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An Investigation into the Fate of Benzylpenicillin and its Degradation Compounds in Milk Following Intramammary Treatments for Bovine Mastitis

David Roger Hulse

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PHD

An Investigation into the Fate of Benzylpenicillin and its Degradation Compounds in Milk Following Intramammary Treatments for Bovine Mastitis

Hulse, David Roger

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An Investigation into the Fate of Benzylpenicillin and its Degradation Compounds in Milk Following Intramammary Treatments for Bovine Mastitis

by

David Roger Hulse

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

Doctor of Philosophy

Department of Environmental Sciences

Faculty of Science

September 1996

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for Nicki

In the odyssey of this Ph.D. you have been my Penelope

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

Relevant scientific seminars and conferences were regularly attended at which work was presented. External institutions were regularly consulted and the following paper prepared for publication.

Publication:

The simultaneous determination of penicillin-G, penicillin-V, ampicillin, cloxacillin and novobiocin in bovine milk by micellar electrokinetic capillary chromatography. *Analytical Proceedings*, Subject to acceptance.

Conferences attended:

The British Mastitis Conference, 1994

The British Mastitis Conference, 1995

Signed.....

Date 06/06/97

Abstract

An Investigation into the Fate of Benzylpenicillin and its Degradation Compounds in Milk Following Intramammary Treatments for Bovine Mastitis

David Roger Hulse

The presence of antibiotics in bovine milk results mainly from the treatment of mastitis by intramammary infusion. The sale of this contaminated milk is illegal and the milk supply is routinely tested for adulteration by antibiotic residues. At present these tests are unspecific microbiological assays which detect only inhibitory substances and consequently the presence of any inactive degradation compounds is not tested for. Various chromatographic approaches exist in the literature which report the resolution of mixtures of antibiotic compounds from milk. However, the presence of antibiotic degradation residues in milk, which may have serious implications for the health of the consumer, has not been studied.

Benzylpenicillin is the antibiotic responsible for most failures of the regulatory tests and is present in the majority of common mastitis treatments. Although often administered as the procaine salt it degrades rapidly, especially in the presence of β-lactamase enzymes produced by resistant strains of bacteria. This thesis initially describes the development of a chromatographic system for the detection and separation of benzylpenicillin and its major degradation compounds in aqueous solution. HPLC and the relatively recently developed technique of Micellar Electrokinetic Capillary Chromatography (MECC) were both successfully employed to perform this function.

An important part of the research, however, was the development of an efficient milk extraction and sample clean up technique, complicated by the complexity of the milk matrix and also the instability of benzylpenicillin. HPLC was found to be unsuitable for the resolution of the degradation compounds from milk because of background interference despite extensive sample clean up by methods reported in the literature and developed during the course of this work. MECC on the other hand provided the necessary resolution for the detection of these compounds without the need for extensive sample manipulation. The final novel procedure was a combination of acetonitrile extraction coupled with solid phase clean up which took into consideration the presence of proteins, carbohydrates and lipids; the major constituents of the milk matrix.

The preparation and identification of the major aqueous degradation compounds of benzylpenicillin was achieved and confirmed by NMR and FTIR studies. The action of β-lactmases (penicillinase) on penicillin to produce benzylpenicilloic acid and its subsequent epimerisation from the 5R,6R diastereoisomer to the 5S,6R epimer is also reported.

Once the optimum milk extraction and purification methods had been found, they were applied to milk collected from cows treated for mastitis with two different infusions containing benzylpenicillin. From these *in-vivo* samples it was apparent that only one penicillin degradation compound, 5R,6R benzylpenicilloic acid, was present in milk following intramammary treatment. When the benzylpenicillin was administered as the procaine salt the benzylpenicilloic acid was detectable in the milk during the entire 72 hour withdrawal period.

Contents

Chapter 3 Capillary Electrophoresis. Methods and Results.

List of Tables

Chapter 1 Introduction.

Chapter 2 High Performance Liquid Chromatography. Methods and Results.

Chapter 3 Capillary Electrophoresis. Methods and Results.

Chapter 4 Preparation and Identification of Benzylpenicillin Degradation Compounds.

Chapter 5 *In-vivo* Milk Studies.

List of Figures

Chapter 1 Introduction.

Chapter 2 High Performance Liquid Chromatography. Methods and Results.

Chapter 3 Capillary Electrophoresis. Methods and Results.

Chapter 4 Preparation and Identification of Benzylpenicillin Degradation compounds.

Chapter 5 *In-vivo* Milk Studies.

List of Abbreviations

a.u.f.s.	Absorbance Units Full Scale
BCP	Bromocresol Purple Indicator
CE	Capillary Electrophoresis
^{13}C	Carbon 13 NMR
^{13}C-Dept.	Carbon 13 dept. NMR
CMC	Critical Micelle Concentration
CMT	California Mastitis Test
CZE	Capillary Zone Electrophoresis
DCM	Dichloromethane
DMSO	Dimethyl Sulphoxide
EOF	Electroosmotic Flow
FTIR	Fourier Transform Infra-Red
GC	Gas Chromatography
^{1}H	Proton NMR
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
id.	Internal Diameter
IDF	International Dairy Federation
IPA	Isopropyl Alcohol
IR	Infra-Red
i.u.	International Units
KBr	Potassium Bromide
LOD	Limit of Detection
M	Molarity
MECC	Micellar Electrokinetic Capillary Electrophoresis
mg	Milligrams
mgl^{-1}	Milligrams per litre
min	Minutes
ml	Millilitres
mm	Millimetre
MMB	Milk Marketing Board
MS	Mass Spectrometry
nm	Nanometre
PCG	Benzylpenicillin
ppm	Parts per million
RP	Reverse Phase
rpm*	Revolutions per minute (radius of the centrifuge head was 20cm for all experiments described herein)
SDS	Sodium Dodecyl Sulphate
SCC	Somatic Cell Count
TBA-HSO_4	Tetrabutylammonium Hydrogen Sulphate
TBC	Total Baterial Count
TLC	Thin Layer Chromatography
UK	United Kingdom
UV	Ultra Violet
VIS	Visible
WHO	World Health Organisation
δ	Chemical Shift

$[\alpha]$	Specific Rotation
µg	Micrograms
µl	Microlitres
t_r	Retention Time
t_m	Migration Time

Chapter 1

Introduction

1.1 Mastitis. A multifactorial infectious disease.

Mastitis is defined as an inflammatory response of the mammary gland (Klastrup, 1985).

The vast majority of bovine mastitis cases are of an infectious nature caused by the invasion of the udder by pathogenic micro-organisms. Non-infectious mastitis, which includes traumatic and toxic causes, produces an indistinguishable inflammatory reaction, altering the balance between the natural defence mechanisms of the teat and the numbers and pathogenicity of the micro-organisms (IDF, 1987[b]).

The development of mastitis depends extensively on its severity and duration (e.g. acute or chronic). Pathogens enter via the teat duct and multiply. The by-products of bacterial growth and metabolism irritate the tissues and cause an inflammatory response (Schalm *et al.*, 1971). These changes and the increased permeability of the inflamed tissue result in a change of composition of the milk. Concentrations of sodium, potassium, chloride, proteins, enzymes, somatic cells and other products of the secretary process all change. With increasing severity of the disease the composition of milk becomes similar to blood serum. In subclinical mastitis, changes can only be detected by laboratory tests, whereas in clinical mastitis they can be detected by visual examination of the milk and/or the udder. Many environmental factors affect the infection rate and severity of existing mastitis cases. For example, milking machines may introduce bacteria to the teat orifice or alter the teat canal resistance to their invasion (IDF, 1987[c]).

Any mastitis classification necessitates information on the cause, a measure of the spread and severity of the disease. Any such characterisation can only be arbitrary as there is no exact borderline between a healthy gland and the various pathological states. Nevertheless the following classification is generally accepted:-

Infectious clinical mastitis

Pathogens are detected in aseptically taken milk samples and macroscopic inflammatory changes are found in the secretion and/or udder tissue.

Infectious subclinical mastitis

Pathogens are detected in aseptically taken milk with inflammatory changes detected only by laboratory methods. There are no visible changes in the milk composition or udder tissue and consequently the infection may remain undetected for long periods (Craven *et al.*, 1984).

Non-specific clinical mastitis

Pathogenic micro-organisms cannot be detected but there are macroscopic inflammatory changes in milk and udder epithelial tissue.

Non-specific subclinical mastitis

Pathogens are not present and inflammation in the milk and udder tissue can only be detected by laboratory tests.

Healthy Udder.

Without evidence of any of the above. In the uninfected gland, teat canal colonisation is characterised by pathogens multiplying in the teat duct or at the teat orifice, but not the udder sinuses or epithelium which shows no signs of inflammation (IDF, 1987[a])

The on-set of mastitis infection will always be subclinical but often develops into a clinical form with the time of transformation varying from hours to months depending upon the cause of the disease and other conditions (Hillerton *et al.*, 1995). Research has shown that a large percentage of subclinical quarters (40-50%) never develop clinical mastitis in the course of a lactation.

1.2 Milk quality. Somatic cell and Total bacteria counts.

As mentioned previously, mastitis causes an increase in the permeability of the blood/milk barrier and consequently the composition of milk becomes increasingly similar to that of blood. Inflammation is characterised by the accumulation of neutrophils and leukocytes of blood origin in the area of injury. Epithelial cells of the mammary gland are subjected to continuous stress undergoing constant replacement and are therefore a natural component of milk. The number of epithelial cells, however, increases dramatically with the on-set of mastitis. The term 'somatic cell' refers mainly to white blood cells produced by the immune system in response to pathogenic

micro-organisms (Baines, 1995). The number of somatic cells per ml of milk is considered to be an adequate indicator of milk quality and as a reliable indicator of mastitis infection (Booth, 1994). Somatic cell levels are generally measured using a Coulter Counter which utilises particle size. The total bacteria count (TBC) per ml of milk is also used in the assessment of milk hygiene and quality. Bacteria are naturally present in a healthy mammary gland but mastitis causes elevated counts (Baker, 1994).

The 1992 privatisation of the Milk Marketing Board forced farmers to negotiate their own contracts with the milk suppliers (Nestlé, Unigate and Milk Marque), each having a slightly different payment scheme based on total bacterial counts (TBC) and somatic cell counts (SCC) for different milk qualities (Pearce, 1995). From January 1998 all milk intended for human consumption will be required to have a cell count below 400,000 cells/ml (Baines, 1994). At present only 74% of the UKs national dairy herd meets this requirement (Williams, 1994).

The economic penalties for poor quality milk are severe and make detection of individual mastitis cases extremely important. Potentially huge losses for the milk producer have led to increasing amounts of research into the detection of both clinical and sub-clinical mastitis. The California Mastitis Test (CMT) is the most common mastitis detection method, using a surfactant added to the milk which, if the cell count is elevated, coagulates (Fishwick, 1995[a]).

1.3 Mastitis control programs.

The national dairy herd contains approximately 2.5 million milking cows and in 1994 the losses to the dairy industry due to mastitis were estimated at £90 million (Berry, 1994) . Such estimates take into account reduction in milk yield, treatment costs and the milk discarded following antibiotic treatment (this will be discussed in more detail in section 1.5). Such financial incentives and the threat to human health infected milk poses has led to mastitis control programs of which dry cow therapy and teat dipping are an integral part. These control measures are ineffective however without the commitment of the herdsman (Barefoot, 1994). The economics of dairy farming have also been affected recently by the BSE crisis.

Mastitis vaccines are a recent development available for use in some countries. Being specific to particular types of infectious mastitis they remain additional to existing control practices (Hillerton, 1994). Vaccination with live *Str. uberis* has been shown to reduce the incidence of clinical mastitis and improve the SCC and TBC (Finch and Leigh, 1995).

1.3.1 Teat dipping.

The teat canal remains open for up to 2 hours after milking allowing the invasion of micro-organisms and increasing the risk of mammary infection (Genus, 1995). Many measures in dairy management can be implemented to reduce this exposure including clean housing, isolation of infected cattle, antibiotic therapy, efficient cleaning of milking machines and application of antiseptic solutions to the teat before and after milking (fig. 1.1). Treatment of the udder after milking with antiseptic dips such as Iodophors, Chlorhexidine or sodium hypochlorite is a widely accepted practise in the UK dairy industry (Dodd, 1985), dramatically reducing the occurrence of teat lesions and consequently the number of clinical mastitis cases (Shearn and Morgan, 1994).

Fig. 1.1 Diagram to show the administration of teat dip following milking (Genus, 1995).

Teat spraying is also widely used by farmers but is not regarded as being as effective as dipping, mainly because dipping is less sensitive to the technique employed by the herdsman.

1.3.2 Dry cow therapy.

Mastitis control measures cannot be limited to the lactation period alone. Many cows enter the dry period with an established sub-clinical infection. They are consequently more susceptible to clinical infections which are most common within ten days of drying off and during the ten days before calving. Intramammary infusion of slow release bactericides is usually administered to achieve persistent antimicrobial activity during the dry period (Sol *et al.*, 1994). Dry cow therapy has the advantages that persistent infections are treated annually with the antibiotics in higher concentrations and having a longer residence time in the udder. Consequently dry cow therapy is twice as effective as treatment in the lactation period (Genus, 1995). Contamination of the milk with antibiotics is not a problem providing withdrawal periods are strictly observed.

A major concern over the popular use of dry cow therapy is the increased likelihood of antibiotic resistance which has apparent significance to human health. It may also may be related to the higher incidence of gram-negative infections (Oakley, 1984).

1.4 Mastitis pathogens.

Mastitis micro-organisms represent a broad variety of different species, a summary of which is given in Table 1a. The major pathogens associated with the on-set of mastitis are shown in Table 1b. with their potential risk to human health.

There is little doubt that *Staph. aureus*, of which there are more than 80 strains, is by far the most important species when considering bovine mastitis (Heeschen *et al.*, 1985). It is the cause of contagious mastitis which is mainly sub-clinical and although inactivated by pasteurisation many strains can cause infections and food poisoning in man.

Table 1a. Mastitis pathogens (from Heeschen *et al.*, 1985).

Bacteria:	*Bacillus cereus*		*Serratia marcescens*
	Clostridium perfringens		*Staphylococcus aureus*
	Corynebacterium bovis		*Staphylococcus epidermidis*
	Corynebacterium pyogenes		*Streptococcus agalactiae*
	Corynebacterium ulcerans		*Streptococcus dysgalactiae*
	Enterobacteriaceae		*Streptococcus uberis*
	Listeria		*Yersinia pseudotuberculosis*
	Micrococcus spp.	Fungi:	*Trichosporon*
	Mycoplasma spp.	Yeasts	*Candidia spp.*
	Nocardia spp.		*Cryptococcus neoformans*
	Pasteurella multocida		*Saccharomyces spp.*
	Salmonella		*Torulopsis spp.*

Table 1b. The most important mastitis causing micro-organisms and their potential hazard to human health (from Heeschen *et al.*, 1985).

Species	Incidence in mastitis	Risk to human health
Staph. aureus	+ + +	+ + +
Str. agalactiae	+ + +	+ + +
Str. uberis	+ +	-
Str. dysgalactiae	+ +	-
E. coli	+	?

Staph. aureus are facultatively anaerobic gram-positive cocci. L forms are induced by penicillin where as the G variants are selected when growth of normal forms is inhibited by antimicrobials such as antibiotics to which they are resistant. Failure of penicillin against G variants may be in part due to the production of penicillinase (Owens and Nickerson, 1989). However, once established in the udder *Staph. aureus* is very difficult to eliminate (Platt and Candish, 1994) causing extensive tissue invasion which may lead to abscess formation. A major reason for the failure of penicillin appears to be that the organisms inhabit deep pockets protected by a tissue barrier. The organism produces toxic products such as coagulase which enhances its disease producing properties.

Prior to 1945 *Str. agalactiae* was believed responsible for approximately 70% of chronic mastitis cases (Ayres *et al.*, 1980). Being quite sensitive to antibiotics, especially penicillin, its' importance as a cause of mastitis has reduced. With the advent of penicillin, *Staph. aureus* has replaced *Str. agalactiae* as the organism most commonly isolated from mastitis infections, although the latter has become significant again since 1992 for reasons not altogether clear (Fishwick, 1995[b]). Other Streptococcal mastitis causing pathogens include *Str. uberis* responsible for 30-40% of world-wide clinical cases (Field and Leigh, 1994) and *Str. dysgalactiae*. They are of environmental origin being opportunistic, invading mammary quarters when favourable conditions arise.

The coliforms, gram negative enterobacteria of which *E. Coli* is the most commonly isolated, are currently some of the most important causes of clinical mastitis (Green, 1995). *E. Coli* are naturally present in the intestinal environment and consequently faeces are an important source of coliforms but they can be isolated from soil, vegetation and water (Lucas and Hewett, 1994).

1.5 Antibiotics used in Mastitis Treatments.

Antibiotics have played an integral role in veterinary medicine since the discovery of penicillin and have been involved in far reaching success in the treatment of bovine infections (IDF, 1979). In recent years, however, concern over development of resistance, the production of virulent pathogenic strains and the problems involving residues in foods have resulted in legislative measures (report by the joint committee on the use of antibiotics in animal husbandry and veterinary medicine, 1971). These measures, varying from country to country, attempt to restrict the use of certain antibiotics and to enforce milk withholding periods after application of these drugs. If an animal is treated with penicillin current UK regulations prohibit the marketing of milk from six consecutive milkings or for three days (72 hours) (Ayres *et al.*, 1980). Other antibiotics have different milk residence times and withdrawal periods can be for as many as eight consecutive milkings or four days (92 hours). The regulations were based on the considerations listed below.

(i) Residues in milk are illegal (Seymour and Jones, 1988).

(ii) Marketing of milk containing antibiotics indicates that the animal had a serious infection (Brady, 1988).

(iii) Allergic reaction by sensitized consumers. It is generally accepted that up to 10% of the population has some degree of penicillin allergy (Katz, 1982).

(iv) The inhibition of starter cultures in the production of dairy products such as cheese and yogurt (Hands, 1989).

(v) Development of resistant strains of bacteria as a result of over exposure to antibiotics (Crosse, 1984).

The economic pressures placed on todays farmers make it inevitable that antibiotically contaminated milk will occasionally find its way into the bulk tank intended for human consumption. Neither pasteurisation nor boiling removes all antibiotic contaminants (heating milk to 100°C eliminates only 50% of penicillin) and consequently the MMB, until its break up, routinely tested milk for the presence of antimicrobials (Vautier and Postigo, 1986).

Techniques for the analytical detection of antibiotic residues in bovine milk may be classified into two groups: (i) microbiological and immunological methods which are not specific and (ii) chromatographic methods which are compound specific.

1.5.1 Microbiological Assay.

Microbiological assay using *B. Stearothermophilus* are the most common when testing milk samples for the presence of antibiotics (Senyk *et al.*, 1990). The international dairy federation (IDF, 1987) disc assay is a standard reference method which detects 0.0025 to 0.008 i.u. of penicillin per ml of milk adequately meeting the World Health Organizations (WHO) recommended maximum concentration. Table 1c. lists the WHO's acceptable maximum concentration of some common antibiotics used in veterinary medicine.

Table 1c. Acceptable maximum antibiotic residue concentrations in milk as recommended by the World Health Organization (WHO).

Antibiotic	Concentration (ppm)
Ampicillin	0.01
Bacitracin	1.2
Cephalosporins	0.01
Chloramphenicol	0
Chlortetracycline	0.02
Dihydrostreptomycin	0.2
Erythromycin	0.04
Framycin	0.15
Nafcillin	0.02
Neomycin	0.15
Novobiocin	0.15
Nystatin	1.1
Oleandomycin	0.15
Oxytetracycline	0.1
Penicillins	0.006
Polymixin B	5.0
Streptomycin	0.2
Sulphonamides	0.1
Tetracycline	0.1
Tyrosine	0

In the disc assay procedure a disc of absorbent paper impregnated with a milk sample is positioned on an agar plate seeded with *B. Stearothermophilus var calidolactis*. Following incubation at 55°C for approximately 4 hours, a clear zone around the disc in the otherwise cloudy medium indicates the presence of an inhibitory substance.

The same bacterial strain is used for the on-farm Delvotest in which a nutrient tablet and 0.1ml of the test milk are added to the bacterial spores and Bromocresol purple indicator (BCP) (Oliver *et al.*, 1990). During incubation at 64°C for 2.5 hours acid is produced by the microbes changing the indicator from purple to yellow. This, however, is too long for most farmers who require a result within a milking time.

Most failures of microbiological tests are caused by the presence of β-lactam antibiotics (95-99%) (Hands, 1989). Therefore, the majority of tests are particularly sensitive to penicillin, the presence of which can be confirmed by the use of penicillinase.

1.5.2 Rapid assays.

New developments in response to the industry's demands for rapid antibiotic detection tests include the measurement of specific reactions at the cellular level. Such tests, however, tend to be β-lactam specific and relatively expensive (Hands, 1989).

Enzyme Inhibition.

The Penzyme assay uses the inactivation of the enzyme carboxypeptidase by β-lactam antibiotics as the detection tool. The test produces a pink-orange colour when clear of penicillin or a yellow colour if contaminated. This assay, which takes 20-30 minutes, was developed for both laboratory and on farm use (Grover *et al.*, 1987).

Radioactive Assay.

The so called Charm II™ test, introduced in 1978 by Penicillin Assay Inc., is a radioactive assay which can detect antibiotics in milk. The radiolabelled (^{14}C) antibiotic competes with the antibiotic in the sample for attachment to receptor sites (Bishop and White, 1984). The amount of labeled antibiotic bound to the cells is inversely proportional to the concentration of antibiotic in the test milk. Radioactivity is measured using a scintillation counter. Despite providing an accurate method that takes only 10-20 minutes, the equipment is expensive and rather complex.

Immunoassay.

Immunoassay techniques take 3-10 minutes and involve various methods using antibiotic-antigen reactions. One example uses latex beads coated with an antibiotic which competes for attachment to the antibody receptor site (Hands, 1989). Immunoassays can be applied to many milk products such as pasteurised milk, dried milk and cream and are suitable for a general broad spectrum assay.

1.5.3 Application of antibiotic mastitis treatments.

Antibiotics are used in response to primary and secondary diseases caused by micro-organisms. Depending upon the locality and severity of the disease different forms of application are used and a wide variety of veterinary pharmaceutical preparations are available (Jones and Seymour, 1988). Oral, intra-muscular, intravenous and intrauterine applications of antibiotics are used (McEwen *et al.*, 1991[a]) but intramammary administration of such drugs in mastitis therapy are by far the most common cause of milk contamination (McEwen *et al.*, 1991[b]).

Intramammary products fall into two categories; those that act against a narrow range of organisms and those having a broad spectrum of action. Clinical mastitis requires immediate treatment by the herdsman without prior knowledge of the pathogen involved. It is, therefore, necessary for a broad spectrum of antibiotics to be used. Intramammary administration of aqueous solutions, oily suspensions or ointment type preparations are applied directly through the teat duct (fig. 1.2). The concentration of antibiotics in milk following intramammary application and the duration of excretion are affected by:-

(i) Type of preparation and dosage.

(ii) Time interval between treatment and first milking.

(iii) Absorbency of the udder tissue.

(iv) Milk yield.

Intramammary preparations formulated with a hydrophobic base show essentially longer excretion times than those with an aqueous base. For example, following intramammary treatment up to 20% of penicillin may be excreted at the first milking. Thus a cow, suffering from mastitis, with a reduced yield of one litre of milk from the affected quarter and treated with 300,000 i.u. of penicillin could give milk containing 60 i.u. ml^{-1} (37.5mg l^{-1}) at the first secretion following treatment. Following intramammary infusion, passage of the antibiotics into the blood stream occurs and may result in contamination of untreated quarters. Common intramammary preparations include Tetradelta, Leo Yellow and Streptopen QR.

