum



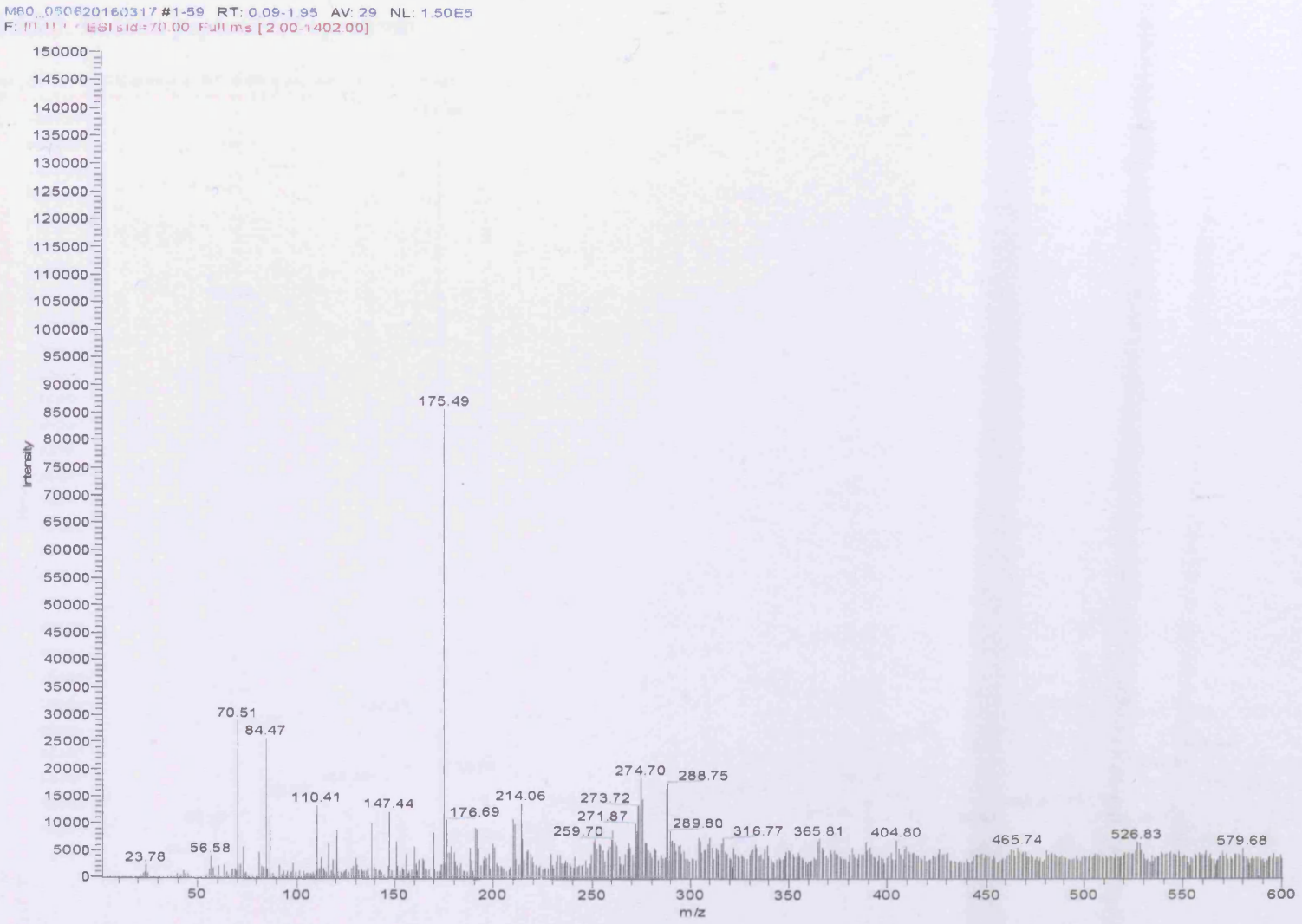
3.2.1.5. Casein peptone C5 spectrum



3.2.1.6. Casein peptone C6 spectrum

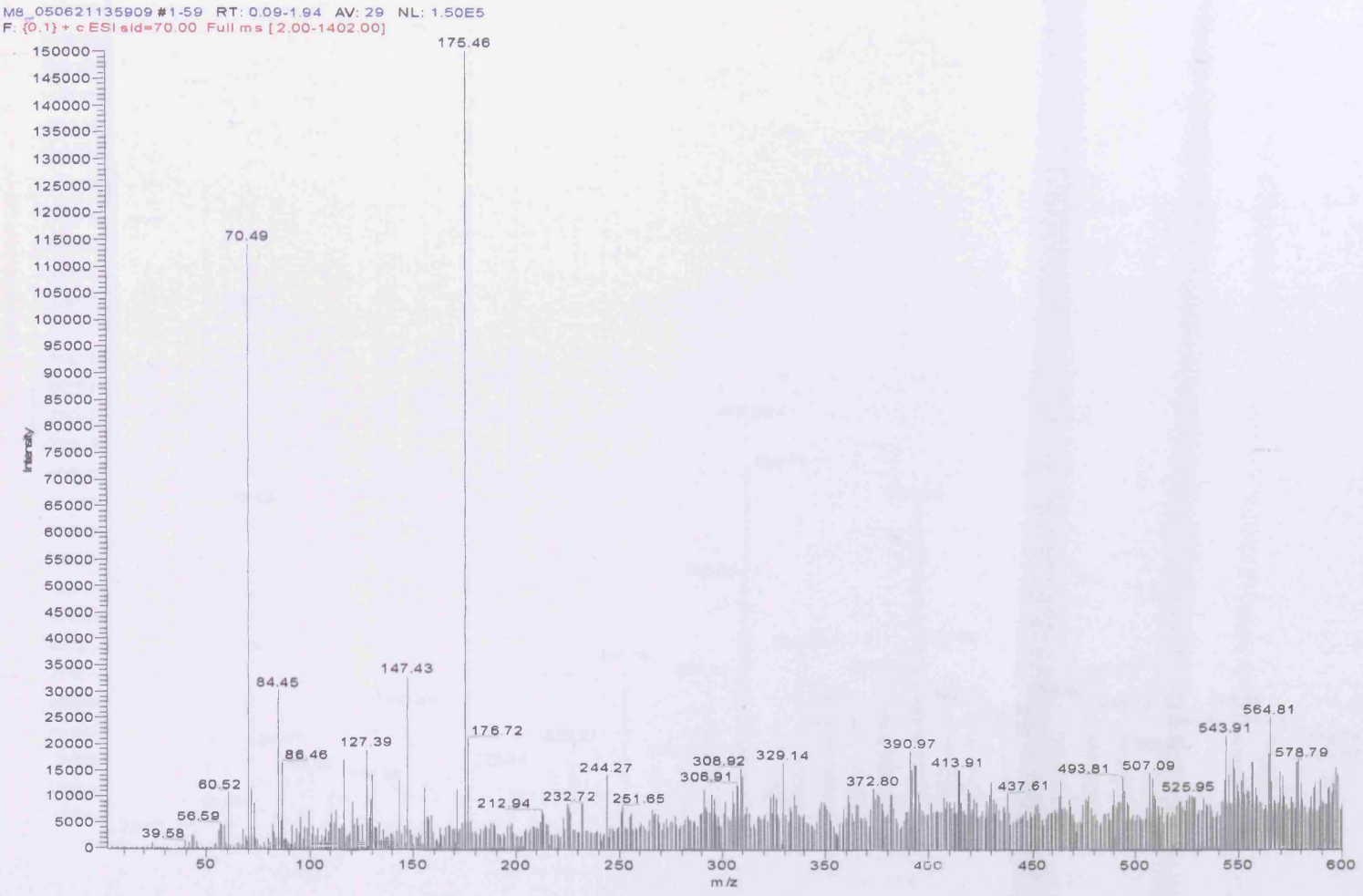


3.2.1.7. Casein peptone C8 spectrum

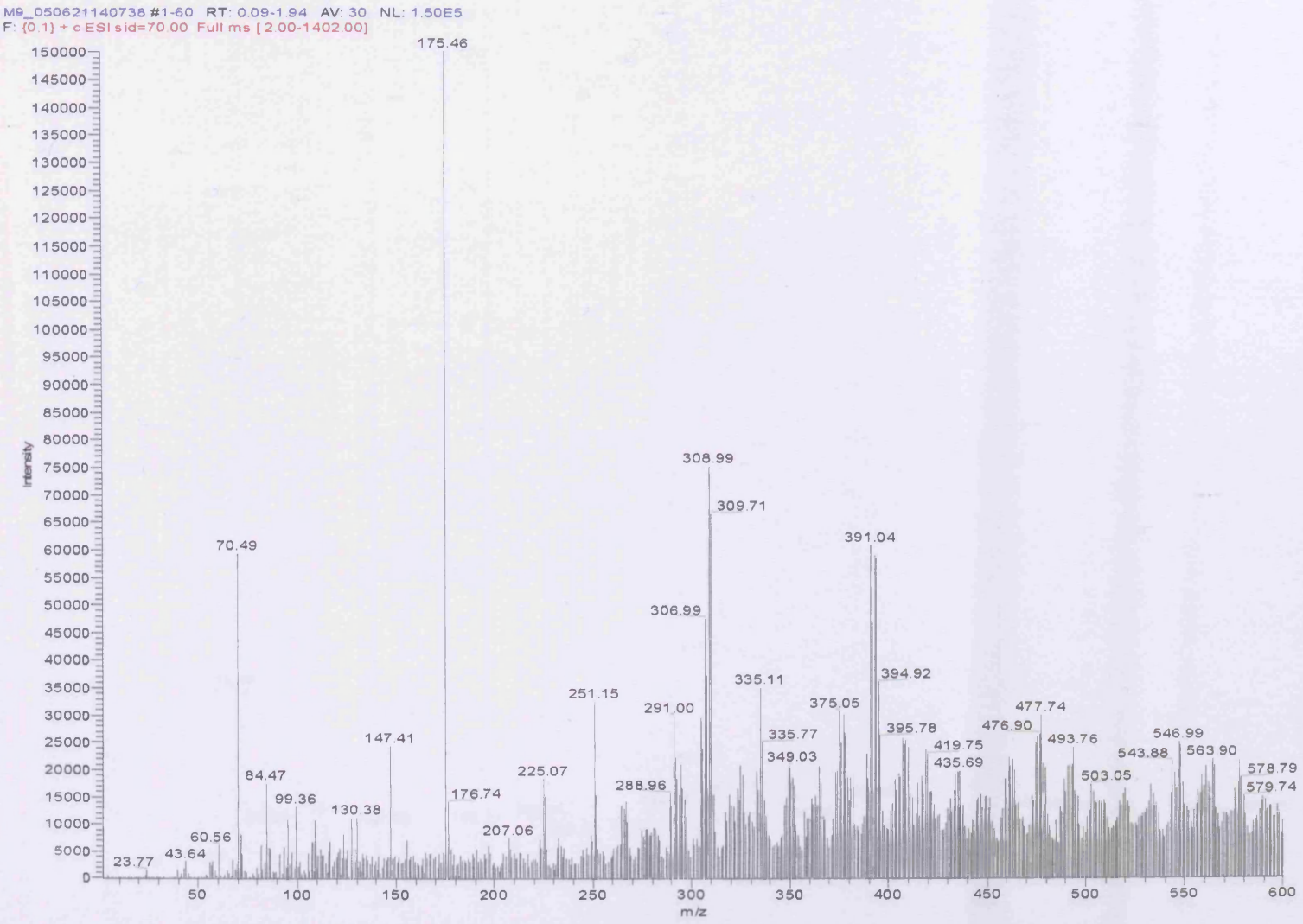


3.2.2. Gelatin peptone spectra

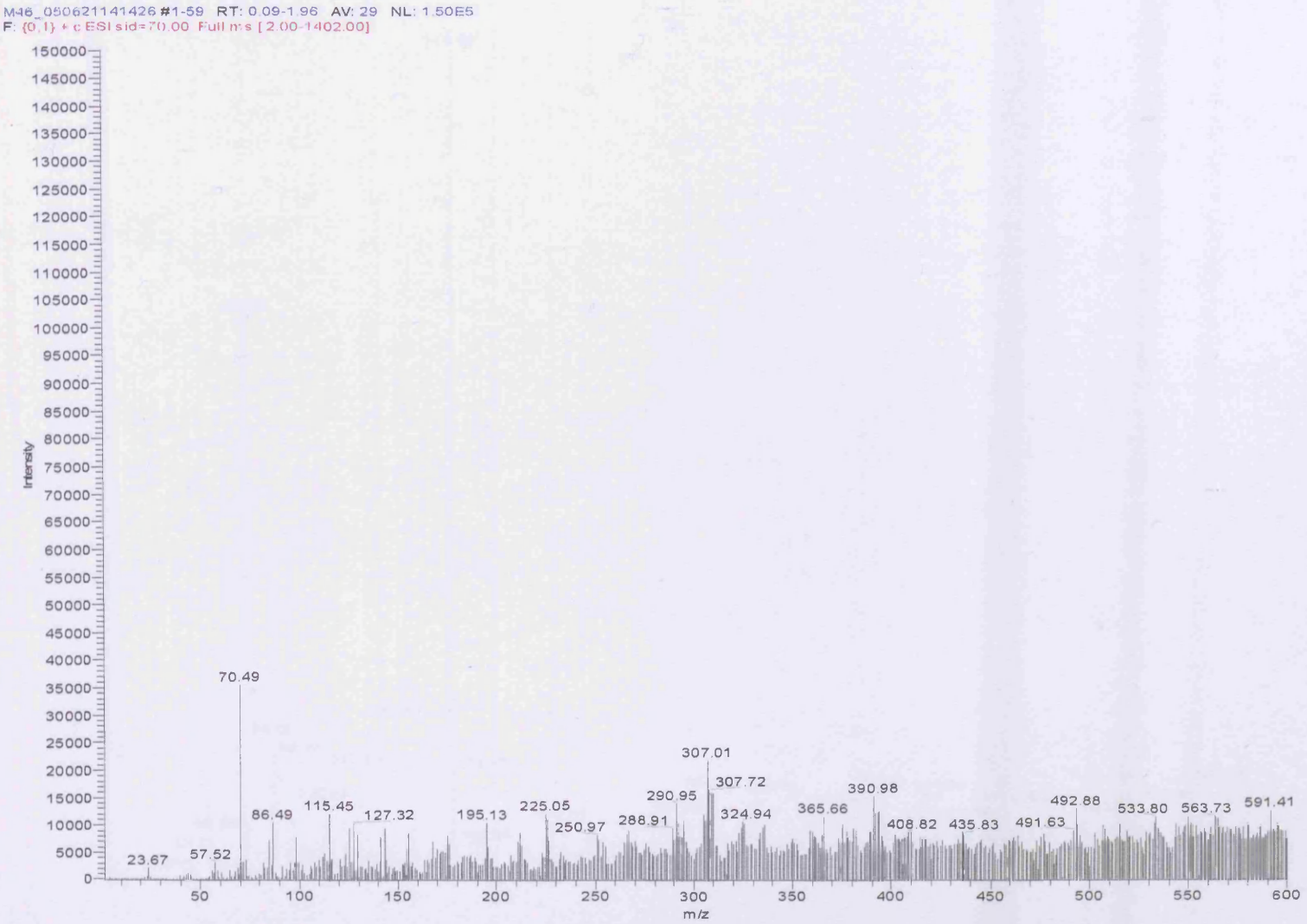
3.2.2.1. Gelatin peptone G1 spectrum



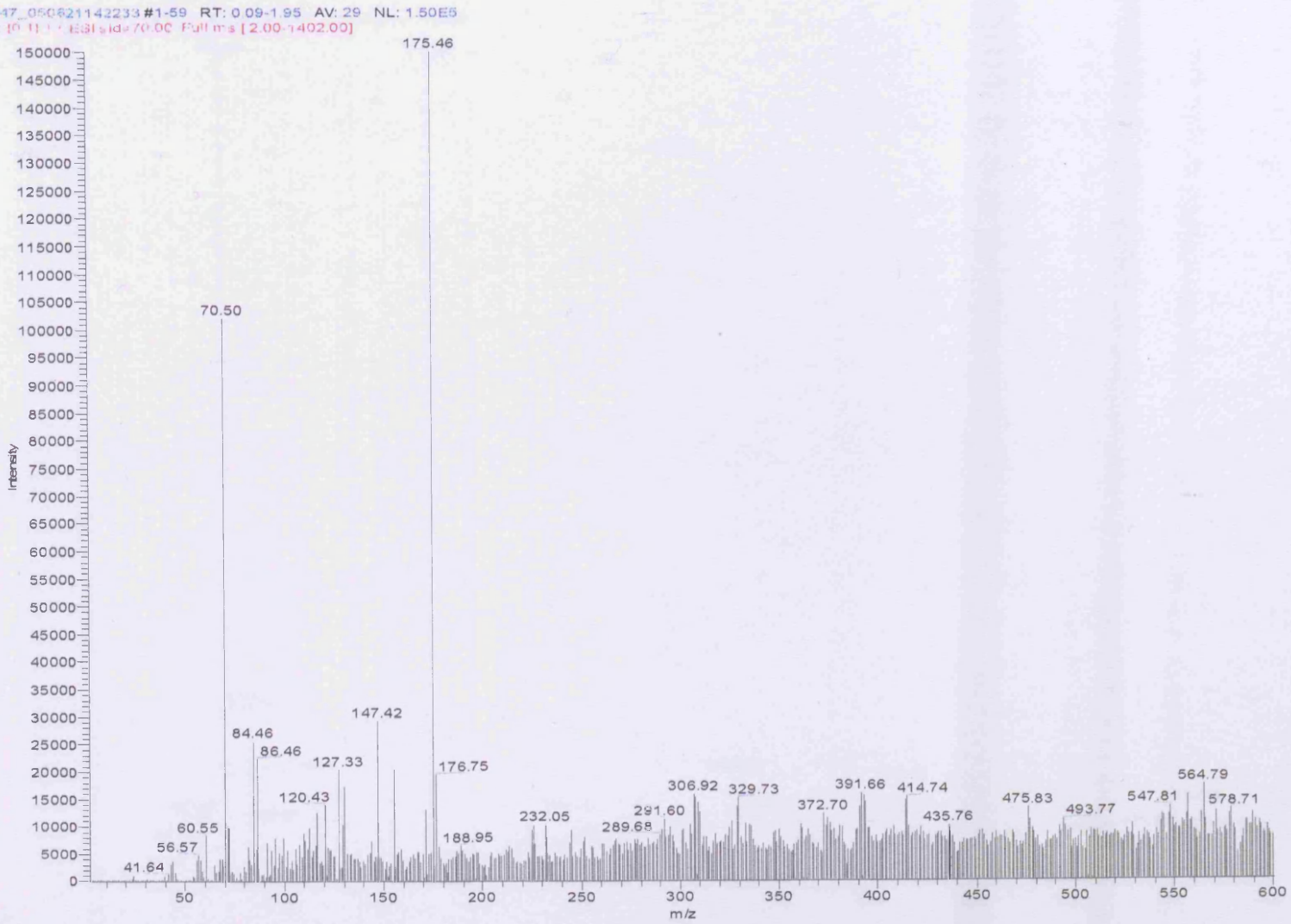
3.2.2.2. Gelatin peptone G2 spectrum