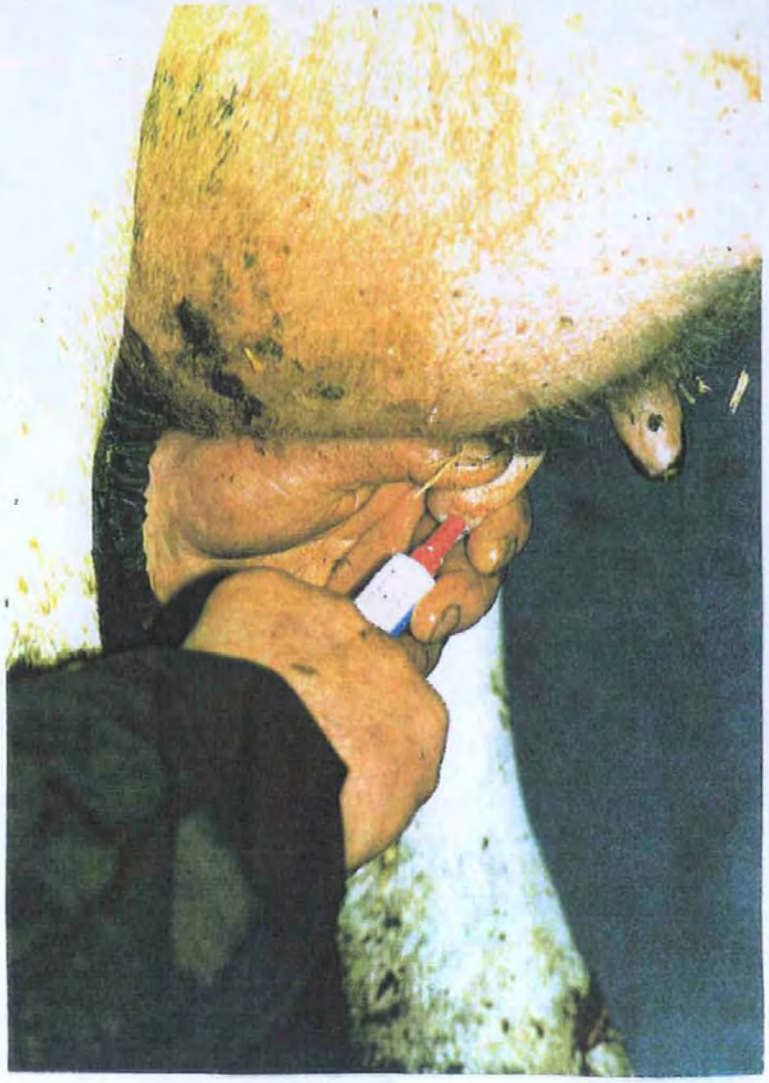


Fig. 1.2 Administration of an intramammary infusion of antibiotics.

Tetradelta.

Tetradelta is an off white oily suspension with each 10ml tube containing:-

Novobiocin (as sodium novobiocin)	100mg
Neomycin sulphate	150mg
Procaine penicillin-G	100, 000i.u. (100mg)
Dihydrostreptomycin sulphate	125mg
Prednisolone. (Anhydrous)	10mg

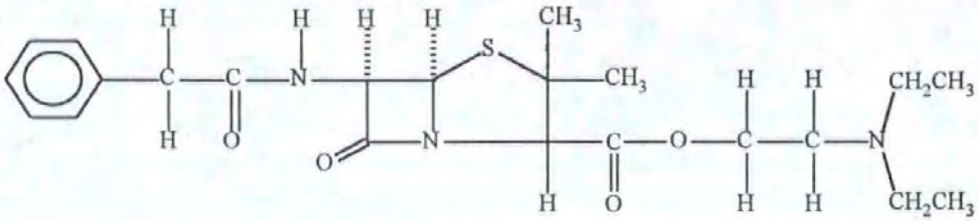
In severe cases, the treatment may be repeated at 24 or 48 hour intervals but milk should not be taken for 72 hours (i.e. the 6th milking) after the last treatment.

Leo Yellow

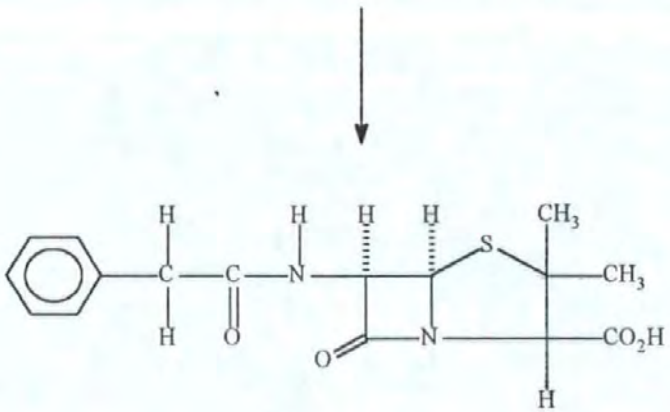
Leo Yellow, produced by Leo Laboratories, is a mixture of the following in an oily suspension.

Streptomycin sulphate	150mg
Neomycin sulphate	50mg
Diethylaminoethylester of Benzylpenicillin (BPC 1954)	150mg

The diethylaminoethylester of benzylpenicillin, when introduced to the mammary gland hydrolyses to benzylpenicillin and diethylaminoethanol as shown in fig. 1.3.



Diethylaminoethylester of Benzylpenicillin (BPC 1954)



Benzylpenicillin

+

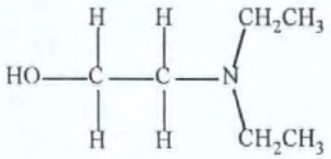


Fig. 1.3 Hydrolysis of the Diethylaminoethylester to penicillin-G

Streptopen QR.

Streptopen is a widely used preparation from Glaxovet containing the following in quick release oily base.

Procaine benzylpenicillin	100mg
Dihydrostreptomycin (as sulphate)	100mg

As for Tetradelta and Leo Yellow, the withdrawal period is 72 hours.

1.5.4 Antibiotic compounds: Chemical structure and mechanisms of action.

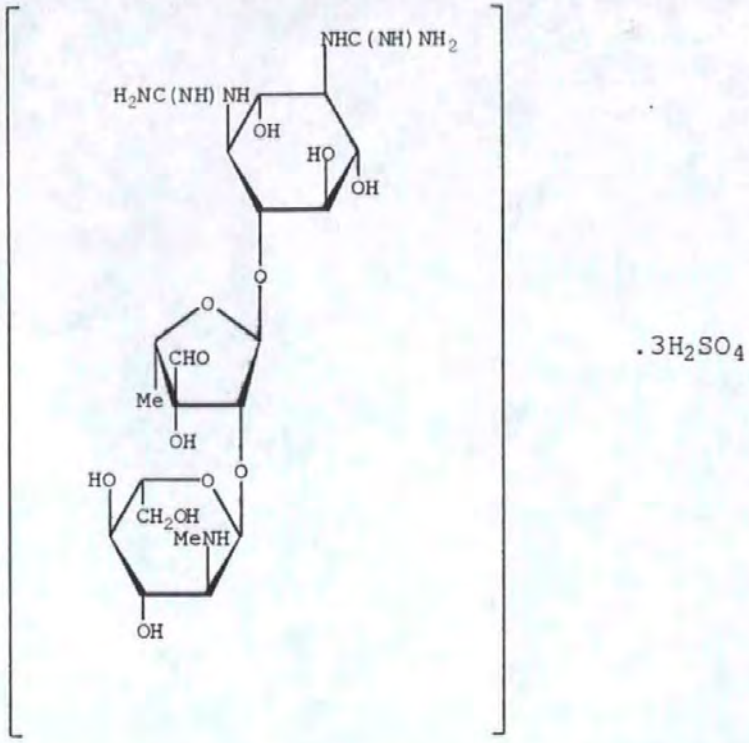
Although the experimental content of this thesis focuses entirely on benzylpenicillin, *in-vivo* samples contained other antibiotics necessitating a brief discussion of the characteristics of the more common varieties.

Aminoglycoside antibiotics.

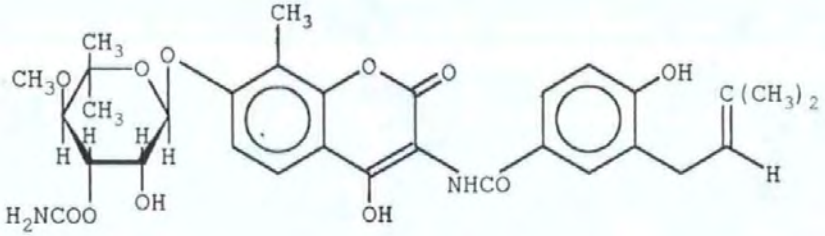
The aminoglycoside antibiotics are clinically useful showing antimicrobial activity against many gram-negative and positive bacteria as well as mycobacteria (Whall, 1981). All of the aminoglycosides are potentially toxic and may persist in milk with apparent risks to the health of the consumer (Shaikh and Allen, 1985). Streptomycin (fig. 1.4), produced by *Streptomyces sp.*, influences the permeability of the plasma membrane, whereas Neomycin falsifies the genetic code and has an adverse effect on protein synthesis (IDF, 1979). Many aminoglycoside antibiotics are UV transparent and have been resolved by applying MECC with indirect UV detection (Ackermans *et al.*, 1992).

Novobiocin.

Novobiocin, produced by *Streptomyces niveus*, is a commonly used bactericide in modern bovine mastitis treatments, owing its antimicrobial activity to its capacity to inhibit RNA and DNA synthesis. A tolerance level of 0.1ppm in milk was set in 1982 (Reeves, 1995). The structure of Novobiocin is shown in fig. 1.4.



Streptomycin A Sulphate



Novobiocin

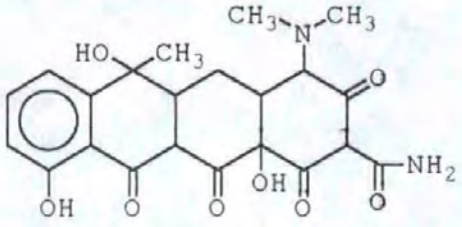
Fig. 1.4 Structures of Streptomycin and Novobiocin antibiotics commonly used in intramammary mastitis treatments.

Tetracyclines.

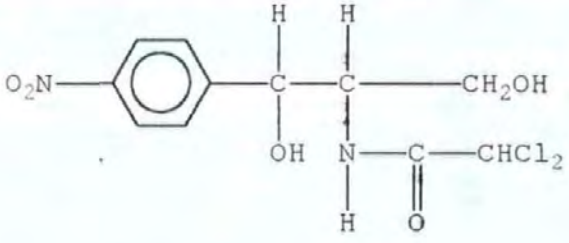
Tetracyclines (fig. 1.5) are often used in the treatment of bovine diseases (Croubels and Van Petegham, 1994). Their mechanism of action consists of inhibition or modification of protein synthesis, although tissue irritation and swelling are common side effects.

Chloramphenicol.

Although chloramphenicol (fig. 1.5) is not approved for use in food producing animals in the United States it has been widely used as an affective treatment for bovine mastitis in the UK (Allen, 1985). First isolated from cultures of *Streptomyces Venezuela* in 1947 it was found that extremely low doses of chloramphenicol can cause aplastic anaemia in humans (Arnold and Somogy, 1985) and inhibition of protein synthesis causing allergic responses in hypersensitive individuals (Long *et al.*, 1990)



Tetracycline



Chloramphenicol

Fig. 1.5 The structures of Tetracycline and Chloramphenicol

1.6 Penicillin.

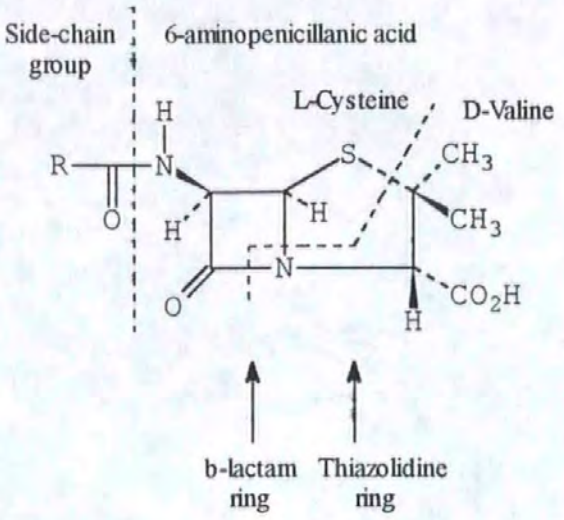
Penicillin was first discovered by Fleming in 1929 and became commercially available in 1943 (Margosis, 1989). It is produced industrially by submerged fermentation using moulds from *Penicillium chrysogenum* and is comprised of a fused β-lactam/thiazolidine structure.

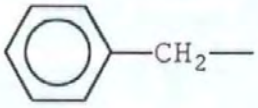
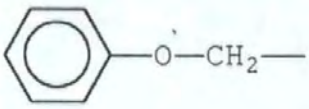
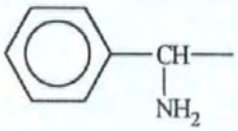
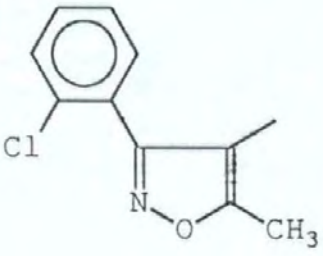
The penicillin molecule consists of a 6-aminopenicillanic acid nucleus and a side chain group. The nucleus contains two amino acids, L-cysteine and D-valine linked to produce a cyclic dipeptide (Hou and Poole, 1971). By reacting 6-aminopenicillanic acid, obtained from benzylpenicillin amidase catalyzed hydrolysis, with a variety of acylating agents other clinically useful antibiotics have been prepared. Fig. 1.6 shows the structure of 6-aminopenicillanic acid and the side chains of commonly used synthetic penicillins.

Penicillin has bactericidal properties against the majority of gram-positive cocci (Morelli, 1994). Acting on the formation of the cell wall it inactivates growing bacteria by binding with the enzymes that catalyse the cross linking of peptide chains (Voet and Voet, 1995). This mechanism of action explains why bacteria with no cell wall, known as protoplasts, are penicillin resistant (Stryer, 1988). The bactericidal action of penicillin lies in the fused β-lactam\thiazolidine structure and any cleavage results in deactivation. The fused structure is much more sensitive to nucleophiles, electrophiles and even water molecules than the simple β-lactam structure. Consequently the antimicrobial activity of penicillin is easily lost especially in the presence of β-lactamase enzymes produced by resistant micro-organisms. The free carboxyl group on the thiazolidine ring is also necessary for microbiological activity, as all penicillin derivatives not possessing this moiety show reduced activity or are totally inactive. Although the penicillin nucleus is essential for antibacterial action the potency of the various related compounds is controlled to a great extent by the nature of the side chain. None of the synthetic penicillins is superior to benzylpenicillin against *Staphylococci* bacteria.

Allergic reactions by humans have been noted more often with penicillins than any other group of bactericidal compounds. Allergies are initiated by antigen-antibody reactions, the antibody having been formed in response to the presence of an immunogenic substance such as penicillin (Schwartz, 1969). Such drugs of relatively low molecular weight are not immunogenic themselves but first must irreversibly combine with a protein, carbohydrate or lipid.

Fig. 1.6 Structure of 6-aminopenicillanic acid and the side chains of common penicillin residues



R	Penicillin
	Penicillin-G
	Penicillin-V
	Ampicillin
	Amoxacillin
	Cloxacillin

1.6.1 Degradation reactions and mechanisms.

The bicyclic β-lactam\thiazolidine structure is sensitive to nucleophiles, electrophiles, oxidizing agents and even water molecules. Fig. 1.7 shows the degradation pathway proposed (Lipczynski, 1987) for benzylpenicillin in dilute acidic and basic aqueous solutions. The proportions of each product are pH dependent (Page, 1992).

Nucelophilic attack reactions.

Benzylpenicillin is extremely susceptible to nucleophilic attack by hydroxyl ions (Hou and Poole, 1971) as shown in fig 1.8.

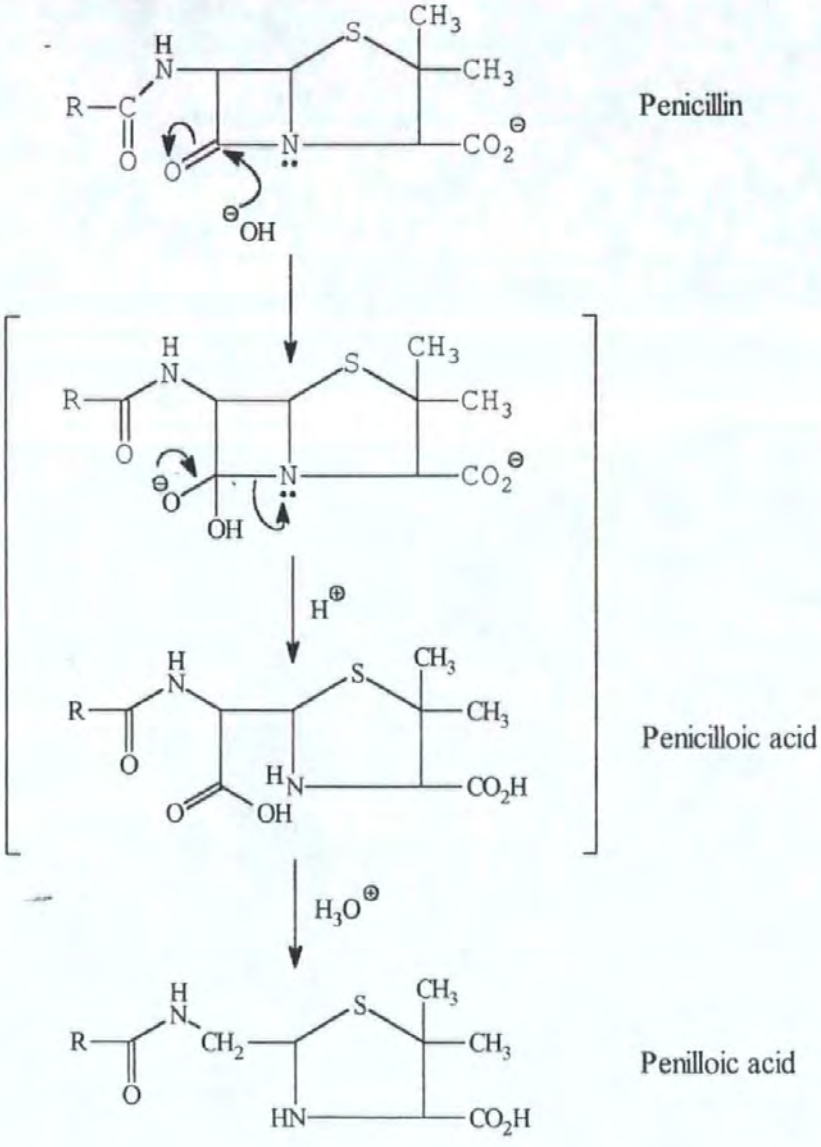


Fig. 1.8 Nucleophilic attack of penicillin to penicilloic acid and hydrolysis to penilloic acid.

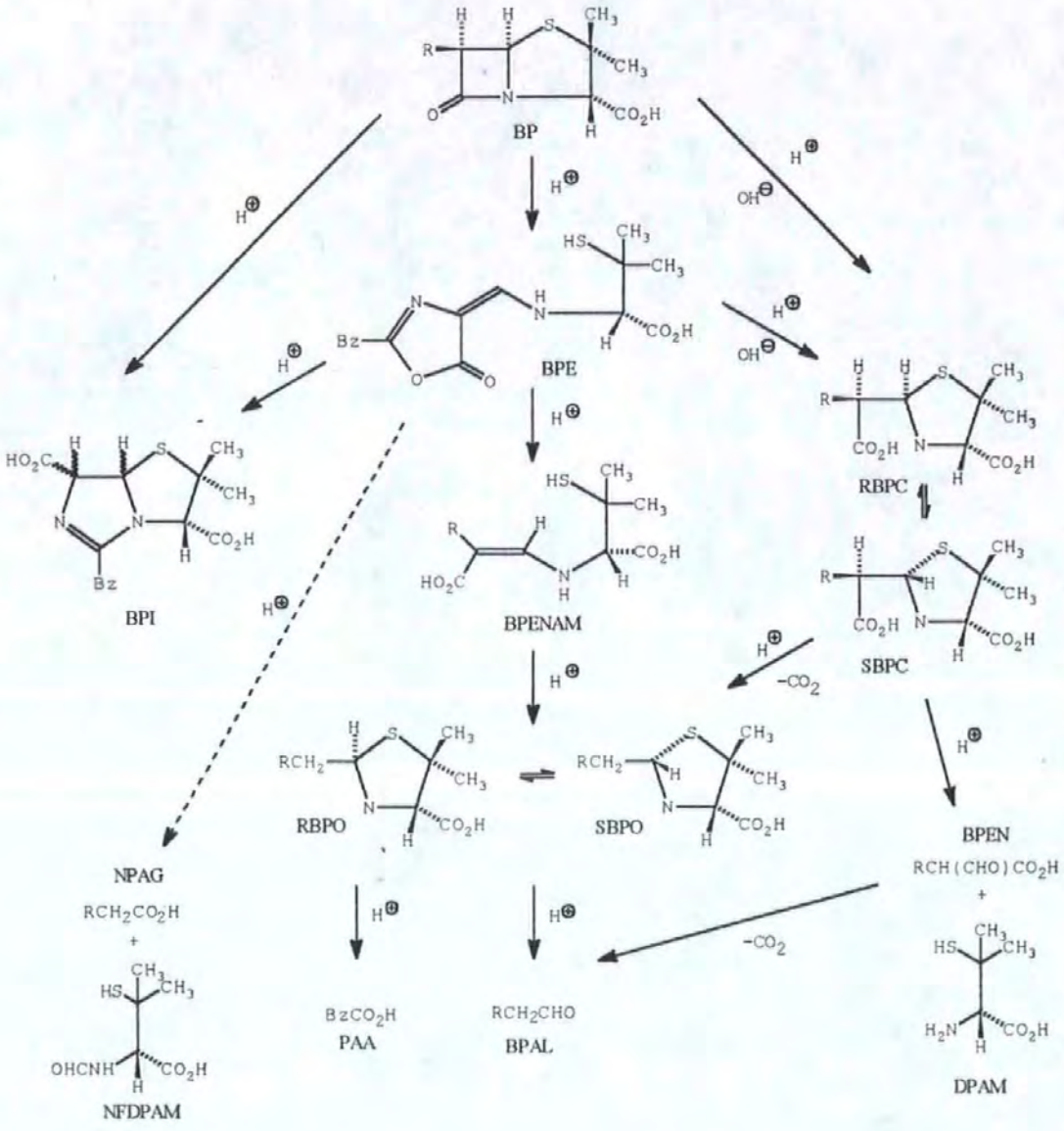


Fig. 1.7 Proposed degradation pathway for benzylpenicillin in dilute acidic and basic solutions. Key: **BP**, Benzylpenicillin; **BPI**, Benzylpenillic acid; **RBPC**, (3S,5R,6R)-Benzylpenicilloic acid; **SBPC**, (3S,5S,6R)-Benzylpenicilloic acid; **RBPO**, (3S,5R)-Benzylpenilloic acid; **SBPO**, (3S,5S) -Benzylpenilloic acid; **PAA**, Phenylacetic acid; **BPE**, Benzylpenicillenic acid; **BPENAM**, Benzylpenamaldic acid; **BPEN**, Benzylpenaldic acid; **BPAL**, Benzylpenilloaldehyde; **DPAM/DS**, Penicillamine disulphide; **NFDPAM**, Formylpenicillamine; **NPAG**, Phenylacetylglycine. R=$C_6H_5CH_2CONH$-; Bz=$C_6H_5CH_2$- . (From Lipczynski, 1987).

The initially hydrolysed product of penicillin is the biologically inactive penicilloic acid. Although incapable of reacting directly with tissue macromolecules to form highly reactive penicilloyl compounds, penicilloic acid has been determined as a minor antigenic agent reacting with the disulphide bonds of proteins (Gebre-Sellassie *et al.*, 1984). It is stable in the form of salts and in neutral solutions, but on acidification it readily loses one molecule of CO_2 to give penilloic acid (Hou and Poole, 1971). Penicillin β-lactamases and certain metal ions such as copper are powerful catalysts of nucleophilic reactions (Kishore *et al.*, 1994).

Electrophilic attack reactions.

Benzylpenicillin is susceptible to electrophilic reactions at the β-lactam nitrogen and the thiazolidine sulphur atom, as shown in Fig. 1.9.

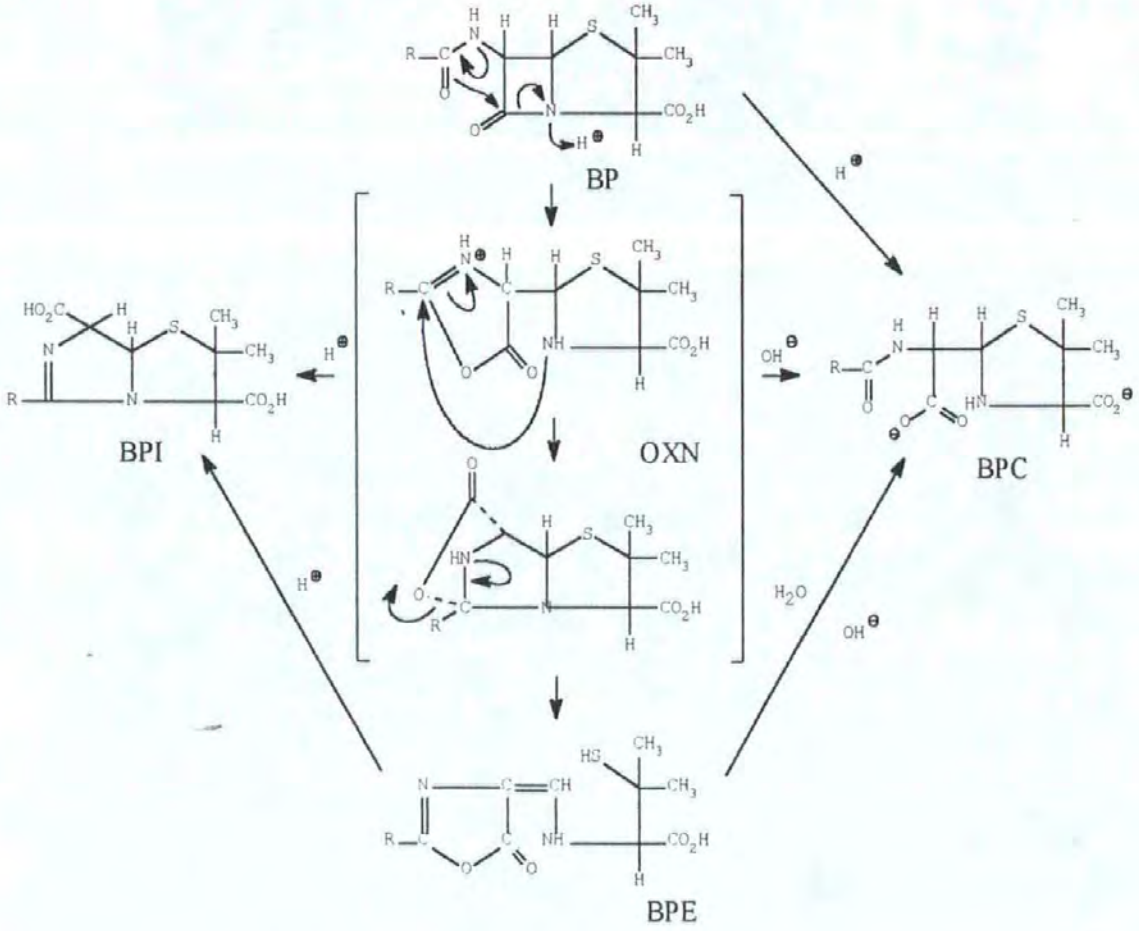


Fig. 1.9 Diagram to show the electrophilic attack on Benzylpenicillin. Key: **BP**, Benzylpenicillin; **BPI**, Benzylpenillic acid; **OXN**, Oxazolone; **BPC**, Benzylpenicilloic acid; **BPE**, Benzylpenicillenic acid. R= $C_6H_5CH_2$- (From Kessler *et al.*, 1983).

In strong acid penicillin is isomerised to penillic acid in a mechanism involving the transient oxalone structure. In weak acid or in neutral solution penicillin undergoes transformation to the extremely unstable penicillenic acid which quickly isomerises to penillic acid or penicilloic acid depending upon the pH of the solution (Page, 1992). The sulphur atom on the thiazolidine ring is also susceptible to electrophilic attack by metal ions.

1.7 Chromatographic analysis of penicillins.

Since the commercialisation of penicillin in 1947 it has been widely used in veterinary medicine to treat microbial infections including bovine mastitis (Boison, 1992). The microbiological tests indicated in section 1.5.1 are nonselective, imprecise (Tyczkowska and Aronson, 1988) and the presence of active metabolites or antibacterial endo- and exo-genic substances can give false positive results (Wiese and Martin, 1989) There has consequently been a great deal of interest focused on chromatographic techniques for the separation and detection of these residues which would provide the necessary qualitative and quantitative information. In contrast to the microbiological methods, chromatographic approaches can provide high sensitivity and separation efficiencies (Bobbitt and Ng, 1992)

Gas Chromatography.

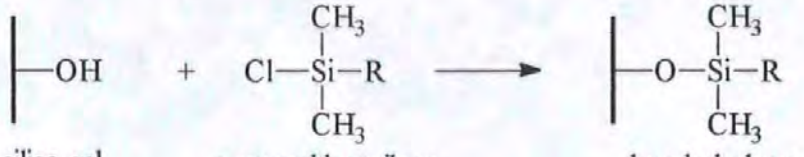
Despite providing adequate resolution for the separation of closely related penicillin analogues, most are insufficiently volatile for direct GC analysis. Electron-capture and thermionic detectors possess high sensitivity that is advantageous to the detection of trace levels of penicillins, but derivatization methods necessary for volatilisation are time consuming and problematic. Meetschen and Petz (1990) methylated penicillins, following sample extraction and purification, with diazomethane to render them amenable to determination by GC and obtained LOD's below 1μg/Kg from *in-vitro* milk samples.

Thin Layer Chromatography.

TLC provides the throughput necessary for screening methods, but poor resolution and high LOD's have limited its application. Neidert *et al.* (1987) used TLC to separate a mixture of fourteen common antibiotics.

High Performance Liquid Chromatography.

Of the many liquid chromatographic modes, bonded phase chromatography is the most common. The usual support for bonded phase is silica with groups linked to its surface by silanisation with organosilanes.



silica gel organochlorosilane bonded phase

Unreacted hydroxyl groups on the silica may cause problems due to chemical reactions with either the sample or mobile phase. This can be averted by "end capping" involving further silanisation with chlorodimethylalkylsilane and chlorotrimethylsilane. In reverse phase applications R, in the reaction above, is a hydrocarbon (typically octadecyl C_{18}), and the stationary phase is therefore non polar. The more polar components of the sample are eluted first and an increase in the polarity of the mobile phase results in an increase in retention times. The major constituent of the mobile phase, in reverse phase systems, is water with miscible solvents added to modify the elution range of the sample (Martin *et al.*, 1988). The predominant factor for retention in RP-HPLC is the hydrophobic interaction of analytes with the stationary phase. Practically all organic molecules possess a region in their structure which is hydrophobic and this explains the wide applicability of this technique (Lough and Wainer, 1995).

The majority of work conducted on the separation of monobasic penicillins has been mainly achieved by reverse phase ion-suppression techniques (Fletouris *et al.*, 1991). However, such mobile phases rely on pH values less than optimum with respect to penicillin stability and suffer from imprecise retention times due to small mobile phase compositional changes. A dramatic decrease in retention time of monobasic penicillins is noted on increasing the pH to 4.5, indicating that ionization of the penicillin molecules occurs above this value making the penicillins less hydrophobic and, therefore, reducing affinity for the stationary phase.

Penicillins can form ion-pairs producing efficient separations with additional modes of control (Bobbitt and Ng, 1992). The type of counter ion and its concentration can be modified to achieve the optimum separation. Moats (1983) and Fletouris *et al.* (1992) used

tetrabutylammoniumhydrogen sulphate (TBA-HSO_4) to resolve penicillins present in milk samples following liquid/liquid extraction, whereas Terada and Sakabe (1985) used sodium-1-heptanesulphonate after solid phase extraction. Nakagawa *et al.* (1982) increased the retention times of β-lactams by associating the ionised amino group of the penicillin with crown ethers. This allowed their resolution from endogenous substances in human urine and plasma.

Capillary Electrophoresis.

Capillary Electrophoresis (CE) is a relatively recently developed technique, employing narrow bore (20-200μm i.d.) capillaries to obtain high efficiency separations. The separations are facilitated by high electric fields often in excess of 500V/cm which generate electroosmotic and electrophoretic flow of buffer solutions and the ionic species in the capillary. CE requires only minute amounts of sample, consumes limited volumes of reagents (<10ml of carrier electrolyte for 24 hours analysis) and is automated for precise quantitative measurements. A basic CE system is shown in fig. 1.10 and modern instruments offer computer control of all operations, pressure and electrostatic injection, an autosampler, temperature control and heat dissipation system (Weinberger, 1993).

The fundamental process that drives CE is the electroosmotic flow (EOF) and is a consequence of the charged wall of the capillary. The non coated silica capillaries typically used for CE separations have ionizable silanol groups in contact with the carrier electrolyte. The major influence on the EOF is the pH of the background electrolyte, and can be adequately illustrated by imagining a zwitterion such as a peptide in low and high pH systems. As shown in fig. 1.11, at high pH the ionization of the negative silanol groups at the capillary wall is large resulting in the formation of an electrical double layer. Anions from the electrolyte are repelled from the wall region, whereas cations are attracted as counter ions. The ions closest to the wall are tightly bound and are immobile even under the strong electric field. Further from the wall is a compact and mobile region with a predominantly positive character. The mobile positive charges migrate in the direction of the cathode when the voltage is applied. Since ions are dissolved in water, the electrolytic fluid is dragged by the migrating charge and this so-called EOF is transmitted through

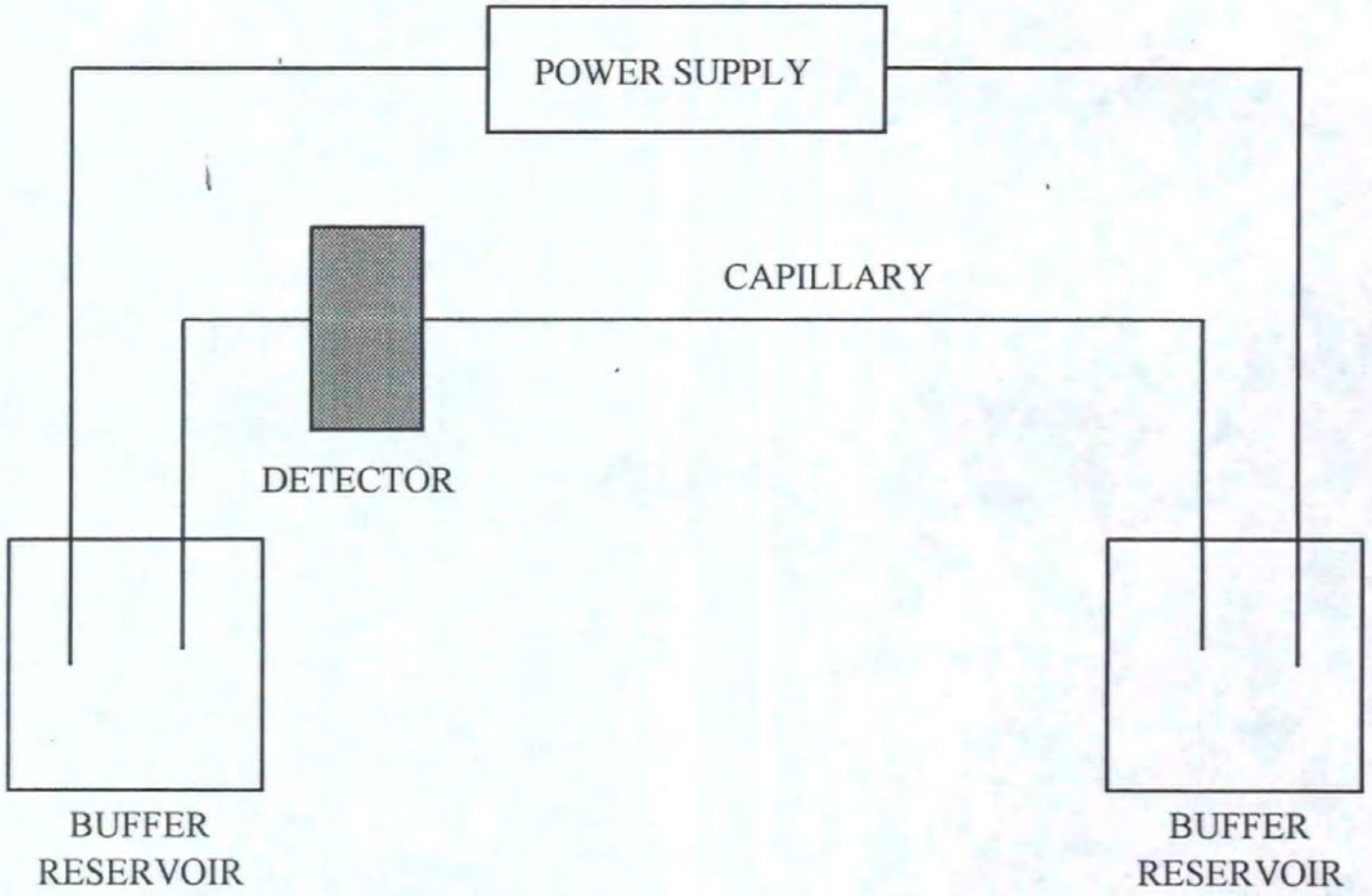


Fig. 1.10 Schematic of a basic CE system (adapted from Weinberger, 1993).

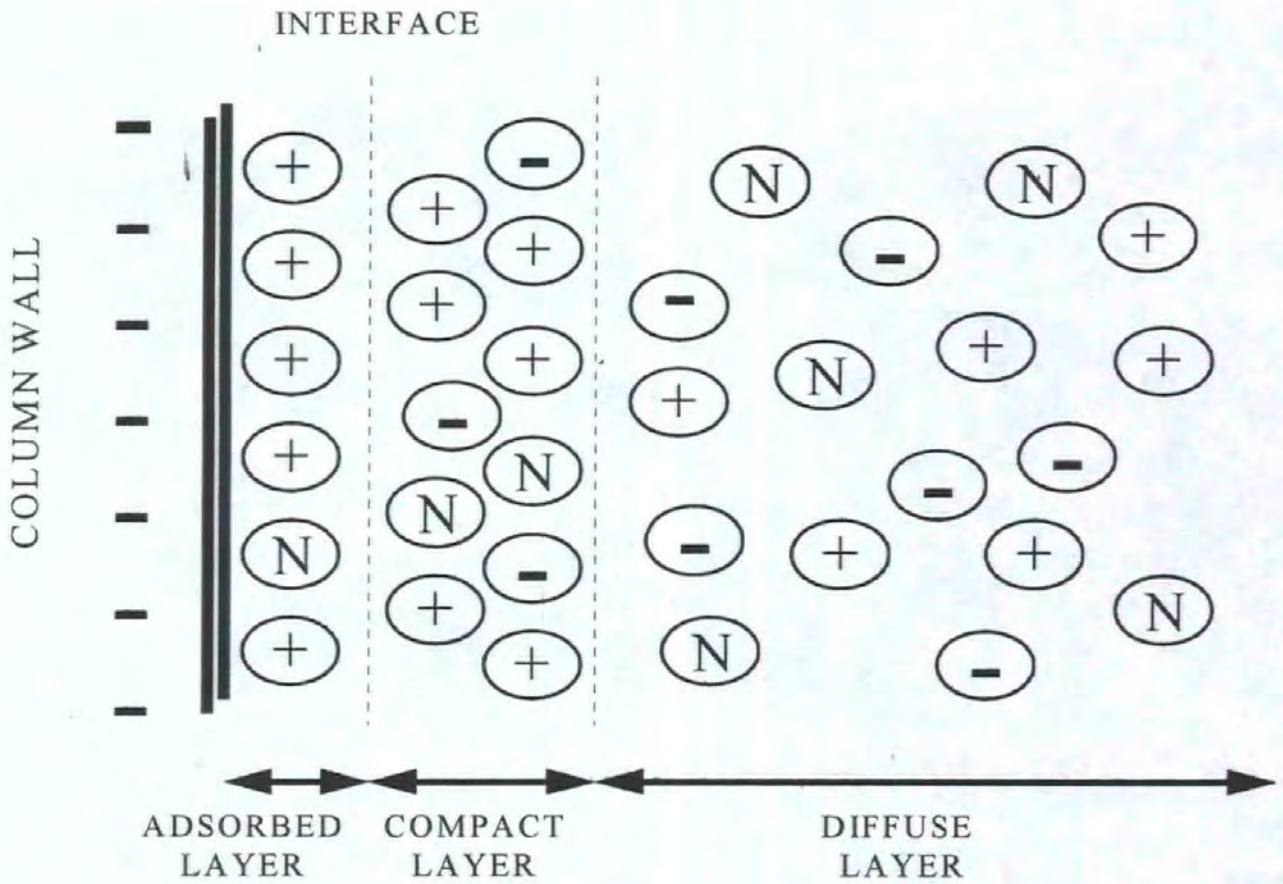


Fig. 1.11 Representation of the electrical double layer at the capillary wall fundamental to electroosmosis. Where N are Neutral molecules. (adapted from Wienberger, 1993).

the diameter of the capillary, thought to be due to hydrogen bonding or Van der Waals interactions between the buffer constituents. Thus the higher the pH the greater the ionization of the silanol groups, creating a strong zeta potential and therefore attracting more cations to the wall and resulting in a stronger EOF. The zwitterion, which at high pH will have a net negative charge, will be attracted electrostatically to the anode, however, the EOF is the dominant force and the analyte will migrate towards the cathode. At low pH the electrophoretic movement is towards the cathode because of a net positive charge on the zwitterion and the EOF is reduced due to the reduced ionization of the silanol groups at the capillary wall. In untreated silica capillaries, most solutes migrate towards the cathode regardless of charge when the electrolyte is above pH 7.0. At acidic pH most zwitterions and cations will also migrate towards the negative electrode.

Capillary electrophoresis comprises a family of techniques, listed below, that have dramatically different characteristics.

1. Capillary zone electrophoresis (CZE)
2. Isoelectric focusing
3. Capillary gel electrophoresis
4. Isotactophoresis
5. Micellar electrokinetic capillary chromatography (MECC)

The first four modes of CE have all been researched and reported on extensively but since all work during this thesis was conducted using MECC, this technique will be discussed in detail.

Micellar Electrokinetic Capillary Electrophoresis (MECC).

The use of micelle forming surfactant solutions allows separations resembling reverse phase LC, with the advantages of CE. Micelles are aggregates of long chain (10-50 units) surfactants which have hydrophobic tails and a hydrophilic charged head. The micelle forms as a consequence of the hydrophobic effect to reduce the free energy of the system. Above a certain surfactant concentration, known as the critical micelle concentration (CMC), the aggregate is fully formed (Terabe, 1992[b]). The four major classes of surfactant are anionic, cationic, zwitterionic and nonionic. Sodium dodecyl sulphate (SDS) used in all experiments

described in this thesis, is an example of an anionic surfactant with a CMC of 8.1×10^{-3} M (at 25°C) and is the most widely used surfactant in reported MECC work.

Micelles having very strong solubilizing power and have the ability to organise analytes at the molecular level. Fig. 1.12 shows a schematic representation of the separation principles of MECC when an anionic surfactant, such as SDS, is employed. The micelle migrates towards the positive electrode by electrophoresis. The EOF, being the stronger force, transports the micelle and the bulk solution towards the negative electrode at a retarded velocity. When an analyte is incorporated into the micellar solution a fraction of it is dissolved into the aggregate and it migrates at the velocity of the micelle. The remaining analyte will be free of the micelle and will migrate at the velocity of the EOF. The migration time of the analyte will therefore depend upon its distribution coefficient between the micelle and the background electrolyte. The greater its solubility in the micelle the longer its migration time. Compounds must migrate at a velocity between the EOF velocity and the velocity of the micelle. This is often referred to as the migration time window (Terabe, 1992[b]).

Temperature has a profound effect on any MECC separation. An increase in temperature causes a reduction in analyte migration time because of the decrease in the distribution coefficient as well as the viscosity of the bulk solution. Since the dependence of the distribution coefficient on temperature is different among analytes, the temperature of the system also affects selectivity. High currents will also cause an increase in temperature and should be avoided.

Although HPLC has been the conventional method for the separation of antibiotics, in recent years, CE methods have achieved considerably more significance. Nishi *et al.* (1989) used MECC to separate seven penicillins and nine cephalosporins and Yeo *et al.* (1991) employed CE with photodiode-array detection to resolve various β-lactam and aminoglycoside antibiotics. Despite CE having large potential for the analysis of penicillins in food matrices because of the high separation efficiencies and multiple separation modes, no work has been published on the separation of β-lactam compounds from milk, although high detection limits may be the issue here.