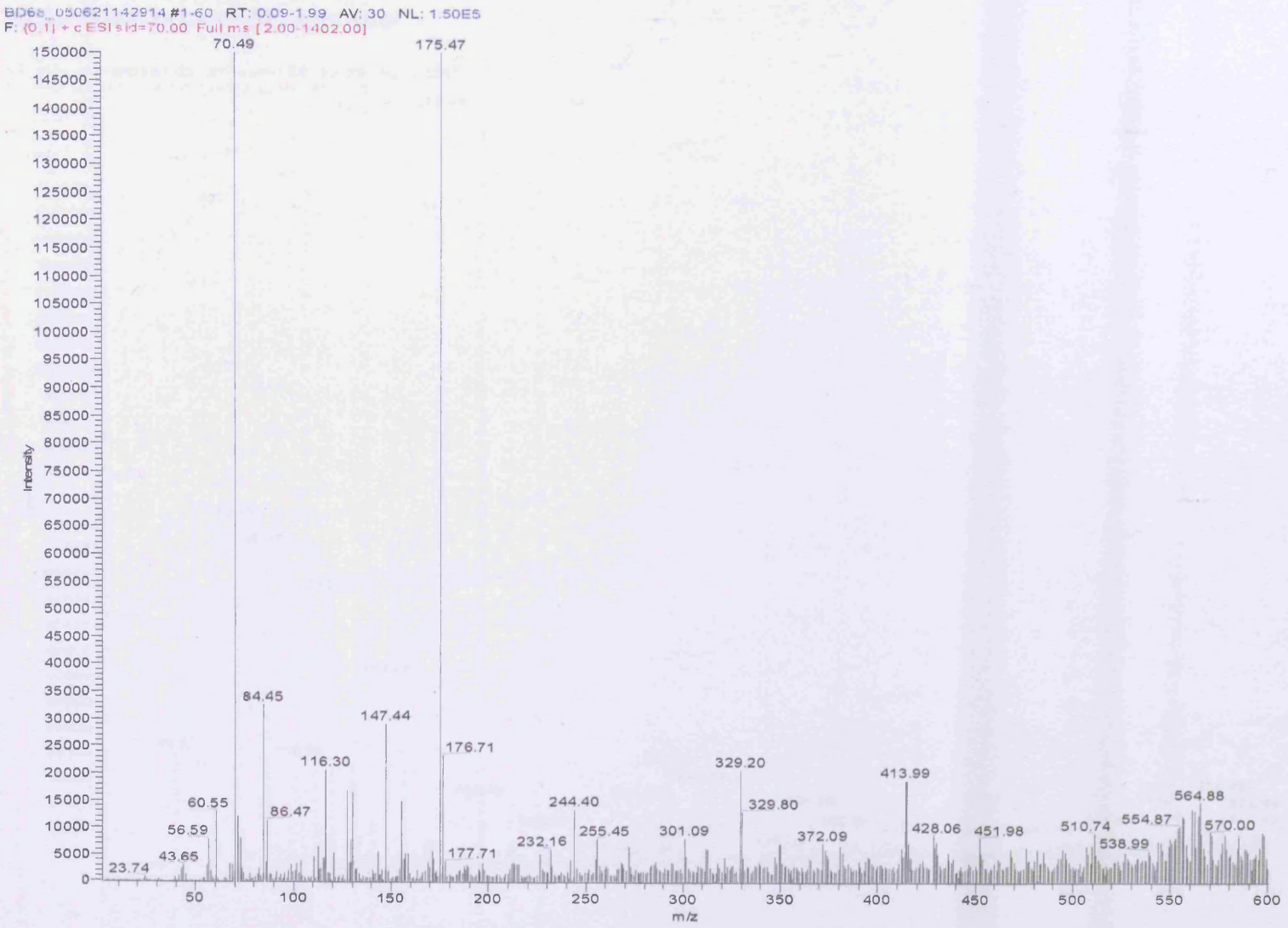
3.2.2.3. Gelatin peptone G3 spectrum



3.2.2.4. Gelatin peptone G4 spectrum

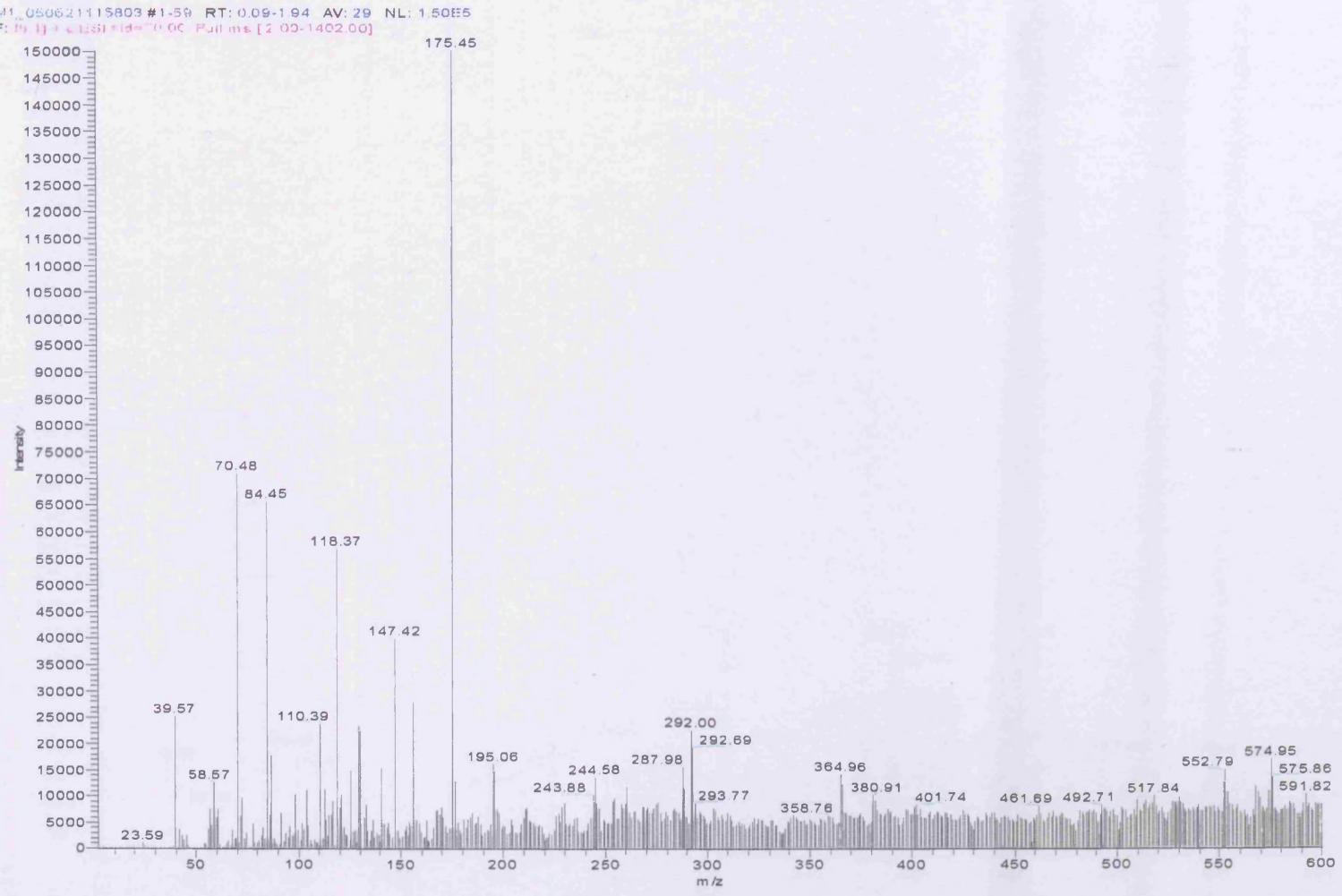


3.2.2.5. Gelatin peptone G5 spectrum

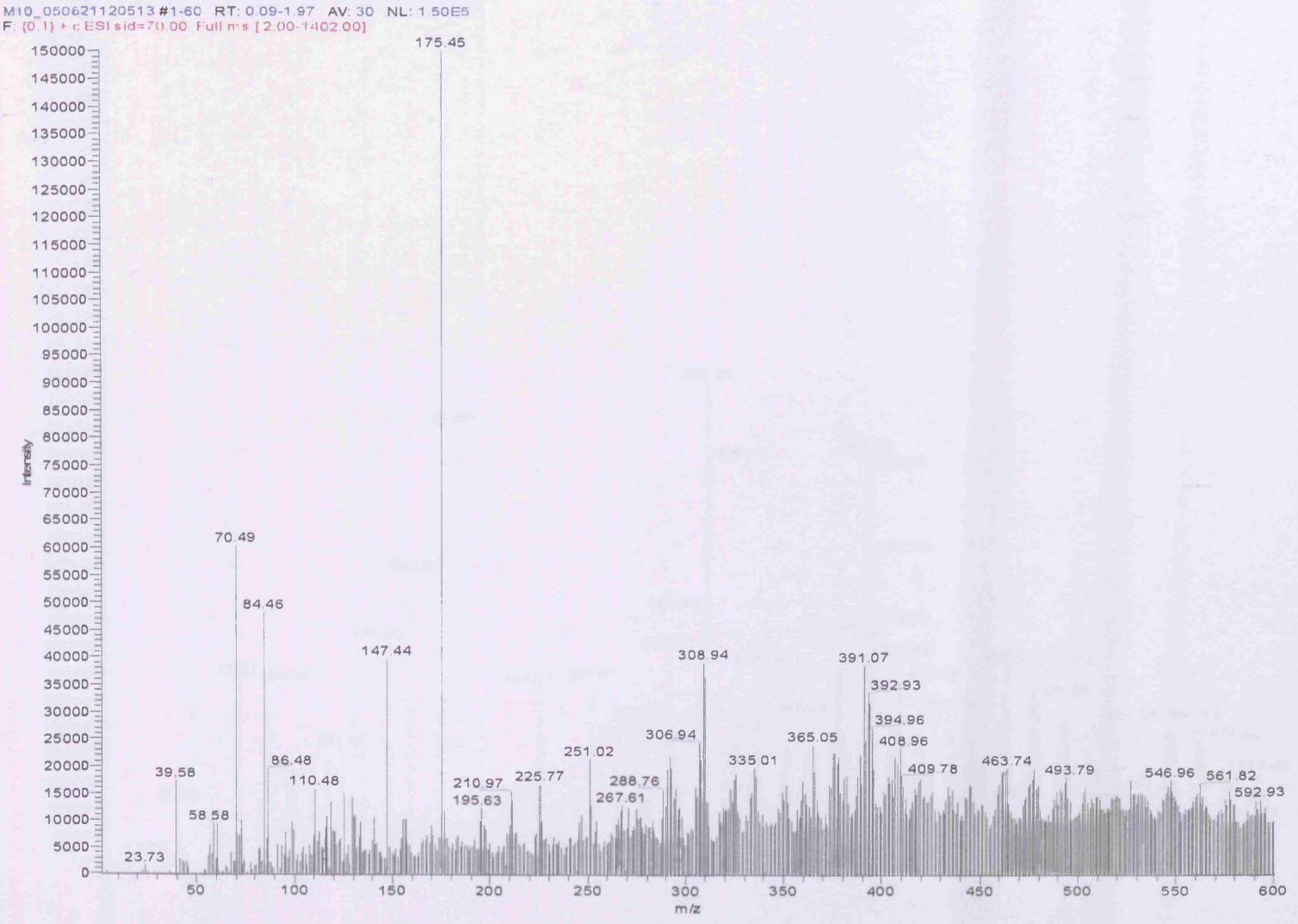