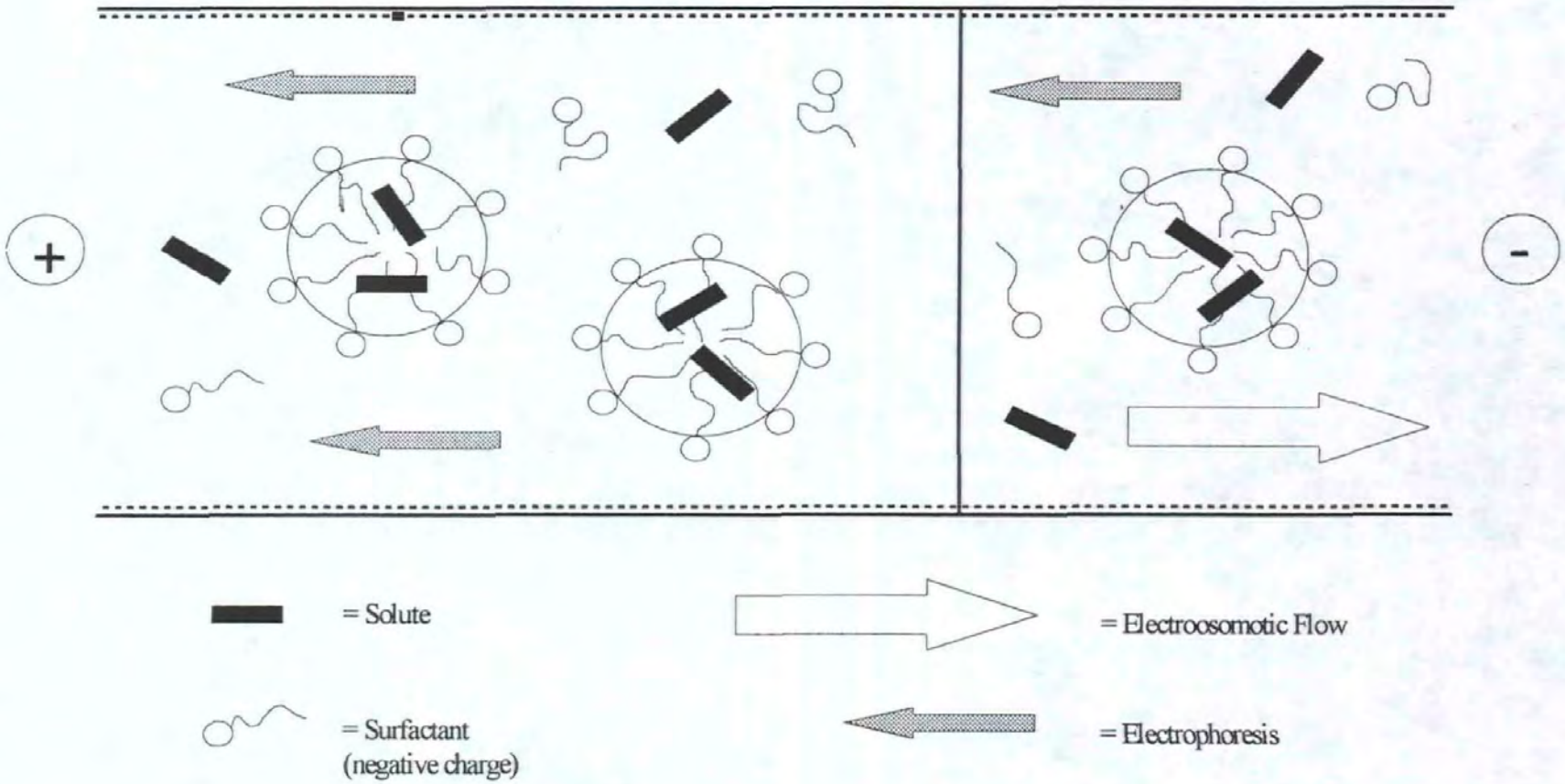


Fig. 1.12 Diagram to show the separation principle of MECC with the detector positioned towards the negative electrode (adapted from Terabe, 1992[b]).

1.7.1 Chromatographic analysis of benzylpenicillin degradation compounds.

Penicillin degradation has been researched in some detail in acidic conditions (Kessler *et al.*, 1981 and 1983). The driving force behind this research was the quest for acid stable penicillins that would not be hydrolysed at the low pH's encountered by orally administered drugs in the human stomach. Lipczynski (1987) reported a RP-HPLC separation of benzylpenicillin and fifteen of its degradation compounds using a mobile phase with a high phosphate buffer concentration. DePaolis *et al.* (1977) subjected benzylpenicillin in animal tissues to different cooking conditions and examined the degradation compounds as their methylesters by HPLC following pre-column derivatization. There appears to be no published work on the direct analysis of antibiotic breakdown residues in biological matrices by any chromatographic system.

1.7.2 Detection modes for the analysis of penicillins.

Penicillin residues do not have a specific or intense chromophore above 230nm and consequently short wavelength UV detection is the most commonly used detection method (Bobbitt and Ng, 1992). A major disadvantage of this occurs when separating penicillin from biological samples, such as milk, since the short wavelength UV detection is insufficiently selective making trace antibiotic analysis extremely difficult because of high background interference (Boison, 1992). Alternatives to UV detection are indicated below.

Derivatization methods.

Chemical reaction of penicillin residues with either a chromophore or fluorophore has extensively been used to enhance their detection from biological matrices, including milk. However derivatization methods add time, cost and complexity to the necessary extraction and clean up procedures. Boison *et al.* (1994) used a previously developed isocratic liquid chromatography method to resolve benzylpenicillin from milk, using the chromophore 1,2,4-triazole-mercuric chloride and UV detection at 325nm. Recoveries were determined to be >70% and the limit of detection was 3ppb.

Munns *et al.* (1985) employed fluorescence detection at 254nm having enzymatically hydrolysed the β-lactam ring on the penicillin. The resulting penicilloate was then reacted with mercuric chloride which liberated the side chain with a terminal aldehyde. This penilloaldehyde was extracted with dichloromethane and fluorolabeled with dansyl hydrazine. This method was used to resolve eight penicillins with average recoveries ranging from 26.4% to 87.7%.

Derivatization techniques may be pre-column (Rogers *et al.*, 1984) or post-column (Haginaka *et al.*, 1988). The main considerations for pre-column derivatization are the effects the derivative may have on the separation and analyte stability; whereas, although post-column derivatization does not affect the chromatographic separation, the reaction must be rapid and must not be affected by the chromatographic conditions.

Polarimetric detection.

Penicillins are chiral which makes polarimetry an ideal detection system. The physical parameter used to describe optical activity is specific rotation [α], which is extremely sensitive to subtle structural variations. Polarimetric detectors, therefore, provide significant specificity without the need for derivatization.

Laser-based polarimetric detection has been applied to the separation of closely related penicillin analogues (Rice *et al.*, 1989) providing an order of magnitude improvement in the mass detectability of the analytes. It has also been used to study erythromycin in milk (Shao *et al.*, 1989) requiring the minimum of sample purification because the specificity of the detector resulted in the recording of few milk matrix components. The mass LOD was found to be at the 10ng level, 15 times lower than with conventional UV/VIS detection.

Mass spectrometry.

Particle beam HPLC-MS has been investigated for antibiotic analysis in food matrices (Voyksner *et al.*, 1990) providing a LOD at the ng/g level. However, the cost and complexity of HPLC-MS instrumentation and the difficulty in obtaining molecular ions are limitations that have prevented widespread application of this technique.

1.8 Milk composition and the changes associated with mastitis.

Milk is a heterogeneous fluid which contains over 100 constituents with an average water content of 80%. Physically, milk is a three phase system consisting of:-

(i) Colloidally dispersed spherical micelles comprised of casein aggregates and calcium and phosphate.

(ii) A lipid phase as an emulsion of finely dispersed fat droplets surrounded by a membrane of lipoprotein.

(iii) An aqueous phase containing proteins, minerals, vitamins and salts.

The major constituents of milk are fat (butterfat), protein (crude protein) and lactose (milk sugar). The concentration of protein and lactose are usually regarded as a single entity called the solid-non-fat. The remainder of the milk solids are collectively known as 'ash' and are generally accepted as making little significant contribution to the solid-non-fat fraction. Table 1d. shows the average percentages, by weight, of the major constituents of milk as indicated in the 1993/94 annual report of the Joint Milk Quality Committee. A certain amount of cellular material is also normally present.

Table 1d. The average percentage composition of milk.

Constituent	Percentage (weighted average)
Butterfat	4.11
Solid-non-fat	8.75
Crude Protein	3.26
Lactose	4.56
Ash	0.75

Only small variations occur due to seasonal effects with the major changes involving the solid-non-fat (proteins, sugars, lactose, minerals) between December and June, and for fat between February and July with May being particularly low when herds graze the young lush grass (Ferns and Roberts, 1984). There are normal changes in milk composition which occur during lactation

and which are generally consistent between individual cows, but are influenced by the time of year when the cow calves. Other factors affecting milk composition include breed, age of cow, nutrition, stage of lactation and milking procedure used (Wong *et al.*, 1988).

With the onset of mastitis, milk composition is adversely affected both quantitatively and qualitatively (Morris and Marsh, 1985). Table 1e. (page 34) shows the constituents of milk and the changes resulting from infection. The viscosity of the milk is usually increased as the somatic cell concentration rises. Increased permeability of the gland to blood components during mastitis brings about elevated milk pH due to movement of bicarbonate ions into the milk. The fat globules become more susceptible to lipolysis due to high concentrations of lipases contained in somatic cells and blood serum. The presence of free fatty acids causes the milk to become rancid. Milk collected from cows with infectious mastitis has a much higher lipase content especially when the invading pathogen is *Staph. aureus* than in non-specific cases.

Changes in milk composition due to direct degradation by bacteria are small. However, a build up of lactic acid has been noted because of the action of *Str. agalactae* on lactose. One of the more obvious manifestations of mastitis is the decrease in yield. The economic consequences of this depression has led to extensive research into the relationship between mastitis and milk yield. Table 1f. shows the milk losses associated with various conditions of infection.

Table 1f. Losses of milk associated with various conditions of mastitic infection (from Woolford, 1985).

Yield losses	Conditions
40%	Whole lactation clinicals
33%	Part lactation clinicals
12-20%	Subclinical
15.3%	Staphylococcal
18.4-26.2%	Varied with pathogen
35%	Infected longer than 3 months

Table 1e. Changes of milk composition associated with mastitis (from Heeschen *et al.*, 1985).

Main components	Normal level	Trend of change
Fat %	3.45	–
Protein %	3.61	–
Lactose %	4.85	–
Fat components		
Free fatty acids (mEqu./l)	0.6-0.8	+ +
Fatty acid pattern C_4 - C_{12}	126.4	+
(mg/g fat) C_{16} - C_{18}	708.4	–
Protein fractions (mg/ml)		
Total casein	27.9	– –
Total whey protein	8.5	+ + +
Caseins (mg/ml)		
α-s_1-casein	13.3	– – –
β-casein	10.6	– – –
χ-casein	1.6	+ +
Whey proteins (mg/ml)		
β-lactoglobulin	4-4.25	– – –
α-lactalbumin	1.03-1.22	– – –
Serum albumin	0.08	+ + +
Total immunoglobin	0.25-0.3	+ + +
Other N-compounds (mg/ml)		
Proteose peptone	1.82	+ + +
Lactoferrin	0.1-0.2	+ + +
Anions and cations (mg/100ml)		
Na	57	+ +
K	172.5	–
Cl	80-130	+ + +
Total Ca	136	– – –
Total Mg	18	– – –
P	26	– – –
Conductivity (mM NaCl)	<52	+ (+)
Enzymes		
Catalase (μmol O_2 /min/ml)	0.08	+ + + +
Lactate dehydrogenase (mU/ml)	300-500	+ + +
Glutamate-oxalacetate-transaminase (mU/ml)	4	+ + +
Alkaline phosphate (U/ml)	191	+ + +
Acid phosphate (μmol/min/ml)	0.063	+ +
Xanthinoxidase (μg/ml)	12	– – –
Lipase (μg/ml)	1.49	+ +
Carboxylesterase (μg/ml)	0.004	+ + + +
Arylesterase (μg/ml)	0.028	+ + +
Lysozyme (μg/ml)	0	+ + + (+)
N-Acetyl-β-D-glucosaminidase (μg/ml)	0.021	+ + +
β-Glucuronidase (μg/ml)	0.012	+ + +
α-Mannosidase (μg/ml)	0.043	+ +
Arylsulphatase (μg/ml)	0.0053	+ + +

<10-fold increased = +
11-100-fold increased = + +
101-1000-fold increased = + + +
>1000-fold increased = + + + +

10% reduced = –
11-25% reduced = – –
26-75% reduced = – –
75% reduced = – – – –

1.9 Milk as a biological matrix

Mastitic milk, as shown in the previous section, contains many compounds which may not only interfere with the chromatographic resolution of analytes, but also have a deleterious effect on chromatographic equipment such as injectors, pumps and column packing material. Once a representative milk sample has been collected, chromatographic analysis of penicillins and their breakdown residues requires that the analytes first be freed from protein. This is followed by development of a sample purification technique, where the presence of carbohydrates, proteins and lipids must be taken into consideration. Other requirements of such a method are that the labile penicillin is not degraded further and recovery of the compounds of interest be made as efficient as possible with the minimum sample manipulation.

Penicillins are usually homogeneously distributed throughout the treated quarter milk (Moats, 1983) and consequently the collection of a representative sample is not a problem. Penicillin isolation from complex matrices combined with protein precipitation has previously been achieved by treatment of samples with methanol (Salisbury *et al.*, 1989 ; Nagata and Saeki, 1986), acetonitrile (Moats, 1983 ; Blanchflower *et al.*, 1994), sulphuric acid and sodium tungstate (Terada *et al.*, 1985 ; Boison *et al.*, 1994) or ultrafiltration (Tyczkowska *et al.*, 1990). Acetonitrile extraction avoids the extremes of pH and extensive protein binding that occurs when using sodium tungstate and sulphuric acid. Treatment of the milk sample with two volumes of acetonitrile is sufficient to precipitate the majority of proteins and any higher proportion causes the formation of a gummy mass at the bottom of the container which reduces recoveries. Ultrafiltration involves the use of molecular mass cut-off filters to remove proteinaceous material. However, this approach is limited by contamination of the sample with low-molecular weight proteins and other matrix compounds that pass through the filter. Poor recoveries are also demonstrated due to protein binding of the penicillins of interest.

Samples extracted by these methods would still contain elevated levels of co-extractants (milk matrix endogenous and exogenous material) causing unacceptable background noise affecting the detection of the compounds of interest. Further purification of extracted samples is therefore necessary. This is usually performed by partitioning analytes into organic and aqueous solvents in so called 'liquid-liquid' partition methods or by adsorption on to solid phases. Back extractions of penicillins into buffered solutions are the commonest approach to further purification of

organic extracts (Moats, 1983). Fletouris *et al.* (1992) obtained further clean up of milk samples by converting β-lactam antibiotics to ion-pairs with TBA-HSO_4 which were then readily soluble in chloroform. Methods described by Moats (1983) and Fletouris *et al.* (1992) were repeated herein and are discussed in more detail in chapter 2.

Terada *et al.* (1985) used Sep-Pak C_{18} cartridges to obtain further sample purification following aqueous extraction and protein precipitation with sulphuric acid and sodium tungstate. Boison *et al.* (1994) used Bond Elute C_{18} cartridges as it was discovered that the Sep Pak C_{18} had <14% carbon loading in comparison to >17% in the Bond Elute version. It was found that a minimum of 17% carbon loading in the packing bed was required to obtain a recovery of >70% penicillin.

An automated liquid chromatographic clean up (Moats, 1990), involving protein precipitation with acetonitrile, was used to obtain recoveries of 92 ± 9% of penicillin with a LOD near to 2ppb. The aqueous fraction of the milk extract was injected onto a PLRP-S column and the penicillin eluted with a 0.01M, pH 7.0, phosphate (A), acetonitrile (B) gradient (A-B 100:0(0-3min)-40:60 (25min)). The narrow fraction of penicillin was collected and rechromatographed on the same type of column at pH 1.9.

A major disadvantage of the referenced methods for the extraction and purification of penicillin is that they all rely on acidification of the antibiotics to effect either protein precipitation or partition into organic solvents. When attempting to investigate the degradation of penicillin following treatment for mastitis, breakdown caused by the extraction and purification methods needs to be kept to a minimum. No references to the extraction and detection of penicillin degradation residues from milk or any other biological matrix were found in the literature.

1.10 Research aims and objectives.

The primary aim of the research was to determine whether milk taken following intramammary treatment using benzylpenicillin preparations was contaminated with its degradation compounds; and if so, whether these compounds persisted in the milk longer than the 72 hour withdrawal period.

Specific aims of the research were;

1 Development of an efficient chromatographic technique for the simultaneous detection and separation of benzylpenicillin and its major degradation compounds.

2 Development of an efficient extraction and purification technique of *in*-vitro milk samples, to enable chromatographic analysis to be effectively executed without causing hydrolysis of the compounds of interest.

3 Identification of the major benzylpenicillin degradation compounds present in aqueous solution by comparison with synthetic standards.

4 Application of the developed chromatographic system and extraction and purification technique to the analysis of *in-vivo* milk samples collected following treatments for bovine mastitis using benzylpenicillin containing intramammary infusions.

Chapter 2

High Performance Liquid Chromatography Methods and Results

2.1 HPLC instrumentation and conditions.

HPLC analyses were performed using a quaternary Perkin Elmer series 410 pump equipped with a UV/VIS photodiode array detector, a manual Rheodyne 7125 injector fitted with a 100µl loop and a Nucleosil C_{18} AB 5 micron column (250×4.6mm i.d.)(Alltech Associates) protected by a disposable guard cartridge of the same material. Analyses were made using a flow rate of 1ml/min and detector settings of 210nm and 0.01a.u.f.s.

2.2 Development of the optimum HPLC system for the resolution and separation of Benzylpenicillin and its degradation compounds in aqueous solution.

Of the common chromatographic techniques, HPLC has been recognised as the superior approach for the analysis of penicillins, having the requisite accuracy, simplicity, sensitivity and speed. Several reported HPLC methods for the separation of closely related penicillin compounds in aqueous solutions were repeated, including techniques utilising the ion pairing agents sodium-1-heptane sulphonate (Terada *et al.*, 1985) and tetrabutylammonium hydrogen sulphate (Fletouris *et* al., 1991), with little success.

Fletouris *et al* (1991) suggested that in a system composed of acetonitrile and 0.02M phosphate buffer (38:62) the capacity factors of all related penicillin compounds were independent of pH between values of 2.0 and 3.5. This suggested that the dissociation of the hydrogen ion of the carboxylate moiety on the thiozolidine ring was suppressed and the pK_a considerably increased from its value of 2.7 in aqueous solution.

In a preliminary investigation attempting to repeat the work of Fletouris *et al.* (1991), the degradation of a 0.2mg l^{-1} solution of potassium benzylpenicillin over a period of 48 hours at room temperature was followed. Using a mobile phase as indicated above, with a final pH of 3.2, analyses were made at 0, 24 and 48 hours with the resultant chromatograms shown in fig. 2.1. After 24 hours the benzylpenicillin peak, at a retention time of t_r 5.60(min), had completely disappeared but two degradation products were evident at times t_r 2.50(min) and 2.95(min). The resolution of the two breakdown compounds was affected by system peaks following the injection.

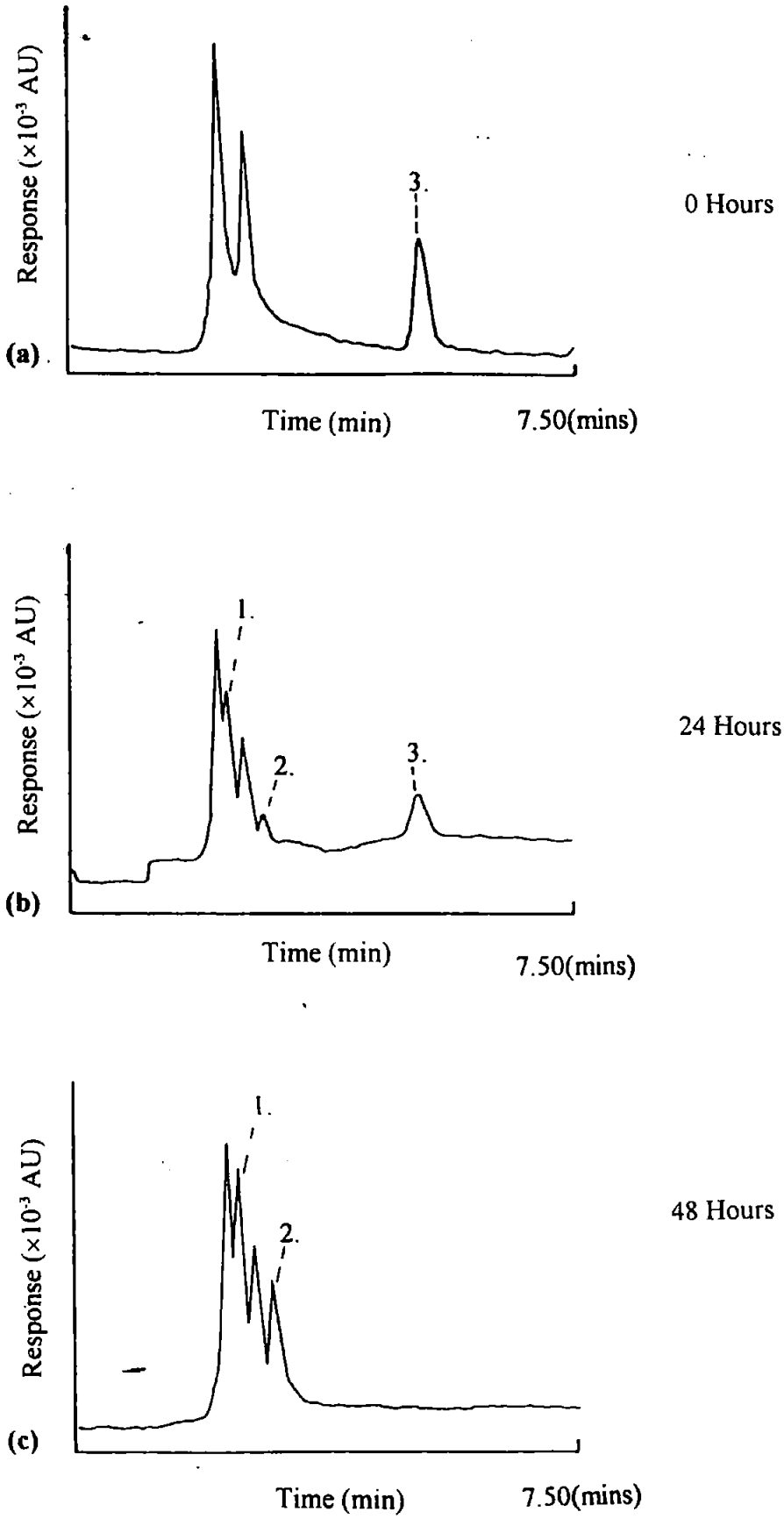


Fig. 2.1 Chromatograms obtained from the analysis of an aqueous solution of 0.2mg l^{-1} benzylpenicillin, maintained at room temperature for (a) 0 hours, (b) 24 hours and (c) 48 hours using a mobile phase 0.02M phosphate buffer:acetonitrile (62:38, pH 3.2). Chromatograms show the gradual disappearance of benzylpenicillin (3) at t_r 5.60(min) and the emergence of two degradation products (1) and (2) at t_r 2.50(min) and 2.95(min) respectively.

The labile nature of penicillin in acidic media make such ion suppression methods unsuitable for degradation monitoring.

A system reported for the separation of benzylpenicillin and its acid/base degradation compounds (Lipczynski, 1987), involving high buffer concentrations which have analogous effects to ion pair concentration on the retention of such compounds, was attempted. Under slightly acidic conditions the high buffering capacity of the mobile phase (0.05M) exceeds the ability of the solutes to modify local pH, thus reducing the dissociation of the carboxylic acid functional group present on penicillin and its degradation compounds. The result is an increase in the concentration of hydrophobic species available for retention by the stationary phase.

Accordingly, a mobile phase of 0.05M phosphate buffer and acetonitrile (80:20)(pH 6.0) was prepared and the degradation of a 10mg l^{-1} aqueous solution of potassium benzylpenicillin at room temperature was investigated. Analyses were performed at 24 hour intervals for a period of three days. The penicillin peak occurred at a retention time of 12.50(min) and after 72 hours eight degradation compounds were observed. The experiment was repeated using a mobile phase containing 17% acetonitrile to improve the separation of the degradation compounds. The resultant chromatogram can be seen in fig. 2.2.

The improved separation of penicillin degradation compounds obtained using the mobile phase of 0.05M phosphate buffer:acetonitrile (83:17, pH 6.0), together with the apparent unsuitability of ion-pairing and ion suppression techniques, resulted in this system being used in attempts to resolve the analytes from *in-vitro* milk samples (as discussed in section 2.3).

2.3 Development of an efficient extraction and clean up procedure for the detection of benzylpenicillin and its degradation compounds from *in-vitro* milk samples.

The fact that milk contains many potentially interfering substances for the chromatographic analysis of penicillin residues has been mentioned previously, but the complexity of the matrix must be emphasised. The elaborate chemical composition of the milk is such that the partitioning of polar molecules between aqueous and non-aqueous phases is not easy to predict.

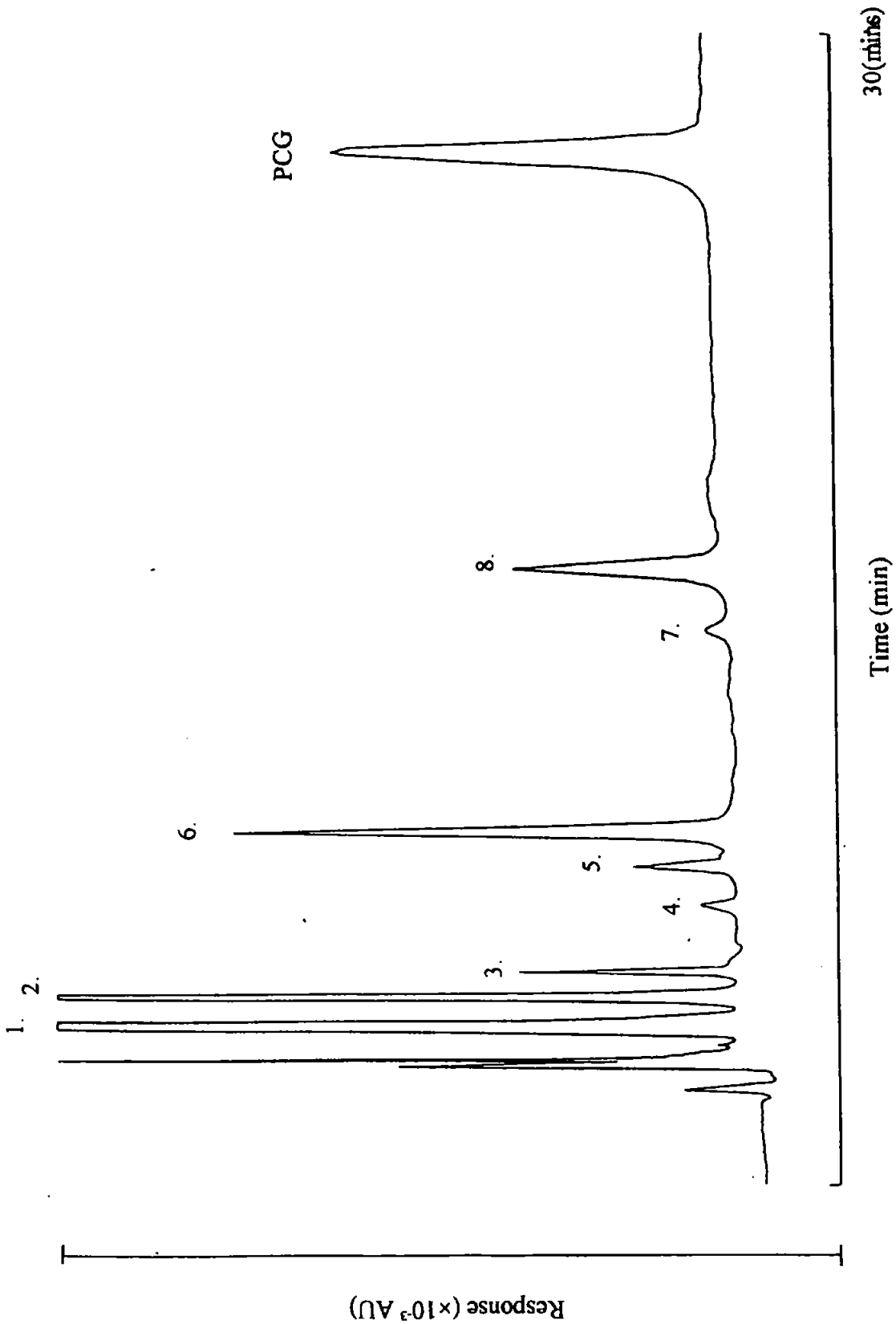


Fig. 2.2 Chromatograms obtained from the analysis of a 10mg l^{-1} aqueous solution of benzylpenicillin incubated at 37°C for 20 hours using a mobile phase of 0.05M phosphate buffer:acetonitrile (83:17, pH 6.0). Eight penicillin degradation compounds were separated at t_r (1) 4.11(min), (2) 4.87(min), (3) 5.53(min), (4) 7.25(min), (5) 8.26(min), (6) 9.16(min), (7) 14.40(min) and (8) 16.04(min). The penicillin (PCG) was eluted at t_r 26.83(min).

The combination of surface active components, salts and lipophilics causes analytes present in the milk to have a different behaviour to that when in a more ideal system. The considerable number of matrix components exhibit a wide range of activities, such as ion-pair formation, which may also impede analytical methods.

2.3.1 Silica-Carte Pre-Cep C18 cartridge.

A Silica-Carte Pre-Cep C_{18} cartridge (SPC) (Waters Chromatography) attached to a 20ml glass syringe was preconditioned with methanol (20ml) and water (20ml). 2% Sodium chloride (2ml) was then washed through the column as penicillins are not retained to any extent from water on C_{18} reverse phase columns (Terada *et al*, 1985), but are absorbed strongly from aqueous salt solutions.

Fresh pasteurised milk (100ml) was inoculated to a concentration of 5mg l^{-1} with an aqueous solution of benzylpenicillin which had been maintained at room temperature for 48 hours so that degradation of the antibiotic had occurred. An aliquot of the spiked milk (20ml) was drawn through the SPC cartridge at approximately 2ml/min. The cartridge was washed with water (20ml) and then eluted with methanol (20ml). A 100µl sample of the eluent was analysed using the HPLC conditions indicated in section 2.2.

The chromatograms obtained showed considerable interference caused by the milk matrix components that effectively masked the area where the degradation compounds had previously been shown to occur (see fig 2.2). Penicillin was detected at a retention time of 24.96(min) as a broad peak with a recovery of only 45% (calculated from the area under the peak in comparison to that produced by the aqueous standard used to spike the milk sample).

The above cartridge clean up procedure as a whole was found to be unsatisfactory. As well as the inadequate clean up and poor recovery of antibiotic residue it was found that the preconditioning with solvents required to activate the cartridge was very slow. Boison (1992) reported that in order to obtain a >70% recovery of penicillin from such C_{18} cartridges a minimum carbon loading of >17% in the packing bed was required. As the carbon loading of the SPC cartridge was <14% the poor recovery of the penicillin could be accounted for.

2.3.2 Acetonitrile extraction.

Moats (1983) reported that treatment of milk samples with two volumes of acetonitrile was effective in precipitating most proteins and some lipids, with excellent recovery of penicillins. Moats also indicated that losses of analyte due to protein binding were minimal. However, organic solvents such as acetonitrile result in the co-extraction of large concentrations of polar and non polar milk matrix components with the compounds of interest, and further clean up is therefore required. This was performed by back extracting the penicillins into buffered solutions as detailed in section 2.3.3.

A preliminary investigation involving acetonitrile extraction without the buffer purification steps was initially undertaken. A milk sample (30ml) spiked with an aqueous solution of benzylpenicillin 5mgl^{-1} maintained at room temperature for 48 hours, was treated with two volumes of acetonitrile. The organic solvent was added at 5ml intervals with vigorous shaking after which the resulting solution was centrifuged at 2500 rpm (* see page xvii) for 10 minutes. The volume of the pale green supernatant layer was reduced to 30ml under reduced pressure, filtered through a 0.46μm nylon membrane and chromatographed under standard conditions.

The results obtained were similar to those previously obtained using the C_{18} cartridge as described in section 2.3.1. Large milk matrix interference occurred between retention times 1.95(min) and 9.50(min) thus making resolution of any degradation compounds impossible (with reference to fig. 2.2). Penicillin was resolved at t_r 24.56(min) with a recovery of 93%.

2.3.3. Acetonitrile extraction with liquid-liquid partition clean up.

The results obtained in the previous section indicated that further removal of interfering milk matrix components was essential for the resolution of penicillin degradation compounds to be achieved. Accordingly the complete procedure as described by Moats (1983) was undertaken.

Acetonitrile (40ml) was added in 2ml increments with vigorous shaking to pasteurised milk (20ml) spiked to a concentration of 10mg l^{-1} with an aqueous potassium benzylpenicillin solution. Following precipitation of the proteinaceous material, the pale green supernatant was

filtered through a plug of defatted cotton wool in the stem of a Pasteur pipette. The filtrate was then shaken with 0.2M phosphate buffer, pH 2.2, (5ml) and DCM (20ml). The low pH buffer effectively reduced the ionization of the carboxylate group on the thiozolidine ring, thus increasing its solubility in the organic phase and concentrating the analytes without the need for evaporation of large volumes of solvents. The lower DCM layer was collected and the upper aqueous layer extracted with an additional volume of DCM (10ml) which was combined with the initial organic phase. Petroleum ether (40-60°C) (40ml) was added to the DCM layer and the organic phase was washed twice with water (1ml) which was discarded. The organic layer was extracted 3 times with 0.01M phosphate buffer, pH 7.0, (1ml) to which diethyl ether (2ml) was added and the mixture vortexed for 10 seconds followed by centrifugation at 1000rpm for 1 minute. The ether was added to the organic phase to facilitate the extraction of the analytes into the aqueous buffered phase. The upper organic phase was discarded and residual ether removed under reduced pressure at 25°C. Saturated ammonium sulphate (3ml) and acetonitrile (2ml) was added to the buffered solution and the mixture was again vortexed and centrifuged at low speed for 1 minute. Ammonium sulphate was used to convert the penicillin to ammonium ions, which enhances its solubility in organic solvents. The acetonitrile layer was transferred to a clean round bottomed flask and combined with acetonitrile (2ml) which was used to extract the aqueous layer. Water (0.8ml) was added and the acetonitrile removed under reduced pressure. The final volume of the extract was adjusted to 1ml by the addition of water and 100µl aliquots injected onto the HPLC column under the standard conditions (See page 40). The procedure used is shown schematically in fig. 2.3.

The chromatogram produced from this procedure, shown in fig. 2.4, showed less interference than previous extractions. However, the recovery of penicillin was only 20% (calculated from the area under the antibiotic peaks on the aqueous standard and milk extraction traces taking into consideration the ×20 concentration factor). This poor recovery of antibiotic may have been caused by rapid degradation as a result of the addition of phosphate buffer, pH 2.2. Fletouris *et al.* (1992) reported a remarkable degradation of penicillin if milk extracts were acidified lower than pH 3.0. Therefore, a compromise must be reached between extraction efficiency and antibiotic stability considerations.

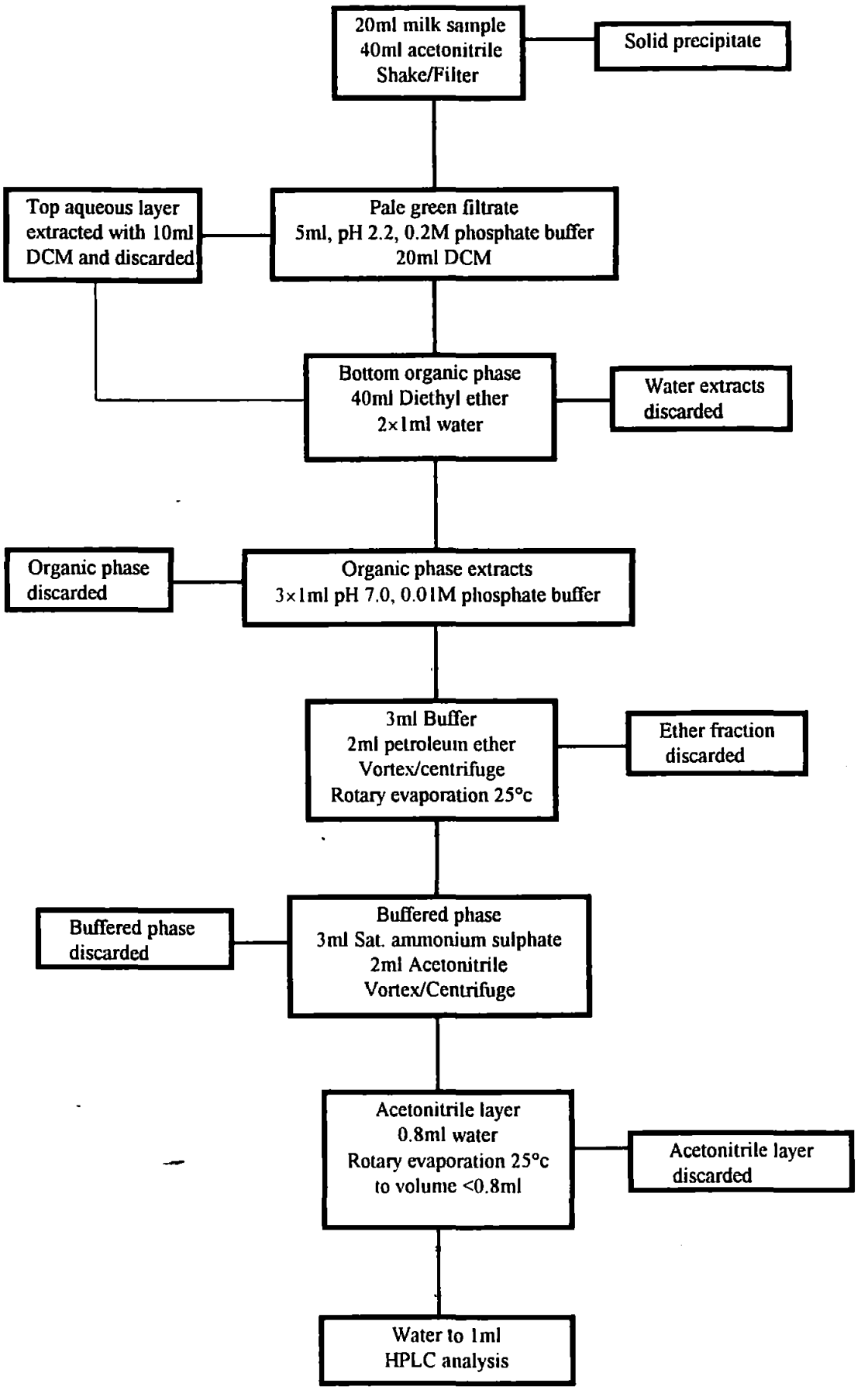


Fig. 2.3 Summary of the extraction procedure described in section 2.3.3. Acetonitrile extraction with liquid-liquid partition clean up devised by Moats (1983).

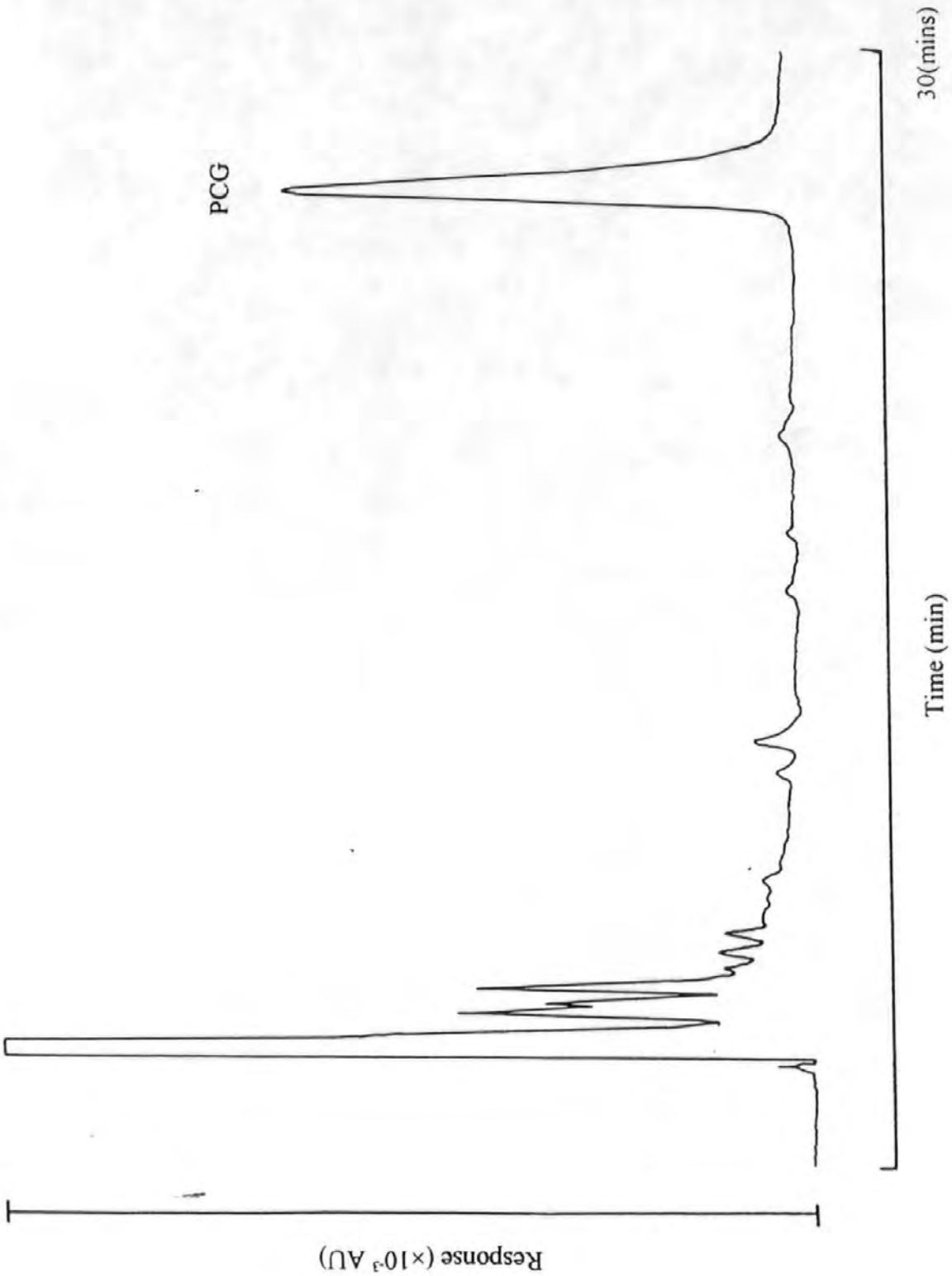


Fig. 2.4 Chromatogram obtained from an *in-vitro* milk sample inoculated with an aqueous solution of 10mg l^{-1} benzylpenicillin and extracted using the procedure reported by Moats (1983) and shown in fig. 2.3. The mobile phase consisted of 0.05M phosphate buffer:acetonitrile (83:17, pH 6.0) and benzylpenicillin (PCG) was resolved at t_r 26.35(min).

The experiment was repeated using an aqueous 10mg l^{-1} benzylpenicillin solution which had been incubated at room temperature for 48 hours, and therefore contained degradation compounds. The resultant HPLC traces showed no evidence of any degradation compounds which appeared to have been lost in the work-up. Consequently the method was abandoned.

2.3.4 DCM extraction with liquid-liquid partition clean up.

The method described by Fletouris *et al.* (1992) was attempted as an alternative. Pasteurised milk (100ml) was inoculated with an aqueous 1000mg l^{-1} solution of potassium benzylpenicillin (1ml) which had been incubated at 37°C for 48 hours. A sample of the spiked milk (7ml) was acidified to pH 3.0 with 0.6M sulphuric acid (1ml). DCM (30ml) was added and the suspension shaken vigorously for 2 minutes and centrifuged at 2500rpm for 10 minutes. Three distinct layers were formed, an upper yellow/green aqueous layer, a middle solid layer and a colourless organic lower fraction. An aliquot of the bottom layer (25ml) was removed using a Pasteur pipette and 0.05M, pH 7.0, phosphate buffer (2ml) added to it. The mixture was evaporated by reduced pressure at 25°C to a volume of 6ml, vortexed and centrifuged for 1 minute at 2000rpm. The aqueous upper layer was transferred to a clean vessel, and the lower DCM phase extracted twice with additional volumes of pH 7.0 buffer (2ml) and the aqueous buffer fractions combined. Saturated ammonium sulphate (1ml) and diethyl ether (3ml) were added to the buffered solution. The suspension was vortexed and centrifuged at 1000rpm for 1 minute. The ether layer was discarded using a Pasteur pipette and any residual ether removed by the introduction of a gentle nitrogen stream. The tube contents were extracted twice with acetonitrile (2ml) and centrifuged at 1000rpm for 1 minute. The combined top layers were mixed with 0.05M phosphate buffer, pH 7.0, (3ml) and the volume reduced to 3ml under reduced pressure. 0.5M aqueous TBA-HSO_4 (0.5ml) was then added to the buffered solution to convert the compounds of interest into ion pairs. This was performed at pH 7.0 since at this pH penicillins are completely ionized and ion pair formation is at a maximum. The conversion of the analytes to ion pairs should afford further clean up of samples since they are readily extracted into chloroform (5ml). The buffered solution and chloroform suspension was shaken vigorously for 2 minutes and centrifuged at 1000rpm for 1 minute. A fraction of the lower organic layer (4.5ml) was separated and evaporated to dryness. Fletouris *et al.* (1992) reported that the formation of ion pairs allowed evaporation of the sample to dryness without any degradation of the antibiotic material. The

sample was redissolved in mobile phase (1ml) and analysed by HPLC. The extraction procedure followed is summarised in fig. 2.5.

The resultant chromatogram is shown in fig. 2.6. The recovery of benzylpenicillin was 73%. However, no degradation compounds were evident and chromatograms still suffered from interference from milk matrix components at retention times expected for the penicillin degradation compounds from previous experiments.

2.3.5 Investigation into the solubility of benzylpenicillin and its degradation compounds in relevant solvents.

From work conducted in sections 2.3.3 and 2.3.4 it was apparent that despite benzylpenicillin and its known degradation compounds being carboxylic acids the latter do not survive the extraction methods employed. An aqueous 10mg l^{-1} solution of potassium benzylpenicillin (5ml) was incubated at 37°C for a period of 24 hours and used in the extractions shown in Table 2a. In each case a volume (100µl) of the aqueous phase was subjected to the usual HPLC conditions.

Table 2a. The solutions used to extract the aqueous degraded penicillin sample to determine the solubility of the degradation compounds in relevant solvents.