3.2.3. Meat peptone spectra

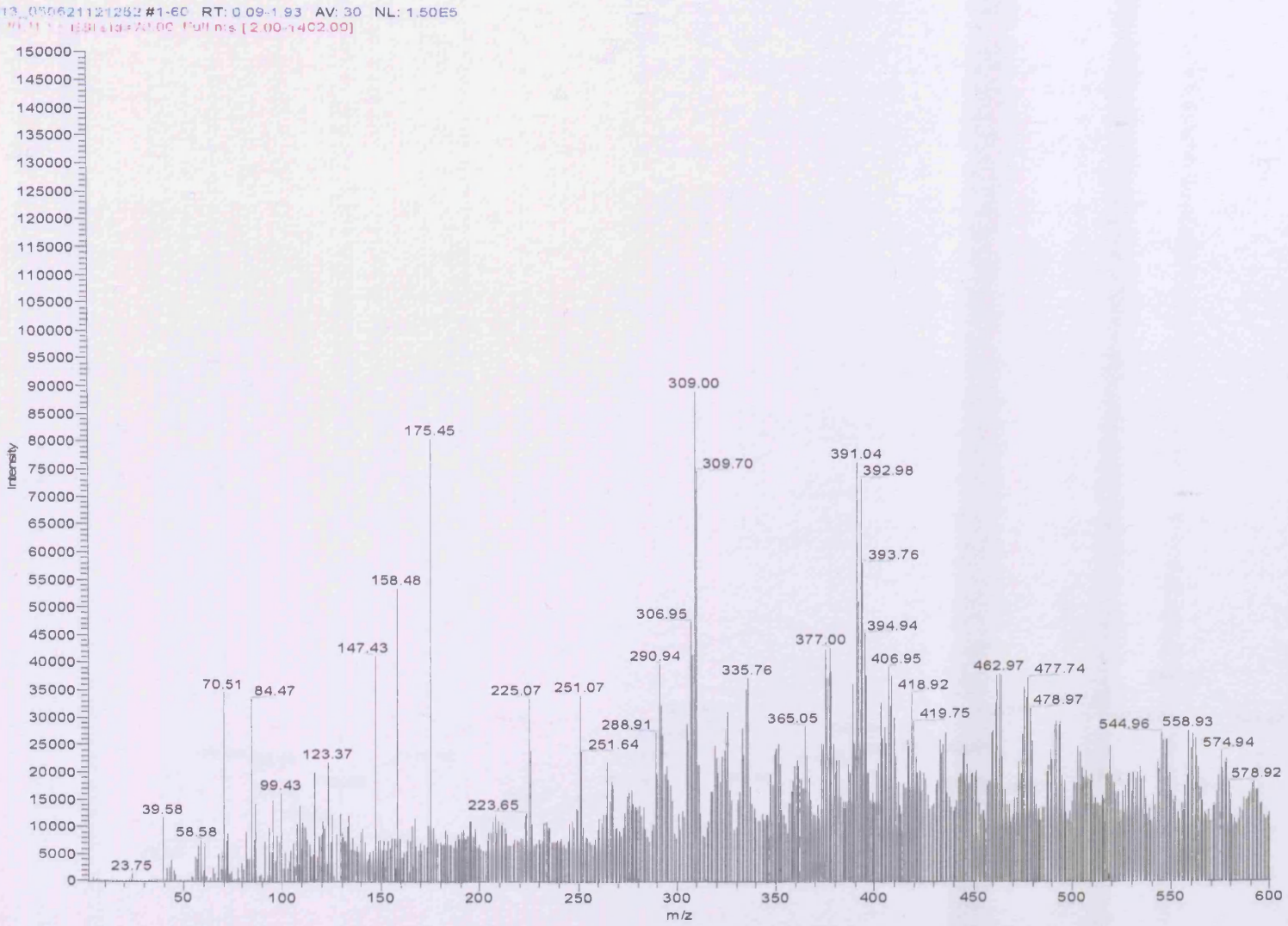
3.2.3.1. Meat peptone M1 spectrum



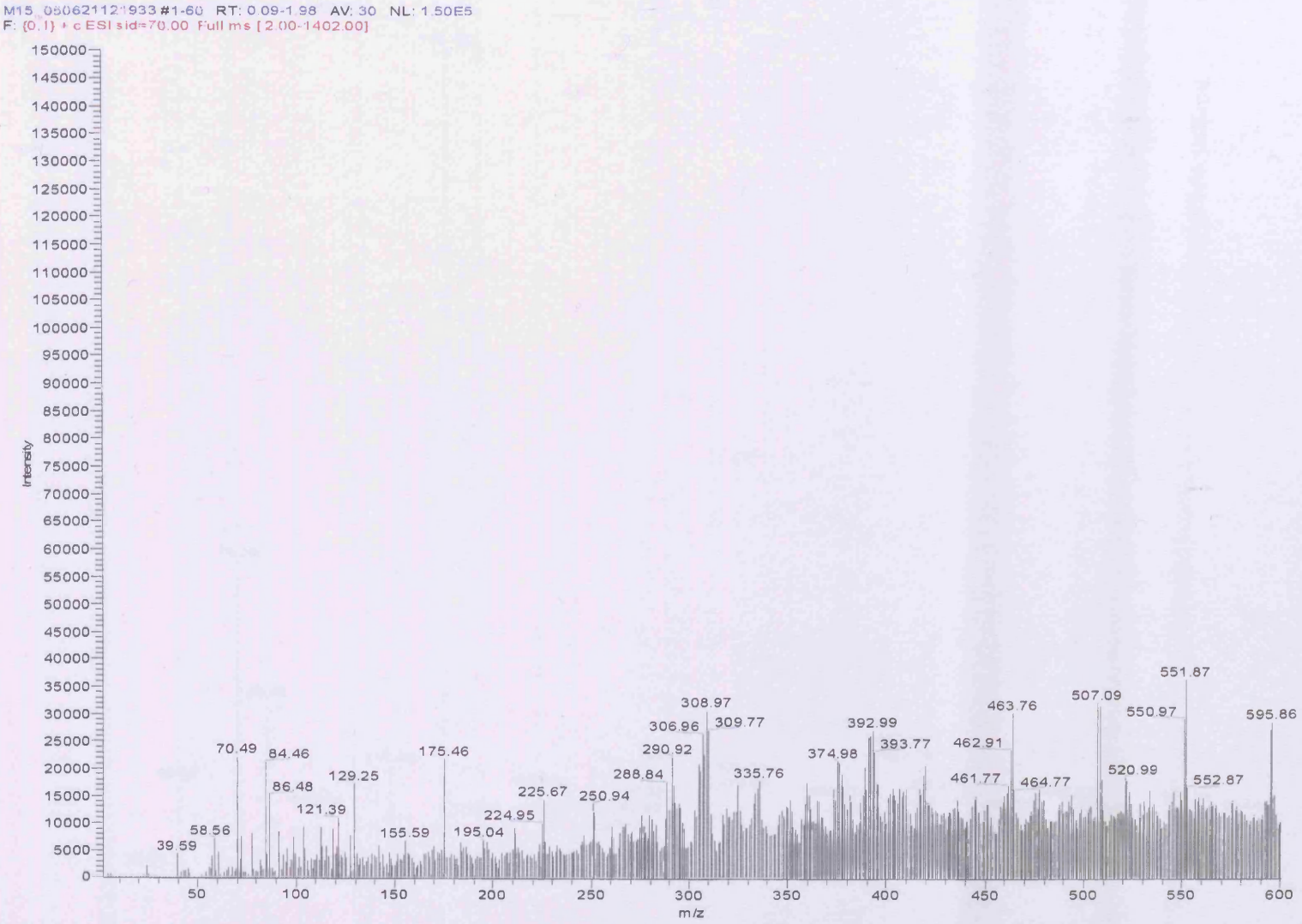
3.2.3.2. Meat peptone M2 spectrum



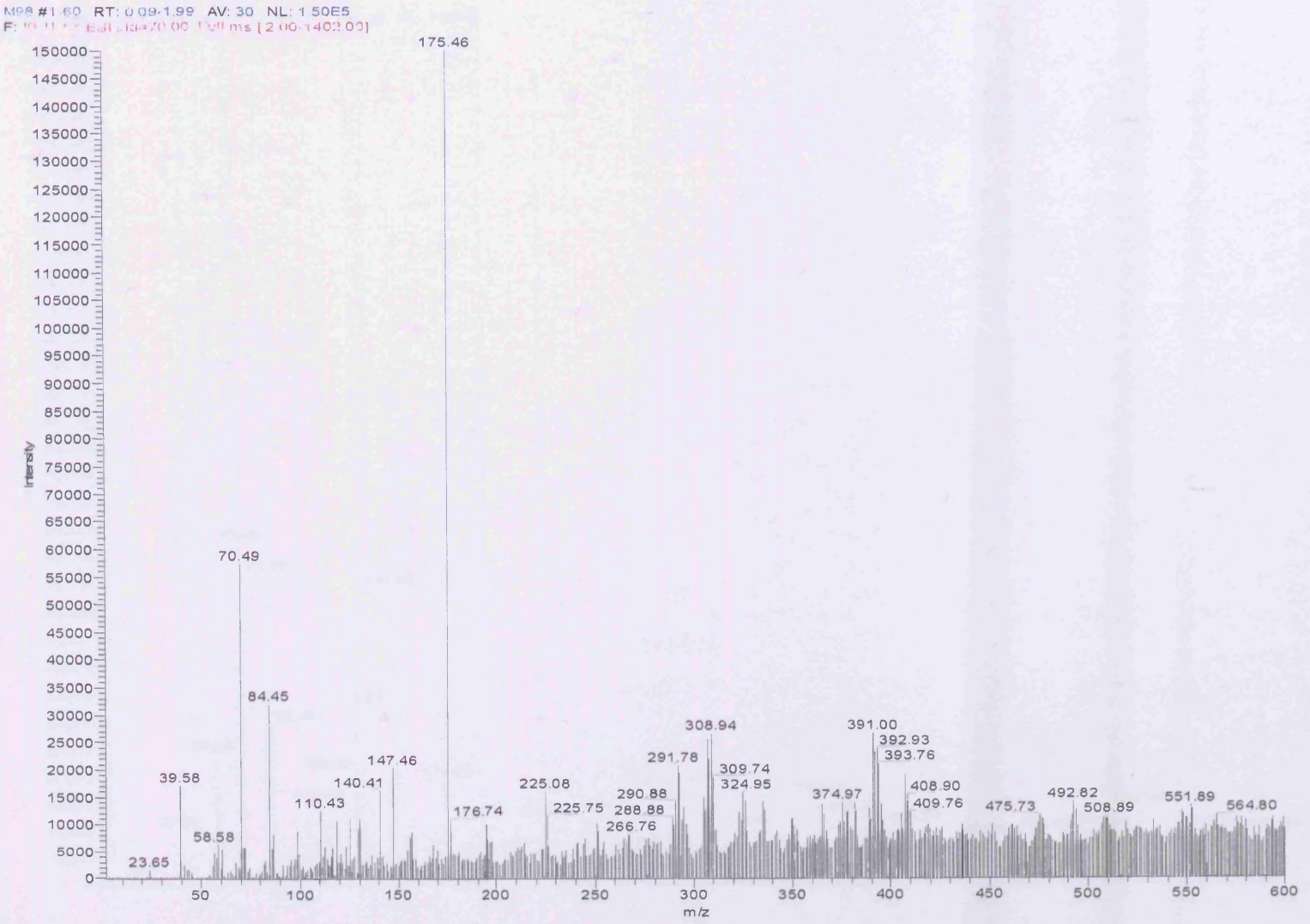