Solution Number	Solvents added	pH
1	5ml DCM	6.32
2	5ml DCM	0.1M H_3PO_4 to 2.0
3	5ml Diethyl ether	6.34
4	5ml Chloroform/1ml TBA-HSO_4	6.37

Neither penicillin or its degradation compounds were found to be soluble in DCM at pH 6.3 because ionization at the carboxylic acid moiety on the thiazolidine ring would be near maximum. When the pH of the aqueous phase was reduced to pH 2.0 rapid degradation of the antibiotic occurred and even at such a low pH the suppression of the ionization of the

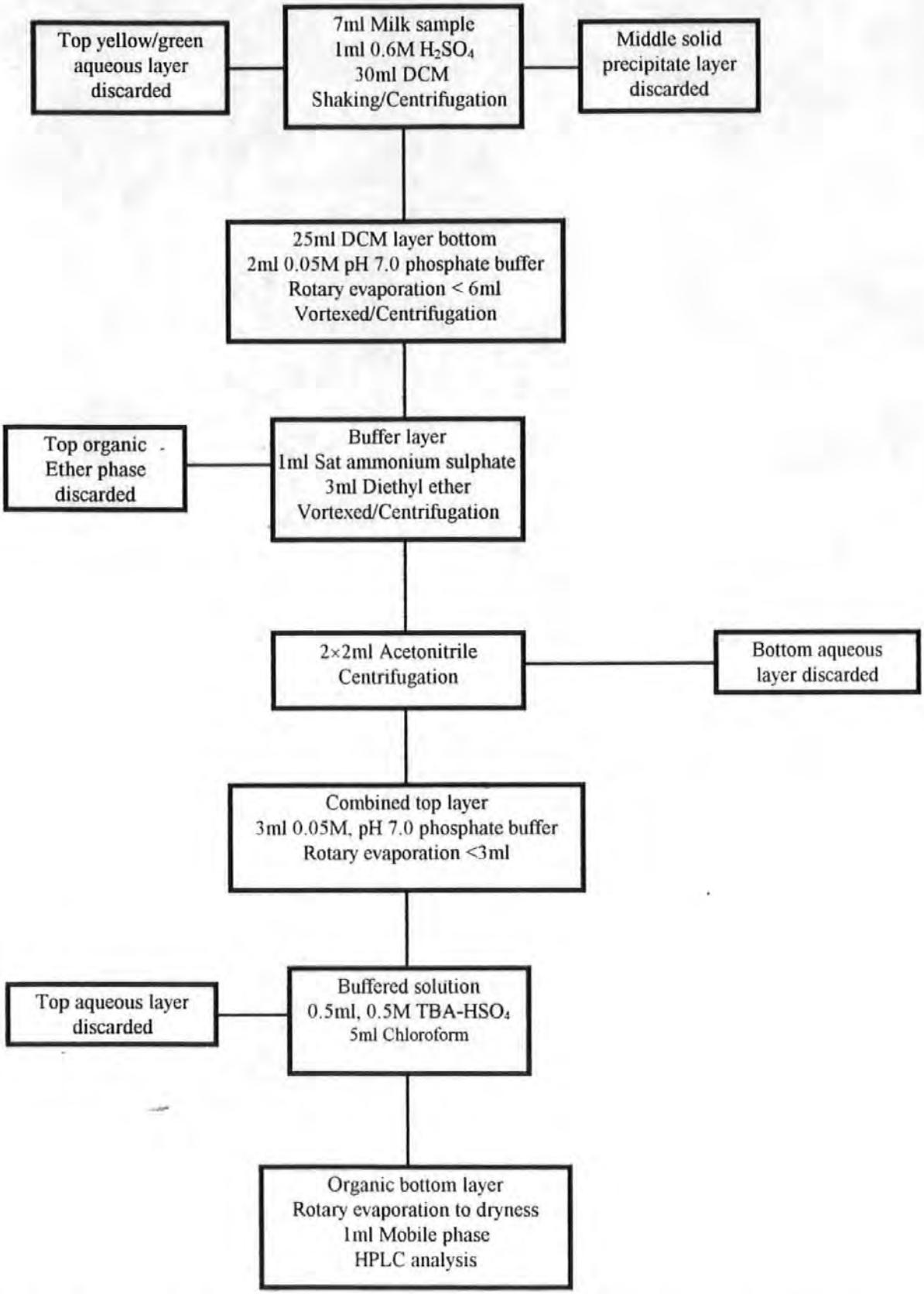


Fig. 2.5 Summary of the extraction procedure as reported by Fletouris *et al.* (1992) and as described in section 2.3.4.

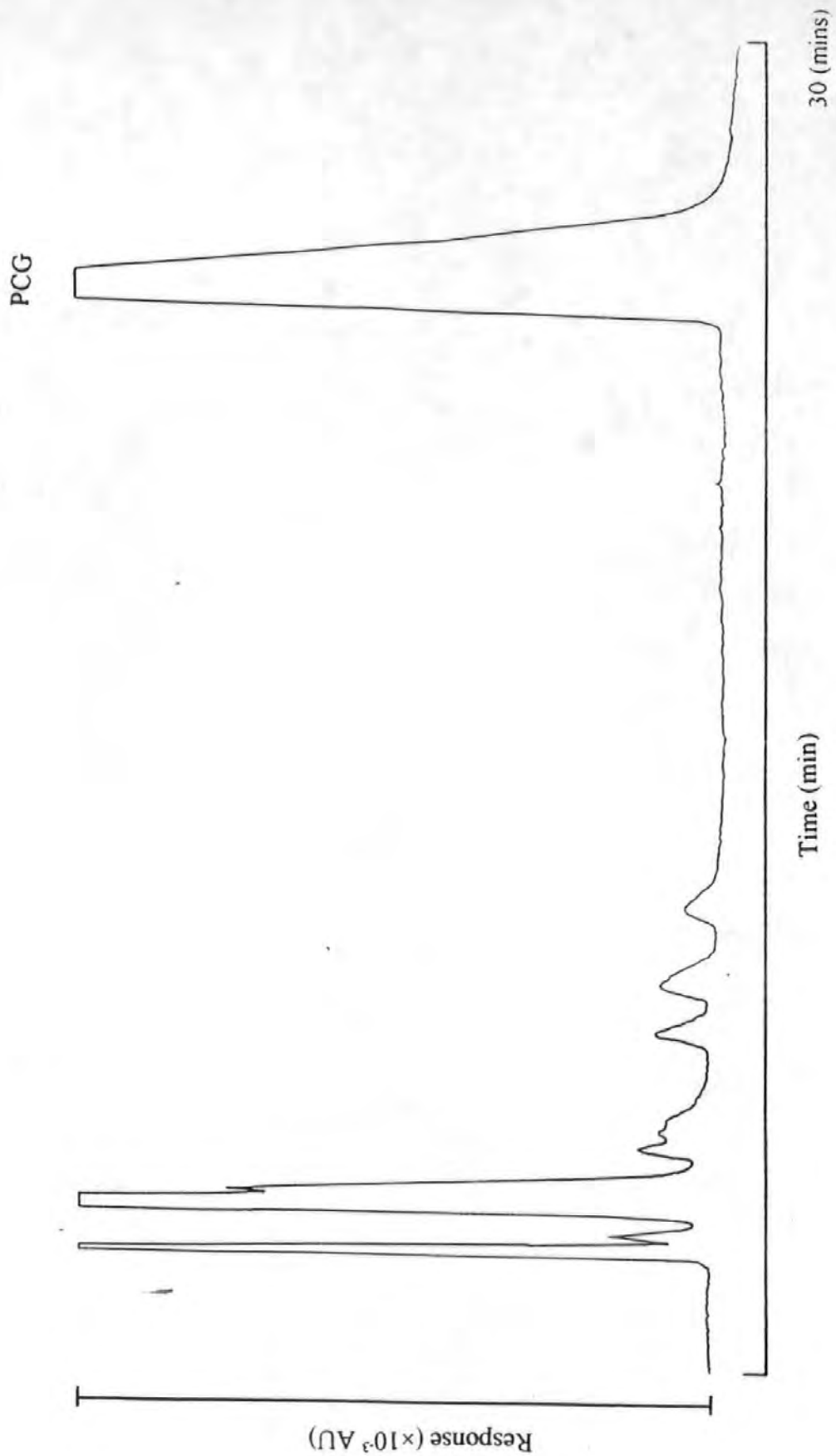


Fig. 2.6 Chromatogram obtained from an *in-vitro* milk sample inoculated with an aqueous solution of 1000 mg l^{-1} benzylpenicillin (1ml) incubated at 37°C for 20 hours and extracted using the procedure reported by Fletouris *et al.* (1992). The mobile phase consisted of 0.05M phosphate buffer:acetonitrile (83:17, pH 6.0). Benzylpenicillin was resolved at tr 25.95(min).

degradation residues was insufficient to render them soluble in the DCM phase. No peaks were evident for the penicillin and since complete acid hydrolysis in such a short time was unlikely, it was concluded that it had been extracted into the DCM organic phase. Thus it appears that the critical point of the procedures described by Moats (1983) and Fletouris *et al.* (1992), is the DCM phase extraction. The penicillin is soluble but its degradation compounds are insoluble in the organic solvent, even at low pH. Neither penicillin or its breakdown residues were soluble in diethyl ether. The antibiotic was completely soluble in chloroform when converted into ion pairs with TBA-HSO_4. However, the degradation compounds were insoluble.

The results reported above explain the reasons for failure of the extraction techniques used in sections 2.3.3 and 2.3.4 since both methods involved a DCM partition step. Consequently the procedures were abandoned.

2.3.6 Aqueous extraction with solid phase clean up.

A procedure reported by Terada *et al.* (1985) is summarised in fig. 2.7. The authors used sodium tungstate, sulphuric acid and water to extract and remove protein from animal tissues and indicated that the method was effective for the isolation of penicillins from milk and other dairy products. As mentioned previously, water extracts fewer milk matrix constituents than organic solvents such as acetonitrile. Consequently only water soluble polar components are extracted from the milk matrix offering more scope for the detection of penicillins, especially the more polar residues, from endogenous material.

The following general procedure was used. Samples (10ml) were mixed with 5% sodium tungstate (10ml) and 0.33M sulphuric acid was added to adjust the pH to 4.0. Following vigorous shaking the solution was centrifuged at 2000rpm for 10 minutes. Any proteins in the sample were denatured and remained at the liqiud liquid interface. The supernatant (20ml) was passed through a basic aluminium oxide column, previously prepared by placing 18g of material into a 30cm×15mm i.d. glass column fitted with a sintered disc to retain the stationary phase, and washed with water (100ml). The solution was allowed to drain by gravity, after which the column was eluted with water (10ml). The colourless eluent (approximately 38ml) was extracted

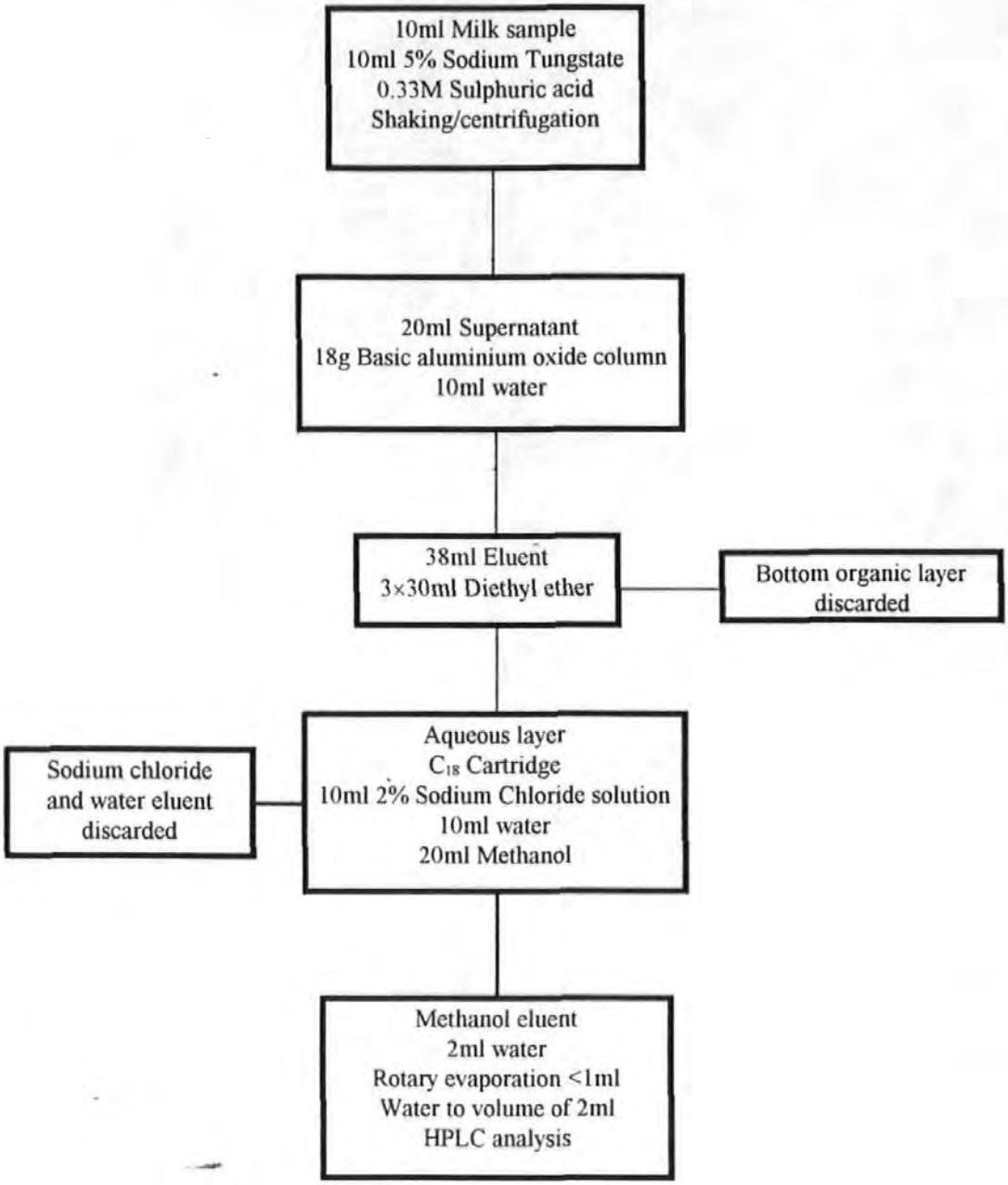


Fig. 2.7 Summary of the extraction method described in section 2.3.6. Aqueous extraction involving solid phase C_{18} and liquid liquid partition clean up as reported by Terada *et al.* (1985).

3 times with diethyl ether (30ml), the organic layers discarded and the aqueous layer combined with 2% sodium chloride solution (10ml).

Using a 20ml glass syringe, the salt solution was introduced onto a silica-Carte C_{18} cartridge which had been preconditioned with methanol (20ml), water (20ml) and 2% sodium chloride solution (2ml). Once the sample had been introduced, the cartridge was washed with 2% sodium chloride solution (10ml) and water (10ml) and then eluted with methanol (20ml). Water (2ml) was added to the methanol eluent and the volume of the solution reduced to approximately 1ml under reduced pressure at 25°C. The volume of the extract was adjusted to 2ml with water and chromatographed using the standard HPLC conditions.

The results obtained when an aqueous 10mg l^{-1} sample of benzylpenicillin, incubated at 37°C for 48 hours, was subjected to the procedure described above are shown in fig. 2.8. Both penicillin and its degradation compounds survived the extraction. However, recoveries were poor (ranging between 10-32% for the degradation compounds) and considerable disturbance was caused by the injection, possibly due to residual methanol in the final extract. A considerable improvement was achieved using this method, since the resultant chromatogram showed that both penicillin and the majority of its degradation compounds had survived the extraction procedure. The method was then repeated on a milk sample spiked with an aqueous solution of 10mg l^{-1} penicillin previously incubated at 37°C for 72 hours. However the presence of degradation compounds was obscured by matrix effects.

2.4 Evaluation of HPLC as a method for the detection of benzylpenicillin and its degradation compounds from milk.

The results obtained using HPLC as a separation and detection tool for penicillin breakdown compounds from *in*-vitro milk samples were disappointing. The degradation products proved to be far more polar than the antibiotic residue and as a consequence could not be partitioned into organic solvents. Milk samples provided a matrix with too many interfering substances for HPLC to resolve the analytes, despite clean up methods aimed at removing protein and lypophilic substances. Liquid-liquid partition methods provided a better clean up of milk samples than solid phase extractions with resultant chromatograms showing less matrix interference.

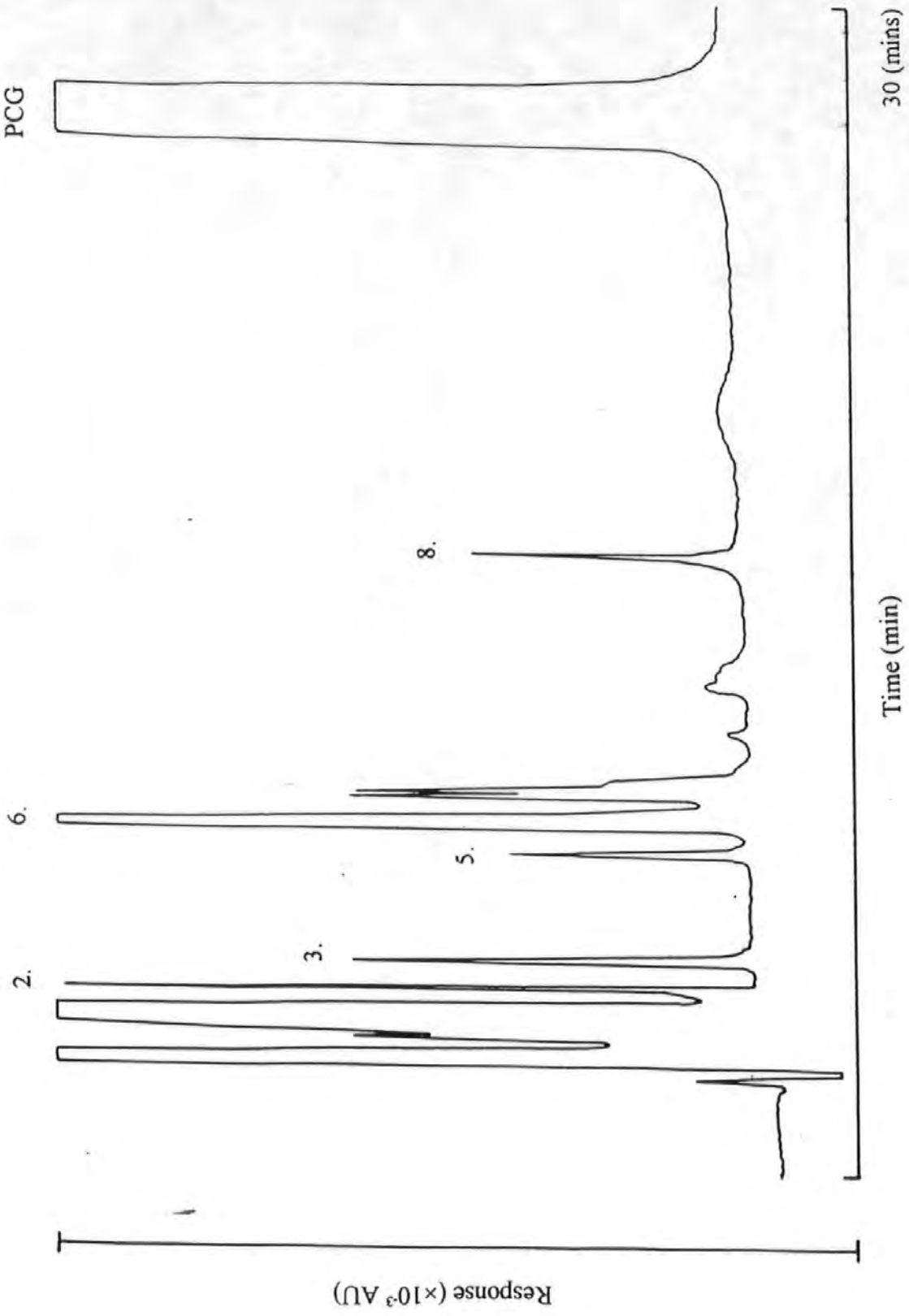


Fig. 2.8 Chromatogram obtained from the extraction of an aqueous 10mg l^{-1} benzylpenicillin sample incubated at 37°C for 20 hours as reported by Terada *et al.* (1985). The mobile phase used was comprised of 0.05M phosphate buffer:acetonitrile (83:17, pH 6.0). Benzylpenicillin (PCG) was resolved at t_r 26.83(min)and the peaks labelled correspond to the penicillin degradation compounds indicated in fig. 2.2.

Various solvents were utilised but the compounds of interest did not survive these procedures. The C_{18} solid phase purification methods failed to provide the necessary window in the milk matrix interference for the detection of penicillin degradation compounds.

Chapter 3

Capillary Electrophoresis Methods and Results

3.0 Evaluation of Capillary electrophoresis as an alternative to HPLC as a method for the detection of benzylpenicillin and its degradation compounds from milk.

The high separation efficiencies, often exceeding 100,000 plates, achievable by CE make it an ideal technique for the analysis of compounds in complicated food matrices. Bobbitt and Ng (1992) reported that the numerous separation modes and mechanisms provided by CE will allow this technique to be used for applications not possible with more conventional chromatographic approaches. Since column efficiencies can be very high in CE, small selectivity differences can provide adequate separations. Relatively poor limits of detection (LOD), due to the narrow optical pathway defined by the capillary, are the biggest restriction to the growth of CE (Altria and Rogan, 1994). When used at an equivalent wavelength it may be necessary to use 2-5 times more concentrated samples for CE to achieve a comparable LOD to HPLC.

The capital costs of HPLC and CE are similar. However, CE consumes limited volumes of reagents and uncoated capillaries are a fraction of the cost of HPLC columns. This makes CE a financially attractive alternative to HPLC, especially when analysing a complex matrix such as milk which contains many compounds that could irreversibly damage costly packing materials. The capillaries used in CE can tolerate injection of sample solutions containing a variety of matrices at a wide pH range. Any material remaining in the capillary can be removed by flushing between analyses. HPLC and CE are both quantitative techniques that can give accurate results; however, a perceived disadvantage of CE which was observed in subsequent experimental work herein, is poor precision. However, this can be compensated for by the use of an internal standard.

A particularly attractive feature of CE is the short set-up time and readily available automation facilities. Buffer vials and capillaries may be changed in seconds - comparing favourably with the removal of HPLC columns and changing mobile phase composition. CE with all of its modes also provides a technique where a wide range of analytes can be separated and the use of low UV wavelengths and indirect UV detection allows the analysis of compounds with less active chromophores. The relative merits of HPLC and CE identified for drug analysis are given in Table 3a (Altria and Rogan, 1994).

Table 3a. Comparison between HPLC and CE for drug analysis (from Altria and Rogan, 1994).

Parameter	HPLC	CE
Practical considerations		
Selectivity options	+++	+++
Preparative operation	+++	+
Solubility restrictions	+++	++
Speed of analysis	+++	+++
Method development	++	+++
Analyte range	++	+++
Operational pH range	++	+++
Detection options	+++	+[a]
Set-up time	++	+++
Performance		
Separation efficiency	++	+++
Accuracy	+++	++
Sensitivity	+++	++
Precision	+++	+[b]
Ruggedness	++	++
Linearity	+++	+++
Method validation\transfer	++	++
Economic considerations		
Simplicity	++	+++
Sample pre-treatment requirements	++	+++
Operation costs	++	++++
Training requirements	++	++
Equipment costs	++	++

a. A large number of detector options have been reported but have yet to be commercialised.
b. Injection precision is considerably improved when using an internal standard.

3.1 Capillary electrophoresis. Instrumentation and conditions.

MECC was performed using a Dionex CES capable of operating at up to 100KV and incorporating on line UV/VIS detection and autosample injection. Fused silica capillary tubes of dimensions 670mm×75µm i.d. (Dionex UK) were used for all analyses. The polymer coating on the capillary tube was removed for UV detection by heat treating a 1cm section 40mm from the cathodic end. The distance that the capillary was inserted into the

detector was 50mm and marked by a cellotape flag. A Dionex A1-450 integrator was used for all data collection and manipulation.

Initial injections were made at a potential difference of 15KV under gravity, with the anodic end of the capillary being immersed in the sample vial for 45 seconds after being raised to a height of 100mm above the detector end. A detector setting of 210nm and 0.01a.u.f.s. were used for all measurements. Electrolyte solutions were filtered through a 0.46μm nylon membrane and degassed under vacuum in Millipore glassware prior to use.

3.2 Determination of the optimum system for the detection and separation of benzylpenicillin and its degradation compounds from aqueous solution.

The buffer, known as the carrier or background electrolyte, provides precise pH control and must be of adequate capacity to resist minor pH fluctuations. A 0.06M sodium borate buffer, of pH 9.0, was used since it has a pK_a of 9.14. Operating at pH values distant from the pK_a can cause excessive conductivity because the buffer component may be fully ionised (Weinberger, 1993). A borate buffer was chosen because of its UV transparency at 210nm and its known usefulness in separating carbohydrates, a major component of the milk matrix (Weinberger, 1993). A high concentration of buffer (0.06M) was necessary to minimise adsorption of milk matrix proteins onto the capillary wall. The added salts probably fill potential adsorption sites (Green and Jorgenson, 1989). The buffer cation also plays a crucial role in the final conductivity of the electrolyte, with lithium and sodium salts being used in preference to potassium since they contribute less to the buffer conductivity.

SDS (0.06M) was the surfactant used for MECC experimental work, being available at high purity and moderate cost. It is also known to be effective in protein and lipid separations (Weinberger, 1993). A high SDS concentration (0.06M) was chosen, almost twice its CMC of 8.1×10^{-3} M, to provide longer solute migration times in an attempt to obtain a better separation by increasing the partitioning of solutes into the micelle.

The pH 9.0 buffer was prepared by titrating the required volume of 0.06M boric acid to 0.06M sodium tetraborate. 0.06M of SDS was added to this solution and the electrolyte

filtered and degassed as reported previously. The solutions were filtered using previously warmed Millipore glassware to reduce the likelihood of precipitation of the SDS surfactant which was observed in temperatures <5°C.

A freshly prepared aqueous 10mgl^{-1} penicillin standard analysed according to the above conditions gave a sharp peak at a migration time of t_m 14.32(min) with a LOD of 0.8mg l^{-1} as shown in fig 3.1.

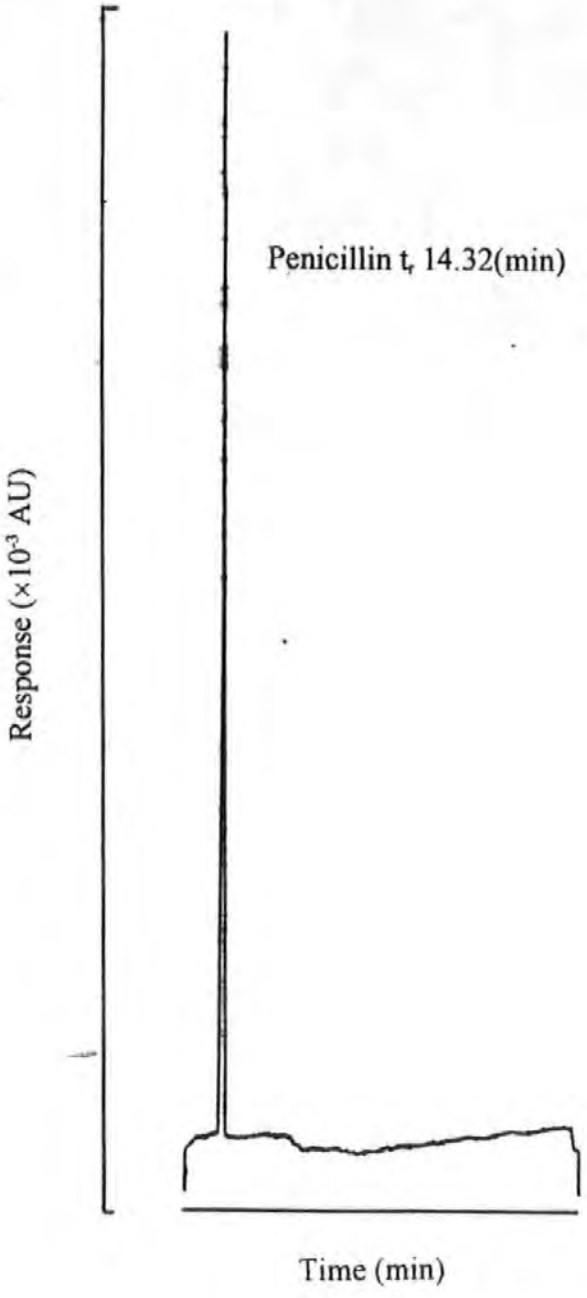


Fig 3.1 Electropherogram obtained from the gravity injection (45 seconds, 100mm) of an aqueous benzylpenicillin standard (10mg l^{-1}) using an electrolyte of 0.06M SDS, 0.06M borate buffer, pH 9.0. The antibiotic was observed at a migration time of t_m 14.32 (min).

3.2.1 Investigation into the effect of adding an organic modifier to the electrolyte.

Organic solvents such as methanol and acetonitrile may be used to modify the migration range of analytes. The use of a solvent in CE may be considered in an analogous way to that in liquid chromatography, as a means of adjusting the partition coefficient of the analytes between the different chromatographic phases (Weinberger, 1993). The result is a reduction in the EOF and is limited by its impact on the micelle aggregate. A concentration of solvent over 25% may cause sufficient disturbance to completely destroy the micelle thus changing the mode of separation to CZE.

Three separate 0.06M sodium borate electrolytes (pH 9.0, 0.06M SDS) were prepared containing 0, 15 and 30% methanol respectively. The detector sensitivity was reduced to 0.1a.u.f.s. as analyses were performed on a fresh aqueous 1000mg l^{-1} penicillin sample. The electropherogram produced by the carrier electrolyte containing 30% methanol showed the penicillin migrating as a broad peak with excessive fronting at a retention time of 32.20(min). The fronting was a consequence of capillary overloading. With the methanol concentration at 30% the electrophoresis mode should be CZE with the SDS aggregates completely disturbed by the elevated methanol content. When the solvent content was reduced to 15% a much sharper penicillin peak was resolved at a migration time of 21.89 (min). However, the most favourable peak symmetry, and therefore the better LOD of 0.8mg l^{-1}, was obtained when the carrier electrolyte contained no methanol as the penicillin appeared at t_m 14.63(min) as a very sharp peak with minimal fronting.

3.2.2 The effect of adding phosphate to the background electrolyte.

Phosphate ions, when added to the carrier electrolyte, affect the conformation of proteins which can impact on the separation (Terabe, 1992[a]). They bind to the capillary wall reducing protein interaction with the ionic silanol groups which would decrease electrophoretic activity (McCormick, 1988). The carrier electrolytes indicated in Table 3b were prepared to pH 9.0 and used in the analysis of a fresh aqueous 50mg l^{-1} penicillin standard. Phosphate-borate blended buffers were prepared by titrating the required volume of sodium dihydrogen orthophosphate to sodium tetraborate.

Table 3b. Four different background electrolytes prepared to pH 9.0 containing 0.06M SDS and used in the analysis of a 50mg l^{-1} penicillin standard.

Solution number	Buffer blend	SDS
1	Sodium borate\boric acid 0.06M	0.06M
2	Sodium borate\Sodium dihydrogen Orthophosphate 0.06M	0.06M Sonicated (30 minutes)
3	Sodium borate\Sodium dihydrogen Orthophosphate 0.02M	0.06M Sonicated (15 minutes)
4	Sodium borate\Sodium dihydrogen Orthophosphate 0.02M	0.05M

Buffer 1 produced a sharp, well defined peak at a migration time 14.56(min). Electrolyte 2 could not be used because the SDS was insufficiently soluble in the buffer solution and when the buffer concentration was reduced to 0.02M, as in solution 3, traces showed disturbed baselines. When the SDS concentration was reduced to 0.05M, as in electrolyte 4, the penicillin was resolved as a narrow peak with excellent symmetry at a migration time of 14.89(min). There was, however, no difference between the results achieved using electrolytes 1 and 4 and it was decided to use the former solution in future experiments since the high concentration of buffer also minimises interactions between solutes and the capillary wall.

3.2.3 Reduction of the operating voltage.

Power to a MECC system may be controlled by one of two ways. Either the voltage can be fixed and the current allowed to fluctuate according to the resistance of the buffer, or alternatively the current can be fixed. All work reported in the following chapters, and most cited in the literature, used constant voltage as the control. The applied voltage should be maintained at a level that is not too high which may cause excessive current. If the voltage

in use is in excess of 30KV it is advisable to control the capillary temperature by blowing nitrogen over the capillary.

A carrier electrolyte of 0.06M sodium borate, pH 9.0, 0.06M SDS was used in the analysis of an aqueous 100mg l^{-1} penicillin standard that had been incubated at 37°C for 96 hours. Measurements were taken at operating voltages of 15KV, as used in previous analyses, and 10KV. The results shown in fig. 3.2 indicate that the lower voltage of 10KV provided a better separation of three major benzylpenicillin degradation compounds from the antibiotic residue. The position of the penicillin, shown in fig 3.2 by the broken line, was found by co-injection of the degraded sample with an aqueous solution of freshly prepared benzylpenicillin (100mg l^{-1}). Despite the peaks being slightly broader a voltage of 10KV was used in subsequent measurements.

In comparison with HPLC, the MECC system described above separated and detected 5 fewer penicillin degradation compounds from aqueous solution at the same wavelength. This was considered to be a consequence of the CE technique having poorer sensitivity due to the reduced sample size available for detection. To prove this, a sample of 1000mg l^{-1} was allowed to degrade under standard conditions and electrophoresed. A total of 8 products were observed. However, since five of these were minor, attention was consequently focused on the 3 major ones.

3.3 The development of an efficient extraction and clean up procedure for the detection of benzylpenicillin and its degradation compounds from *in-vitro* milk samples for analysis by MECC.

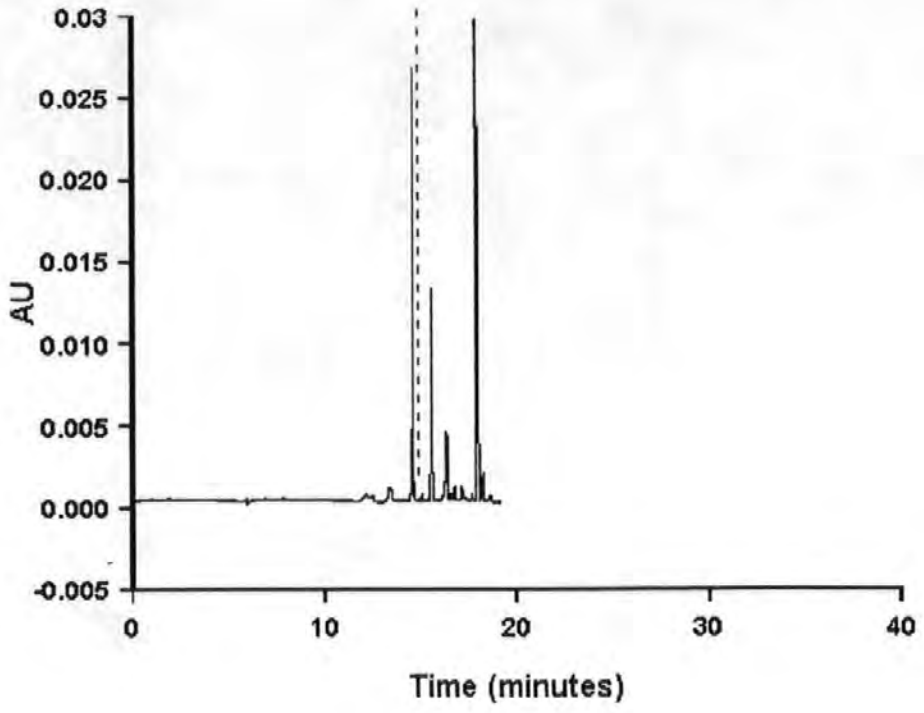
3.3.1 Acetonitrile extraction.

The following three solutions were prepared:-

(a) Milk Blank. Pasteurised milk (90ml) added to water (10ml).

(b) Milk standard (100mg l^{-1}). Pasteurised milk (90ml) added to an aqueous 1000mg l^{-1} solution of penicillin (10ml) that had been incubated at 37°C for 96 hours

(a)



b)

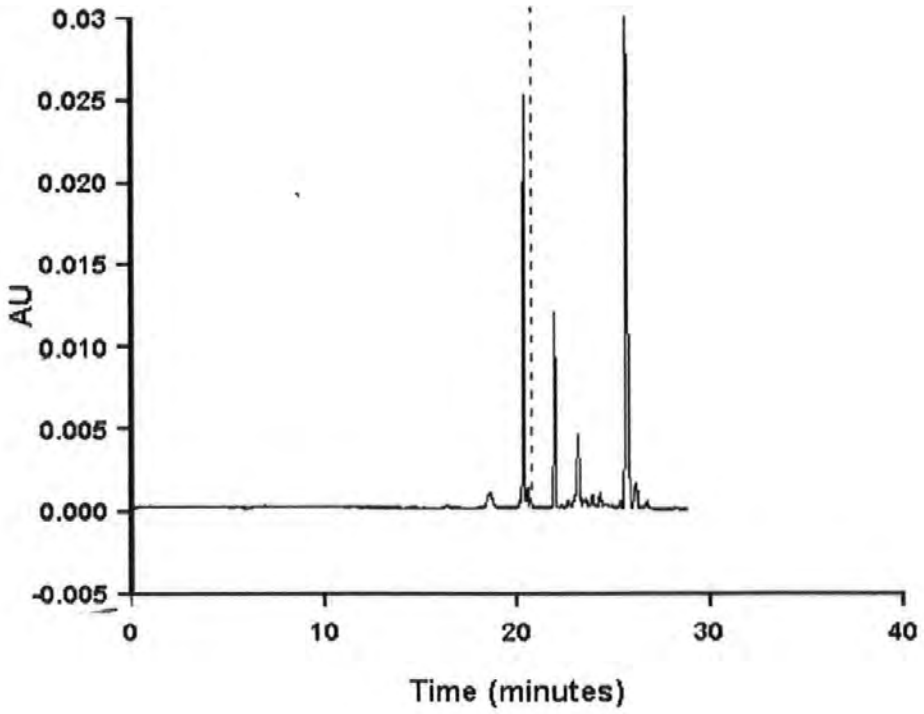


Fig 3.2 Electropherograms obtained from an aqueous standard of 100mg l^{-1} penicillin incubated at 37°C for 96 hours using a carrier electrolyte of 0.06M sodium borate pH 9.0, 0.06M SDS at operating voltages of (a) 15KV and (b) 10 KV. The position of benzylpenicillin is indicated on both electopherograms by the dotted line.

(c) Aqueous standard (100mg l^{-1}). The aqueous 1000mg l^{-1} stock solution (10ml) used to prepare solution (b) was added to water (90ml).

Acetonitrile (10ml) was added in 1ml increments with vigorous shaking to milk samples (a) and (b) (5ml). The solutions were then centrifuged at 2500rpm for 20 minutes, and the pale green supernatant filtered through a plug of defatted glass wool in the stem of a Pasteur pipette. The eluents were electrophoresed under standard MECC conditions and the trace obtained for the spiked milk sample is shown in fig 3.3. Despite the ×2 dilution factor incurred when extracting the milk standard with acetonitrile, three peaks, labelled 1, 2 and 3 in fig. 3.3, were observed that were not evident on the control milk blank.

The electropherogram produced by the aqueous standard (c) was similar to that shown in fig 3.2b with three major degradation compounds evident. The migration times of these peaks however, were not comparable to those on the milk standard electopherogram because of the presence of acetonitrile in the final injection. The procedure could therefore not be used for routine measurements of penicillin degradation compounds in milk samples because of excessive matrix interference and poor analyte recoveries. However, it showed that the MECC method offered the capacity for detection of such analytes from milk if further concentration and purification steps were implemented.

3.3.2 Acetonitrile extraction with liquid liquid partition and solid phase clean up.

As indicated by the results in the previous section, after acetonitrile extraction the milk sample required further clean up and concentration. For this Amberchrom-161m, a high performance version of Amberlite XAD-16 resin, was used. This resin has been used in the purification of small molecules such as peptides and synthetic organics (Laganà *et al.*, 1994; Mañes *et al.*, 1993). It is a nonionic polymer and has properties that provide high selectivity and stability over a large pH range. The Amberchrom resin was activated prior to use by the addition of 1g to 50ml of IPA:water solution (30:70) for a minimum of two hours. The wetted resin was poured into a glass column (15mm×9mm) fitted with a sintered disc and protected by a plug of glass wool. The resin was allowed to settle as the IPA:water solution drained and a plug of glass wool was placed on the top of the column.

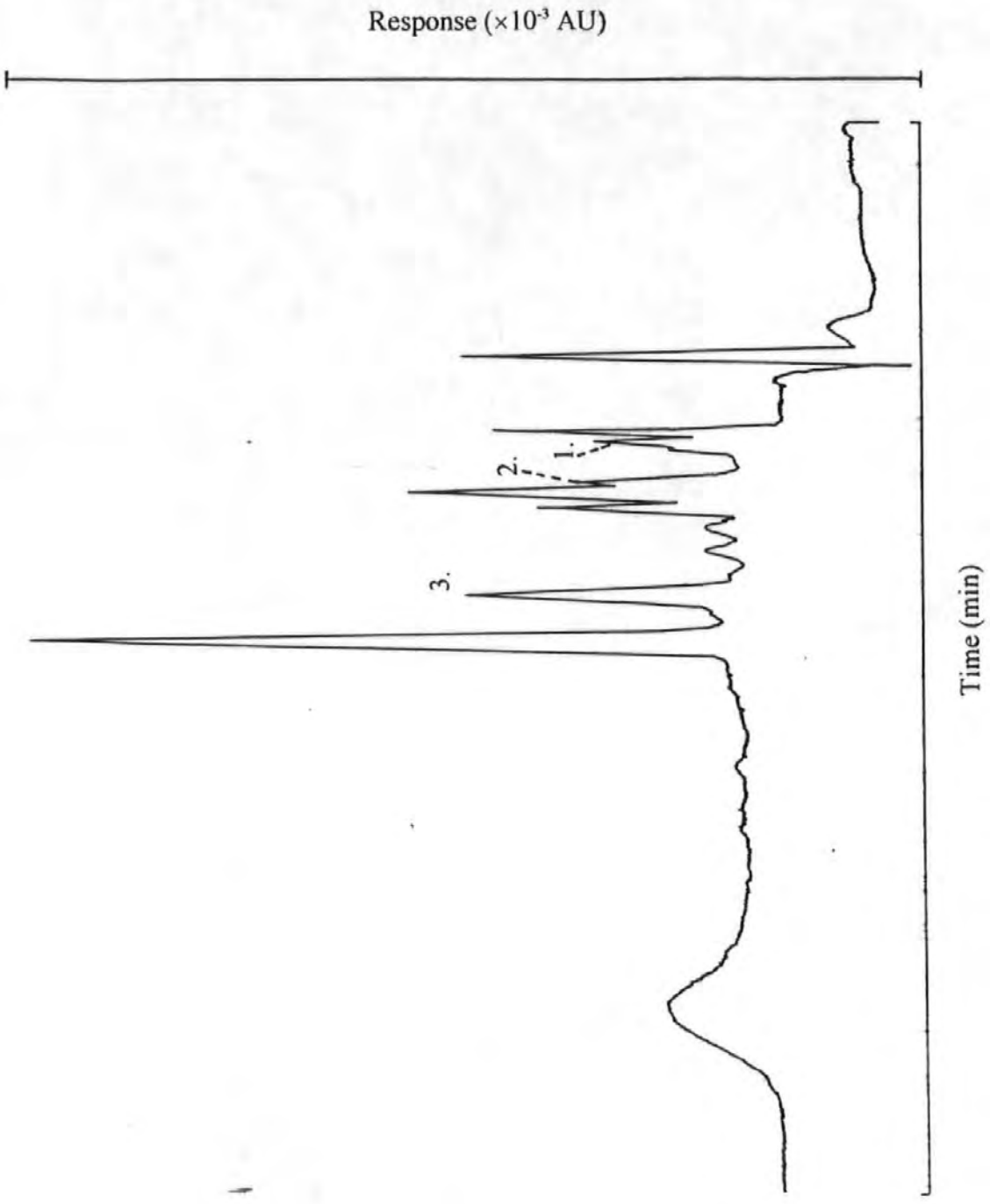


Fig 3.3 Electropherogram obtained from a milk sample spiked to a concentration of 100mg l^{-1} with a solution of penicillin incubated at 37°C for 96 hours and extracted with 2 volumes of acetonitrile. Peaks 1-3 are the major benzylpenicillin degradation compounds not evident on the control.

The column was preconditioned with methanol (30ml) and water (30ml) before use. The glass column was fitted with quickfit adapters, as shown in fig. 3.4, so that a vacuum could be applied to dry the resin packing material.

A pasteurised milk sample was spiked to concentration of 100mg l^{-1} with an aqueous solution of penicillin that had been previously incubated at 37°C for 3 days. An aliquot of the milk sample (4ml) was measured into a stoppered 30ml centrifuge tube and acetonitrile (8ml) gradually added with vigorous shaking. The suspension was sonicated for 10 minutes to homogenize fat globules thereby assisting the recovery of analytes bound in the fat. Following centrifugation at 2500rpm, the pale green supernatant (approximately 10ml) was extracted with DCM (5ml). The top aqueous layer (1.5ml) was removed using a Pasteur pipette and the volume adjusted to 100ml with water. The sample was then applied to the top of the Amberchrom column and allowed to percolate through under gravity. The column was washed with water (10ml), dried by drawing air through it for 1 minute under vacuum and eluted with methanol (10ml). Water (1ml) was added to the eluent and the volume of the solution reduced to approximately 0.5ml by rotary evaporation at 25°C. Water was again added to adjust the final volume to 1.5ml. This purification technique, summarised in fig. 3.5, provided resolution of the three major degradation compounds of benzylpenicillin. However, as shown on the resultant electropherograms in fig. 3.6 the breakdown compound labelled (b) was obscured by a small interfering milk matrix peak which would prevent detection of low concentrations. Recoveries of 25, 54 and 35% (calculated by comparing the areas under each peak to those obtained from an aqueous 100 mgl^{-1} standard produced from the same stock solution as used to spike the milk samples) for the three breakdown compounds was disappointing with losses possibly incurred with the addition of DCM to the acetonitrile extract; the partial miscibility of the two solvents may have resulted in loss of analytes to the organic phase. Recoveries may also have been adversely affected by the large volume of extract introduced onto the column with compounds being eluted before the methanol wash.

The procedure detailed above provided adequate clean up of milk samples for the detection of penicillin and the majority of its degradation compounds. However, resolution of the breakdown compound labelled (b) in fig 3.6 was affected by matrix components and recoveries of analytes were poor. Consequently the method was abandoned.