3.2.3.3. Meat peptone M3 spectrum



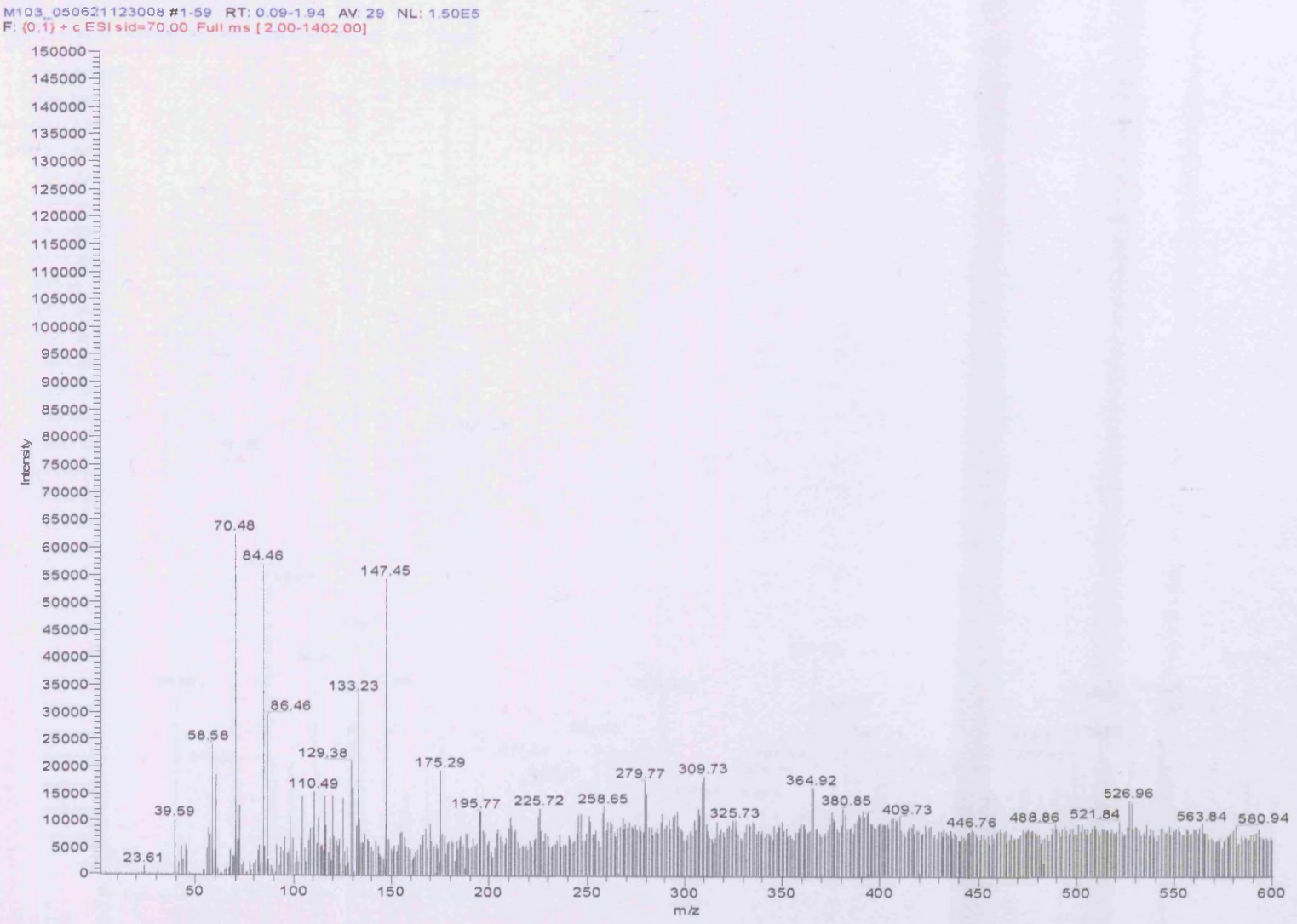
3.2.3.4. Meat peptone M4 spectrum



3.2.3.5. Meat peptone M5 spectrum

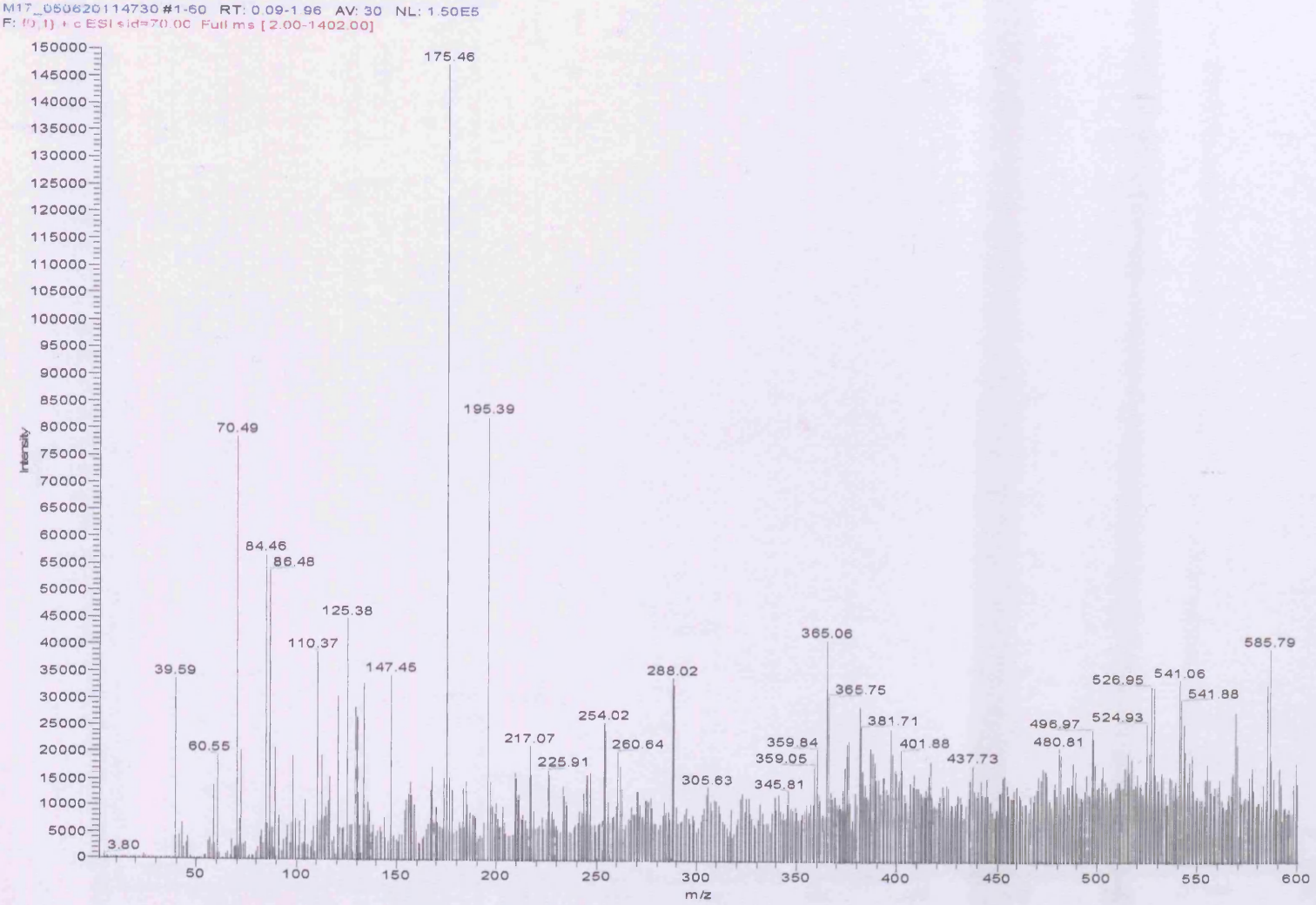


3.2.3.6. Meat peptone M6 spectrum

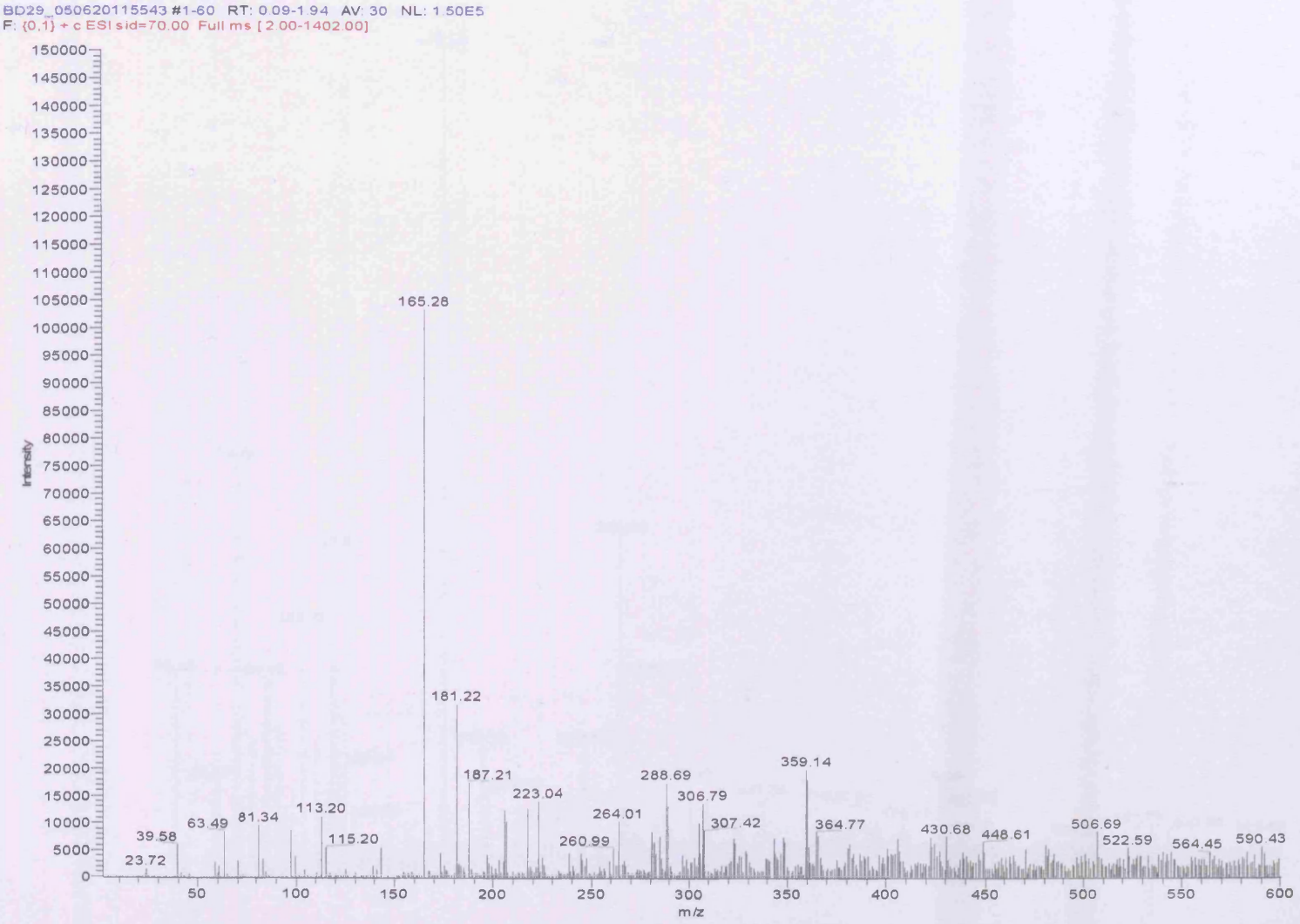


3.2.4. Soy peptone spectra

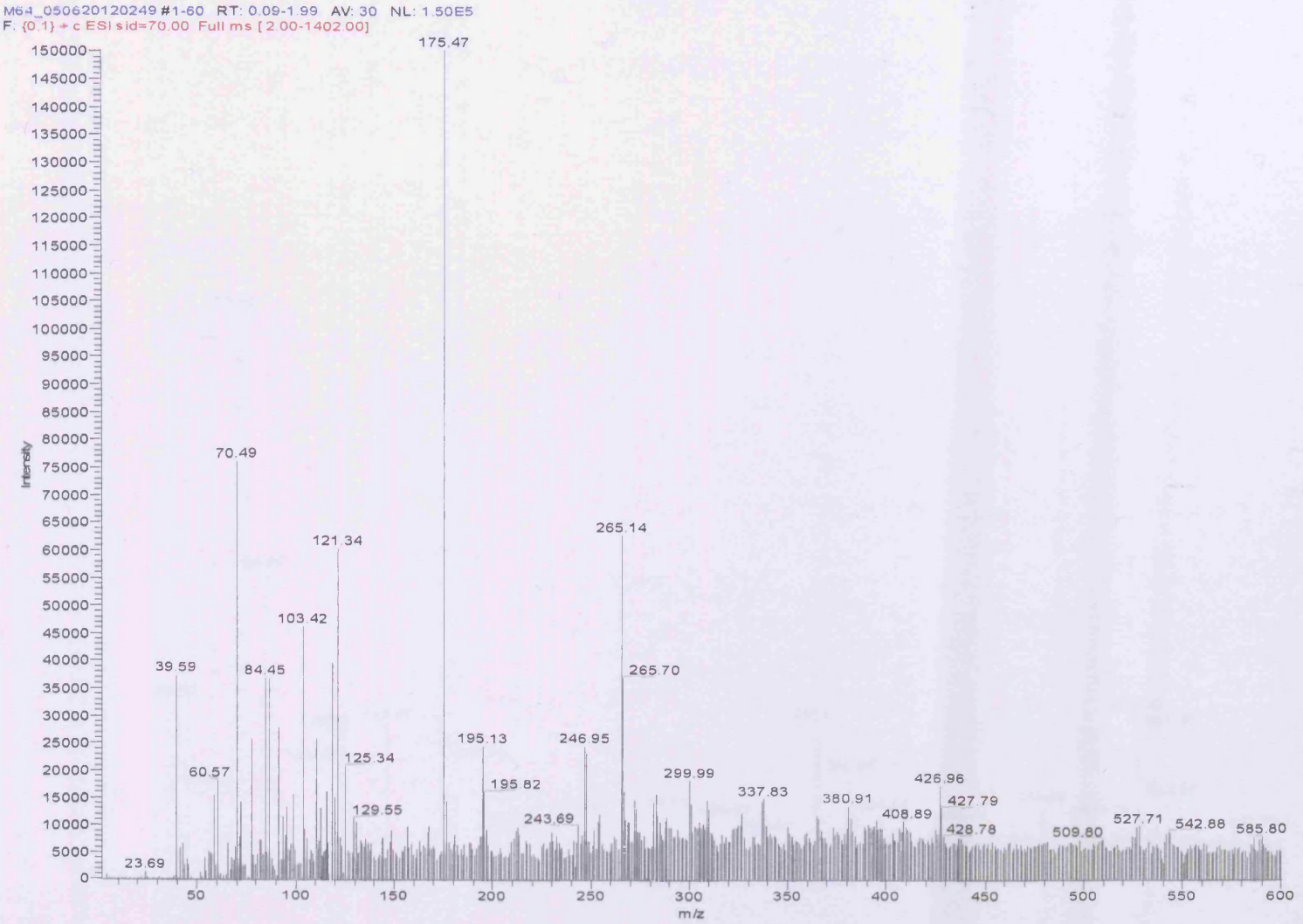
3.2.4.1. Soy peptone S1 spectrum



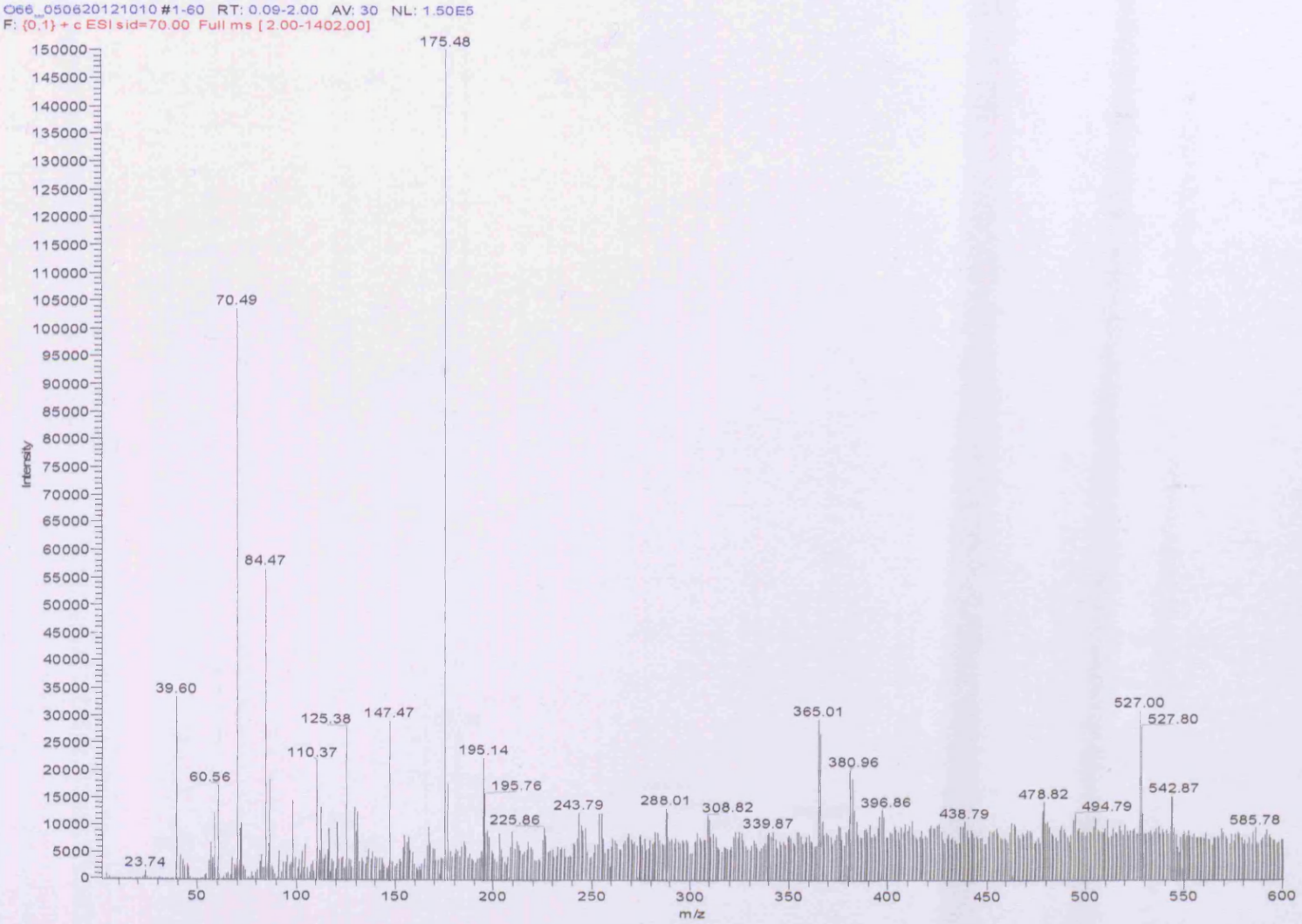
3.2.4.2. Soy peptone S2 spectrum



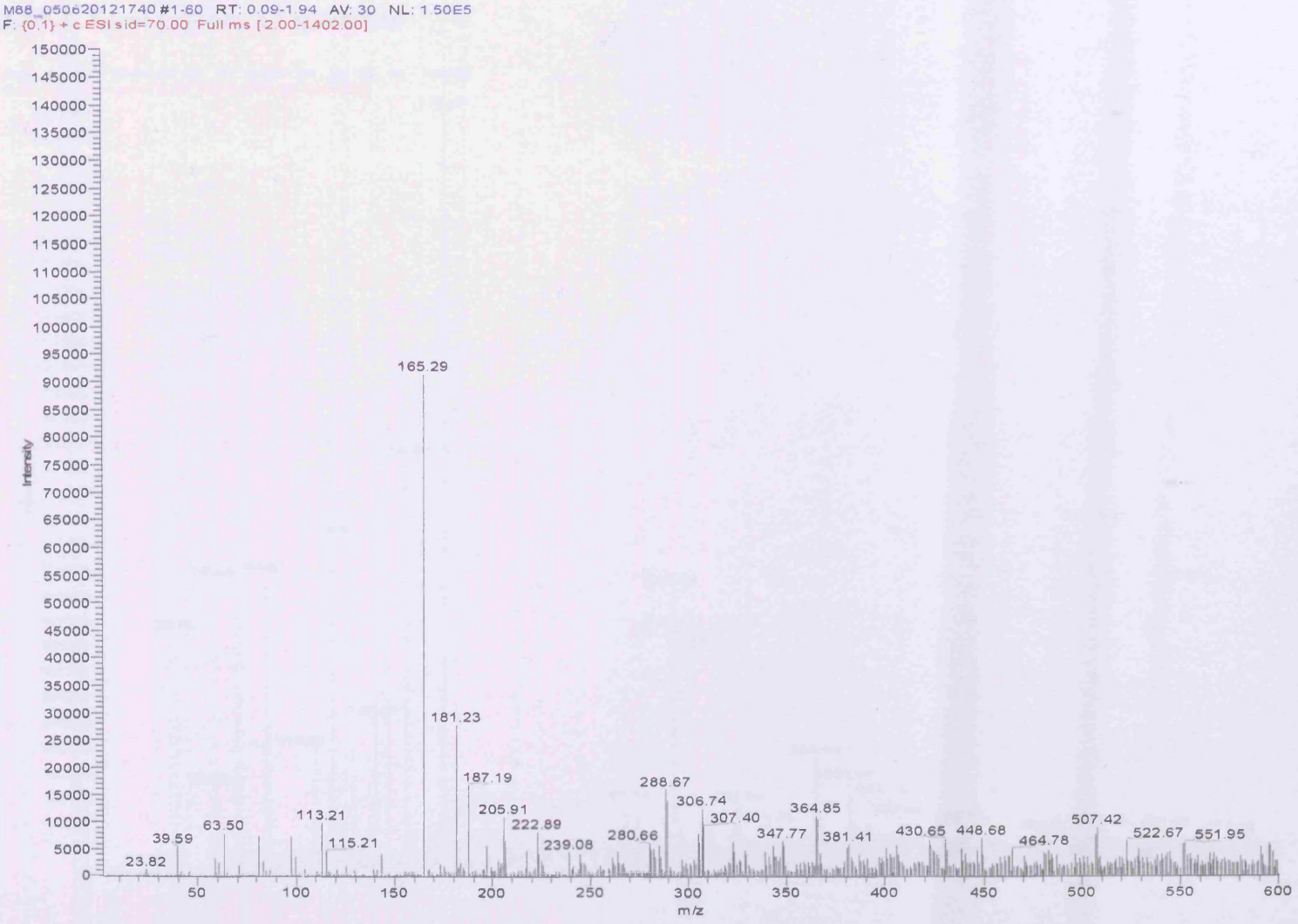
3.2.4.3. Soy peptone S3 spectrum



3.2.4.4. Soy peptone S4 spectrum

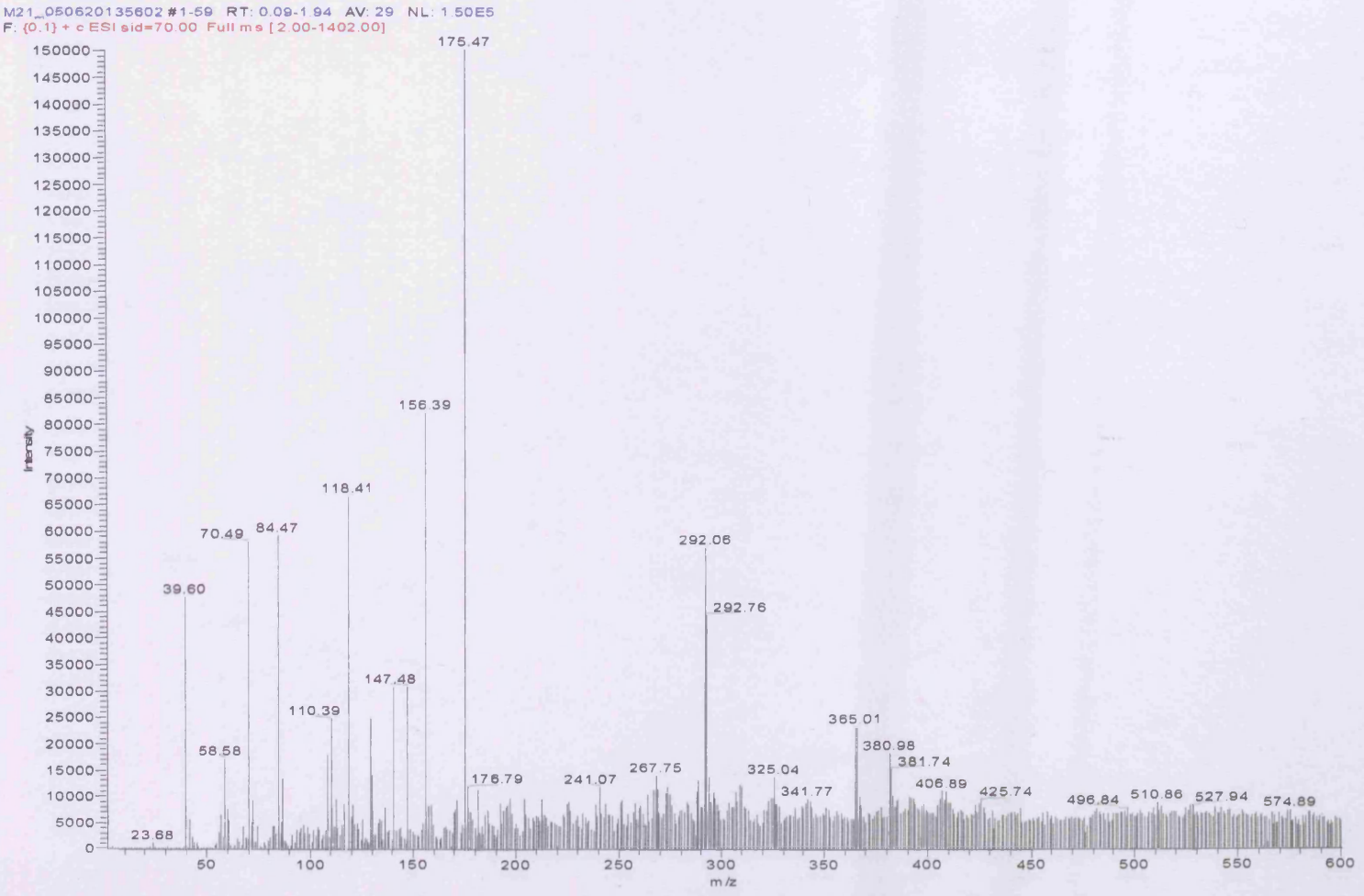


3.2.4.5. Soy peptone S5 spectrum

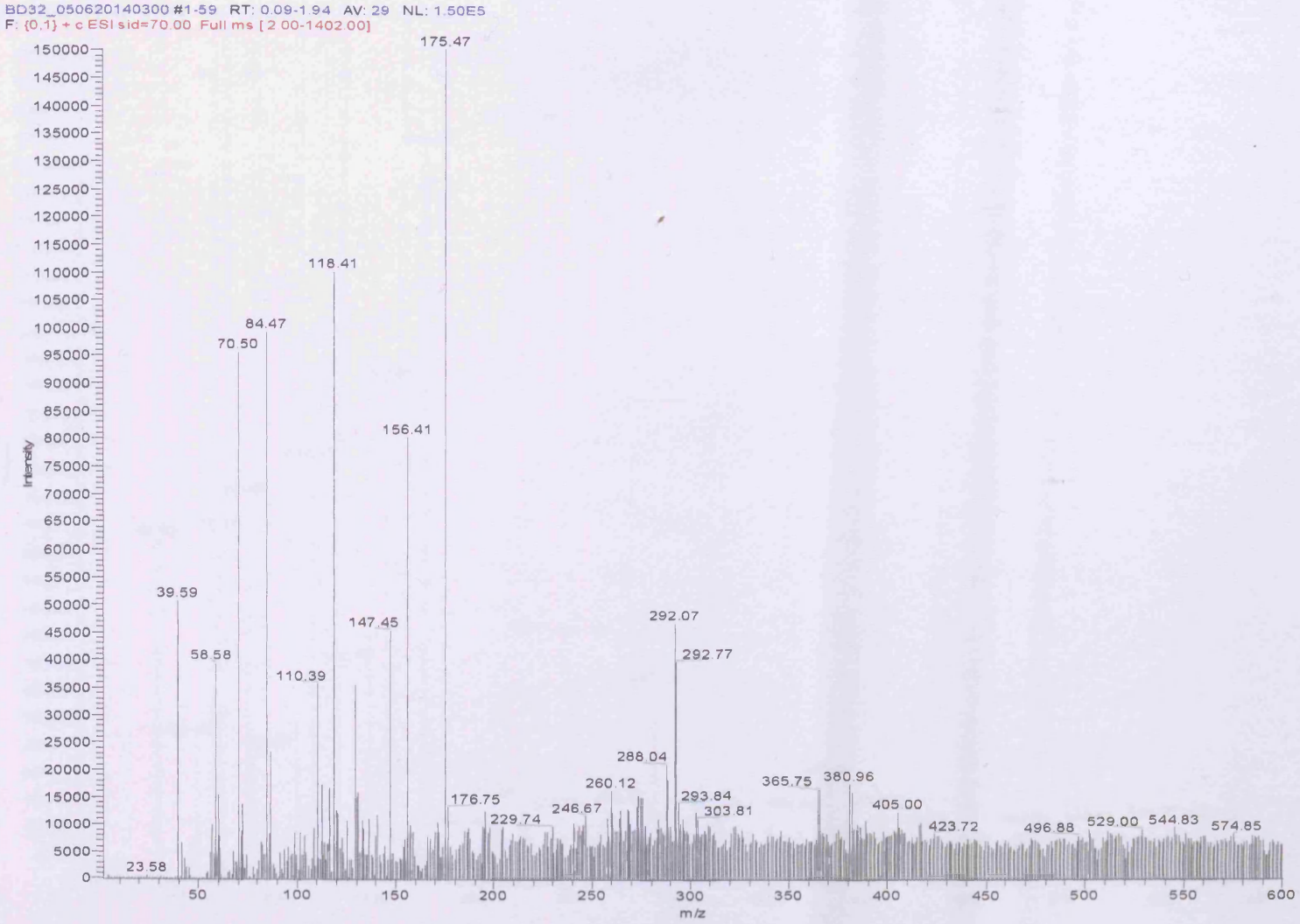


3.2.5. Yeast peptone spectra

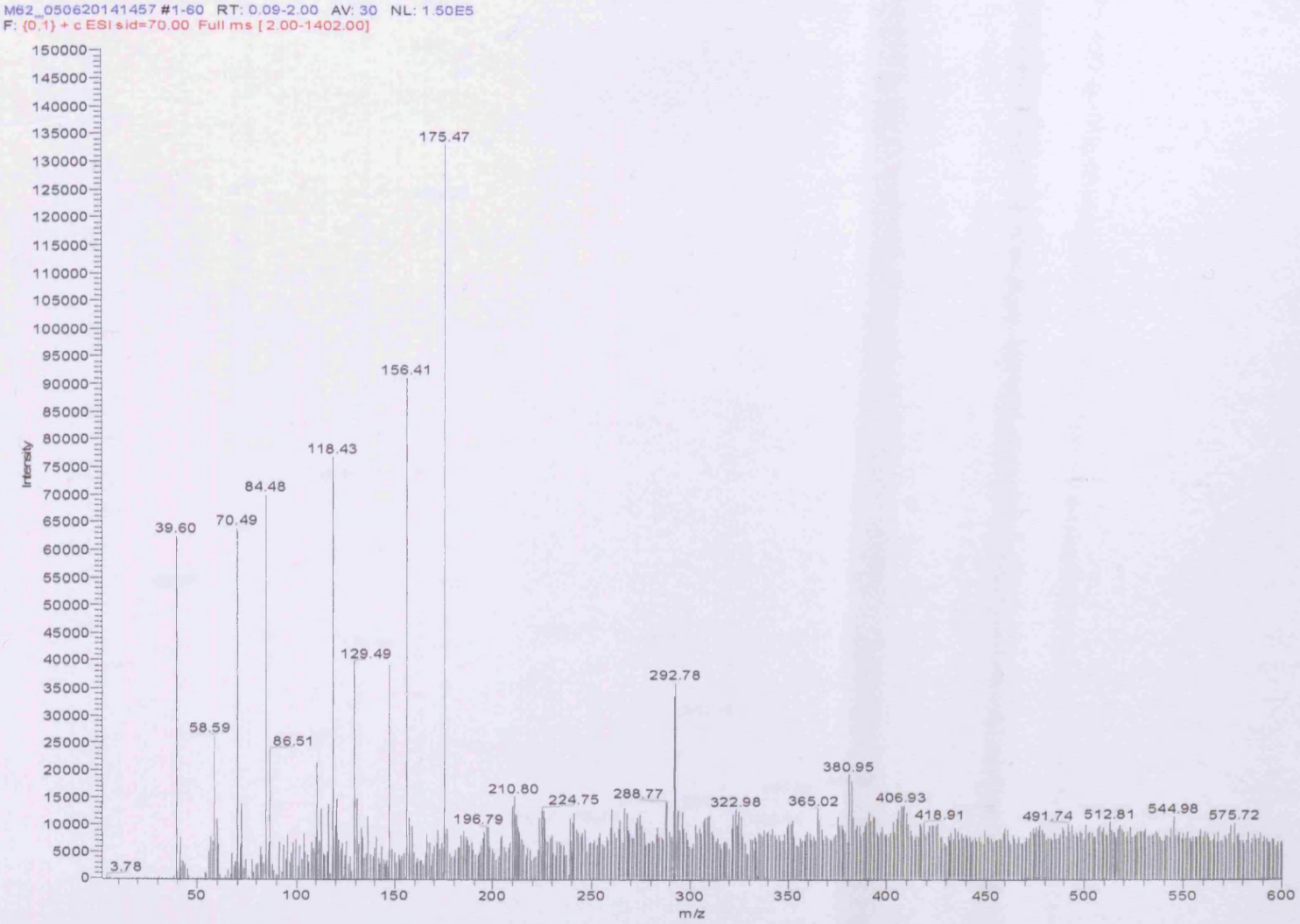
3.2.5.1. Yeast peptone Y1 spectrum



3.2.5.2. Yeast peptone Y2 spectrum



3.2.5.3. Yeast peptone Y3 spectrum



3.2.5.4. Yeast peptone Y4 spectrum