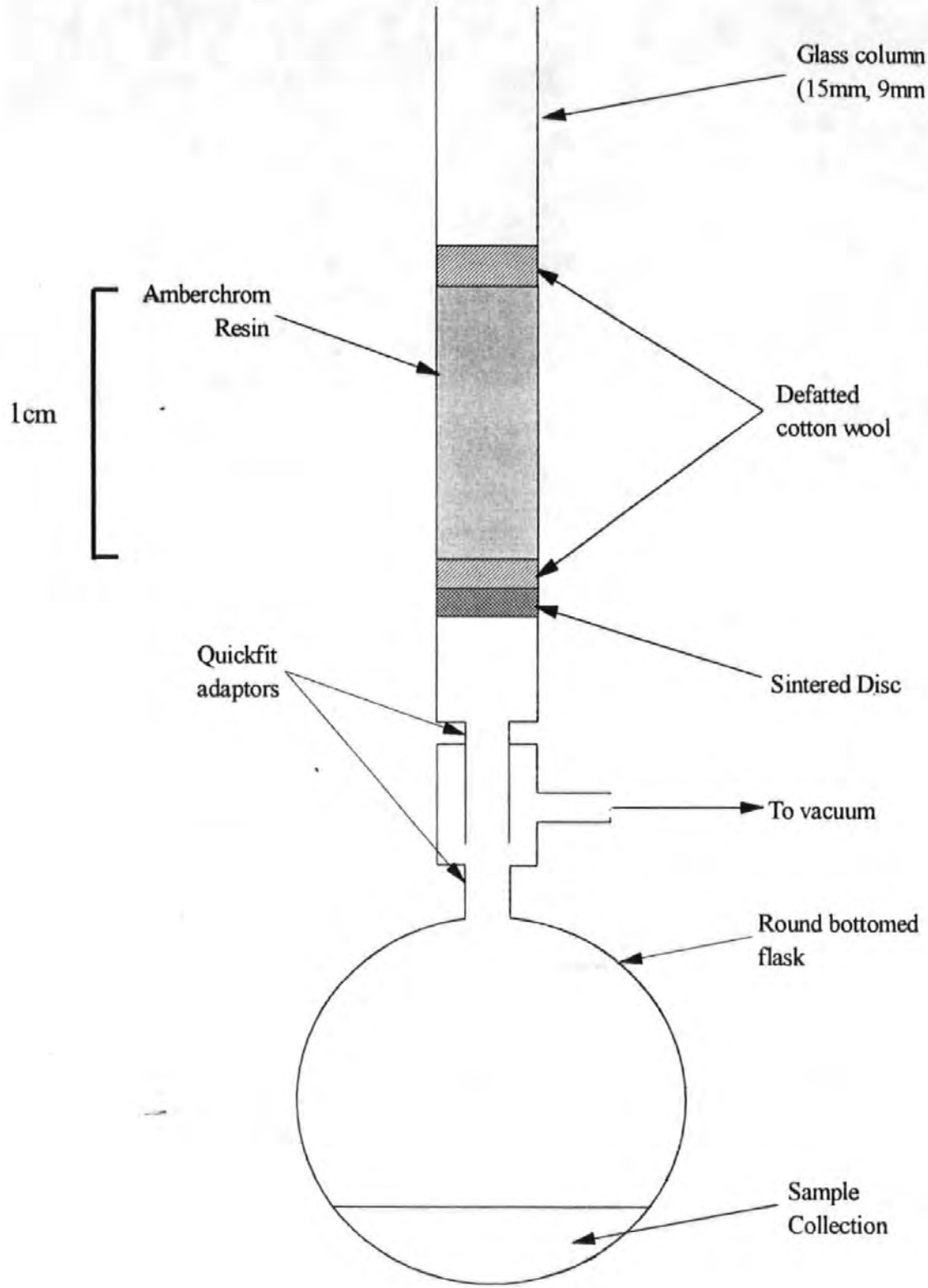


Fig. 3.4 Diagram to show the Amberchrom extraction column used in the purification of milk samples.

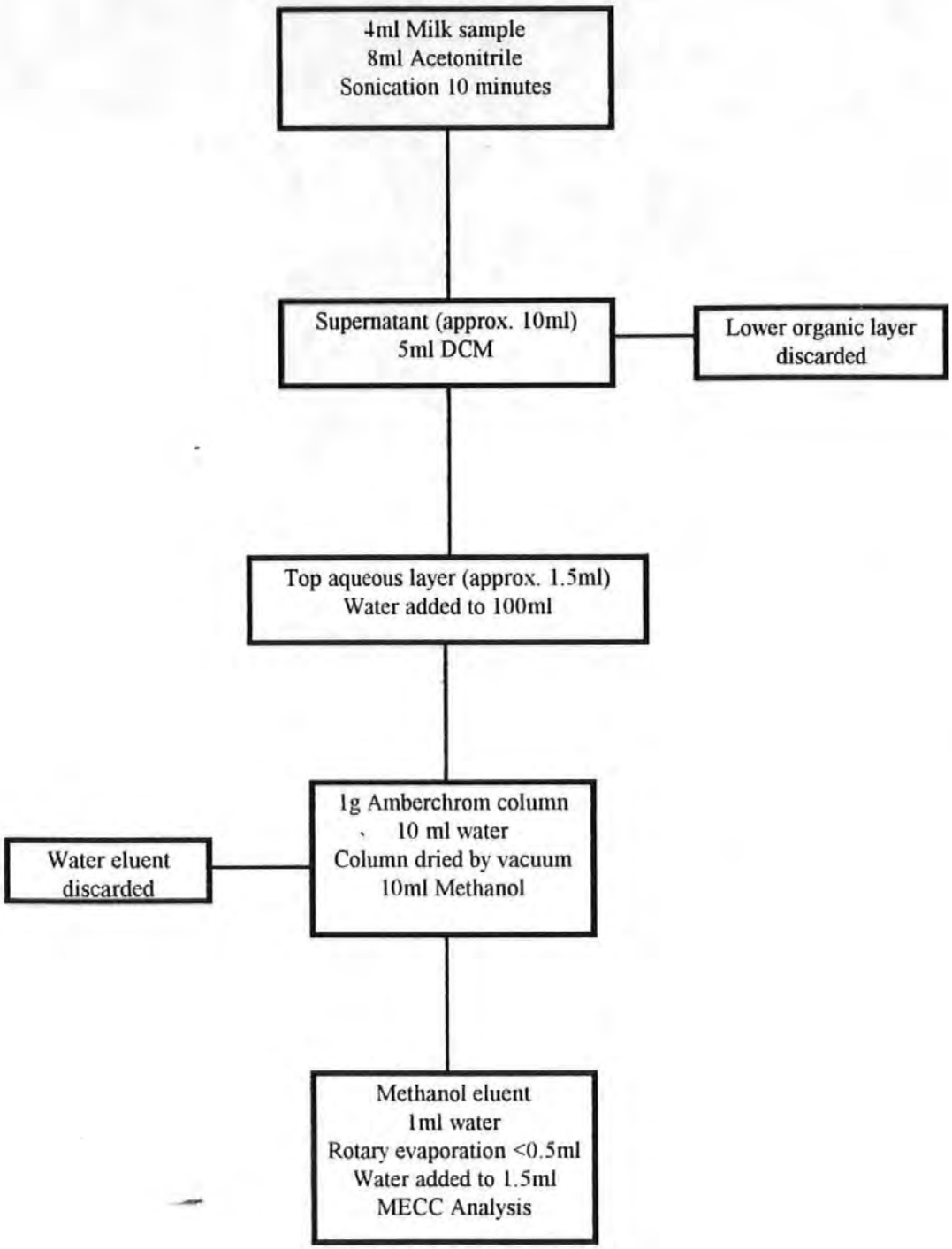
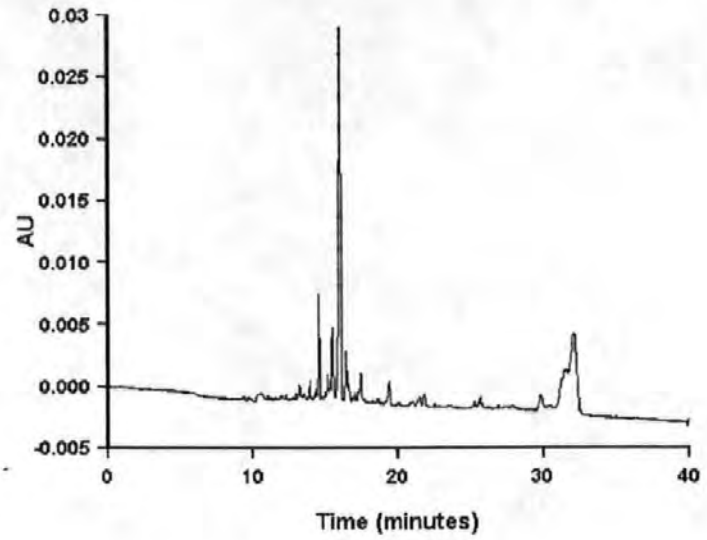


Fig. 3.5 Summary of the extraction method used in section 3.3.2. Acetonitrile extraction with liquid-liquid partition and solid phase purification.

(a)



(b)

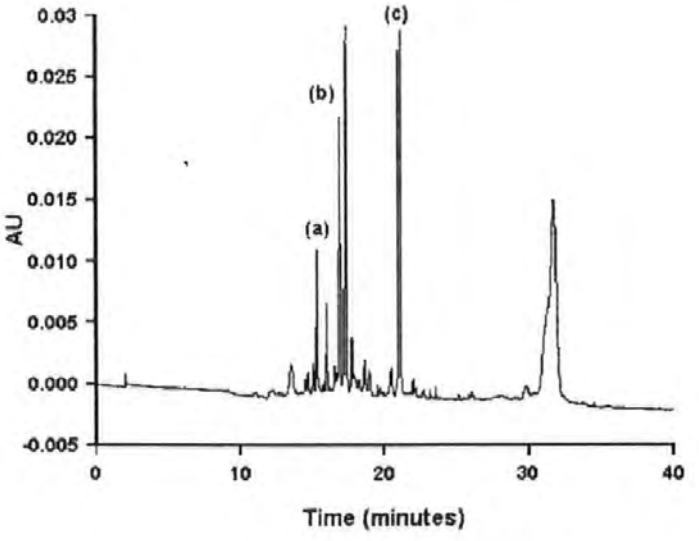


Fig. 3.6 Electropherograms obtained when milk samples were extracted using the procedure indicated in section 3.3.2. (a) Milk blank. (b) Milk sample spiked with 100mg l^{-1} penicillin that had been incubated at 37°C for 3 days. The three major degradation compounds of penicillin, labelled a, b and c, were identified by co-injection with aqueous samples of degraded benzylpenicillin.

3.3.3 Aqueous extraction with solid phase clean up.

As mentioned in section 2.3.2 water only extracts the polar components of the milk matrix and consequently samples contain fewer potentially interfering matrix components than when solvents such as methanol and acetonitrile are used. An aqueous extraction method was therefore devised in an attempt to improve the recoveries of penicillin degradation compounds and provide a better separation of the analytes from the milk matrix. Extractions were performed without acidifying milk samples, thus reducing the risk of further penicillin breakdown. This procedure gave the advantages of using water as the extraction solvent.

A milk sample (5ml), spiked to 100mg l^{-1} with a benzylpenicillin solution that had been incubated at 37°C for 120 hours, was placed into a 30ml stoppered centrifuge tube. Water (5ml) and DCM (5ml) were added in one increment and the tube shaken on an electronic shaker for 5 minutes at high speed. The suspension was centrifuged at 2500rpm for 10 minutes, the upper aqueous layer was separated and filtered through a plug of defatted cotton wool. The filtrate was introduced to a 1g Amberchrom 161-m column and the resin washed with water (10ml). Air was drawn through the column for 1 minute to dry the resin and methanol (10ml) used to recover the compounds of interest. Water (1ml) was added to the methanol eluent and the solvent removed under reduced pressure at 25°C. Water was added to the extract to adjust the final volume to 1ml which was used for injections under the usual MECC conditions. A summary of the procedure is shown in fig. 3.7.

Neither penicillin or any of its degradation compounds were resolved on the resultant electropherograms because of the extensive interference recorded from milk matrix components. From the results it was clear that acetonitrile was the better extraction solvent and was used in all future experiments.

3.3.4 Acetonitrile extraction with solid phase clean up.

In an attempt to improve recoveries of the degradation compounds from milk, sample handling was reduced to a minimum. The DCM extractions of the acetonitrile supernatant described in section 3.3.2 were not included in the following procedure.

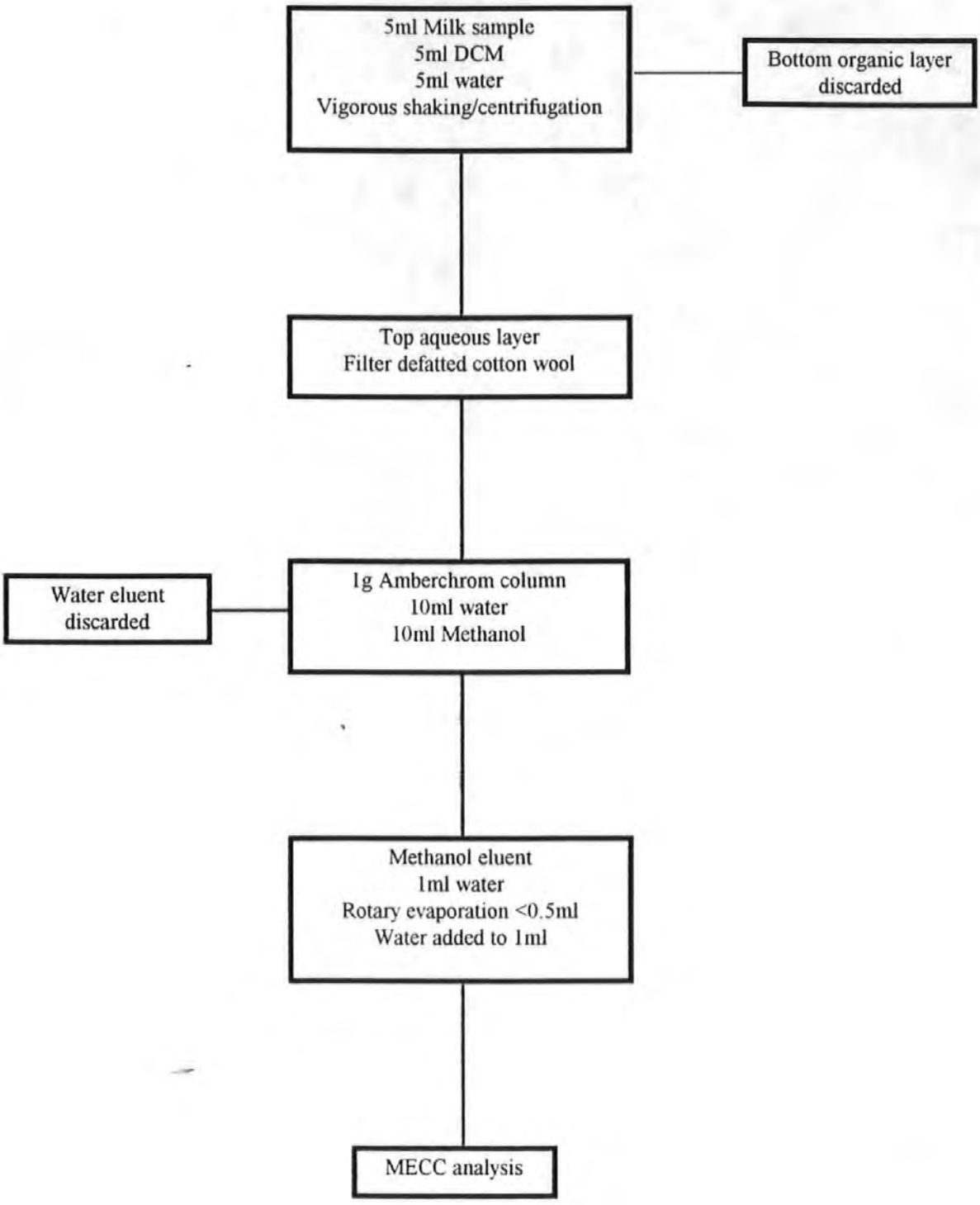


Fig. 3.7 Summary of the extraction and purification technique described in section 3.3.3 involving the aqueous extraction of milk samples.

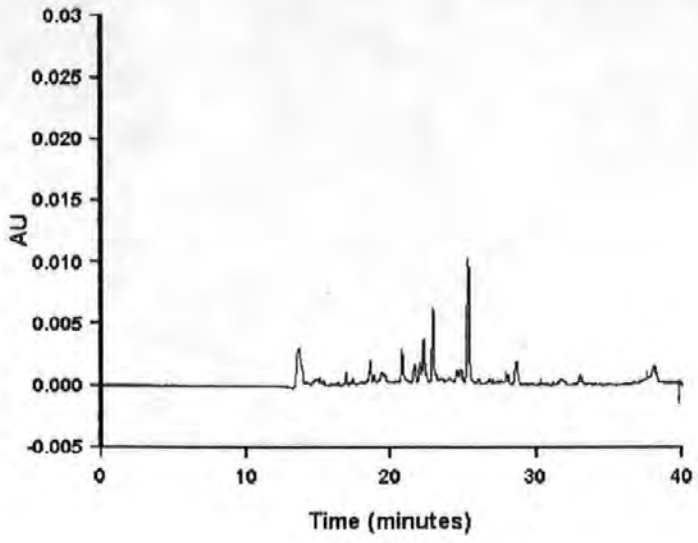
In previous experiments pasteurised milk was used for *in*-vitro studies. To investigate how real samples would affect the results, milk was taken from a cow with a high cell count (600,000 cells/ml). The milk was inoculated to a concentration of 100mg l^{-1} with an aqueous stock solution of penicillin that had been maintained at 37°C for 4 days and thus contained the three major penicillin degradation compounds.

A sample of the spiked milk (3ml) was taken and acetonitrile (6ml) gradually added with vigorous shaking. The solution was centrifuged at 2500rpm for 20 minutes and the supernatant filtered through a plug of defatted cotton wool. The volume of the eluent was reduced to <1ml by rotary evaporation at 25°C after which water (6ml) was added. The solution was introduced to the top of a 1g Amberchrom column and allowed to percolate through under gravity. The column was washed with water (10ml) and dried by drawing air through under vacuum. The column was eluted with methanol (10ml), water (1ml) was added to the eluent and the solution evaporated almost to dryness. The final volume was adjusted to 1ml with water and electrophoresed.

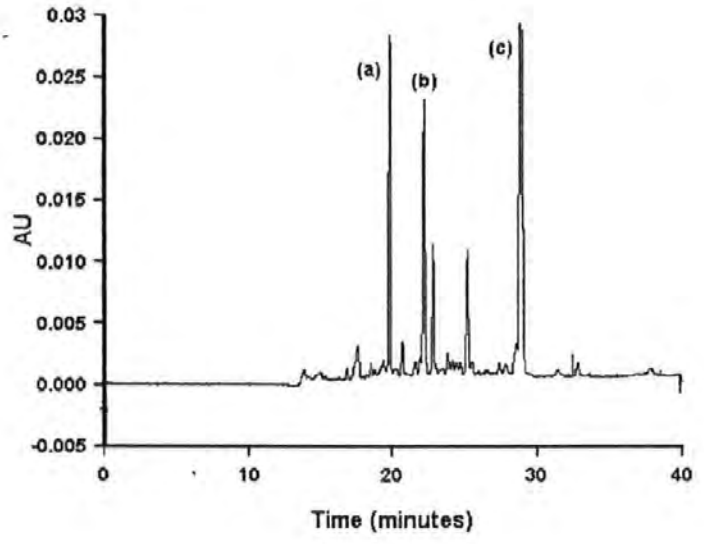
The results, shown in fig. 3.8, indicate that the DCM extractions used in previous methods (section 3.3.2) were unnecessary, providing little additional clean up of the milk samples. Recoveries of 83±2, 89±3, and 82±3% for the three degradation compounds labelled A, B and C in fig. 3.8 were much improved on previous extractions. The procedure was repeated on a water sample containing fresh 100mg l^{-1} potassium benzylpenicillin. Recovery of the antibiotic material was only 49% and as shown in fig. 3.9 a large degradation compound appeared which neither co-migrated with the penicillin or the breakdown compounds previously recorded.

It was apparent that the extraction procedure was causing a rapid breakdown of the antibiotic material resulting in a, hitherto unseen,degradation product.

(i)



(ii)



(iii)

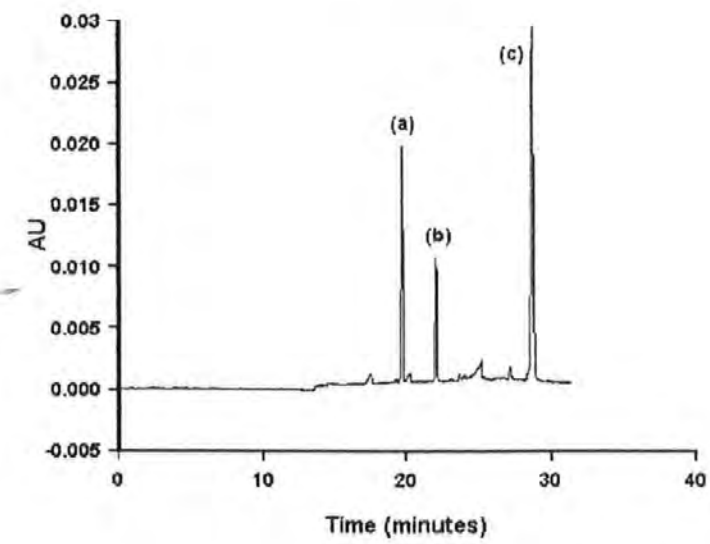


Fig 3.8 Electopherograms obtained from the MECC analysis of (i) Extracted milk blank, (ii) extracted milk spiked standard (100mg l^{-1}) (iii) aqueous standard, as described in section 3.3.4. Penicillin degradation compounds spiked into the milk are labelled (a), (b) and (c).

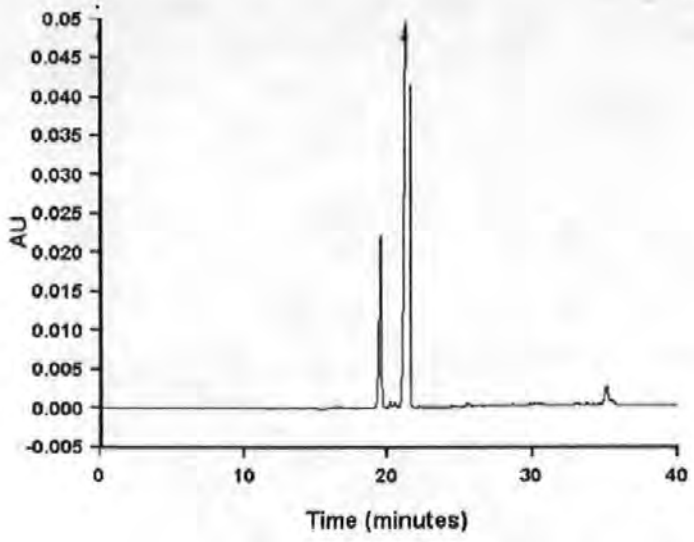


Fig. 3.9 Electropherogram produced by the extraction of a 100mg l^{-1} aqueous penicillin sample as described in section 3.3.4. Penicillin is resolved at t_m 21.58(min) and a previously unencountered degradation compound caused by the extraction procedure at t_m 19.56(min).

3.3.5 Experiments into the origin of benzylpenicillin degradation.

It was necessary to find the origin of penicillin degradation. Processes during the extraction procedure considered likely to cause such rapid degradation were evaporation under reduced pressure, photodegradation or pH changes on the solid phase column.

To examine the first of these possibilities, an aqueous sample of 100mg l^{-1} penicillin (5ml) was added to methanol (25ml) in a round bottomed flask. The solution was reduced to approximately 2ml and the final volume adjusted with water to 5ml before injection into the MECC system. Comparison between the initial 100mg l^{-1} sample and the evaporated sample showed a recovery of 93% of the antibiotic and no degradation was evident.

In an investigation into photodegradation on the Amberchrom resin, two 5ml samples from the same aqueous 100mg l^{-1} fresh penicillin stock were introduced to a 1g Amberchrom column. The first sample was kept in darkness at all times whilst the second control sample was allowed onto the resin in the light. Following the addition of the sample the column was washed with water (10ml), dried by drawing air through under vacuum and eluted with methanol (10ml). MECC analysis showed degradation of both samples indicating

photodegradation played no part in the breakdown. At this stage it was suspected that drying the sample on the Amberchrom column was the cause of the problem.

This was investigated as follows. Two aqueous samples (5ml) were taken from the same fresh 100mg l^{-1} penicillin stock solution and a 1g Amberchrom column prepared in the usual manner. Sample (i) was placed on the column and washed with water (10ml) and the resin dried as described previously. The penicillin was recovered by elution with methanol (10ml). A volume of water (5ml) was added to the eluent and the solution rotary evaporated to approximately 2ml. The final volume was adjusted to 5ml with water. For sample (ii) care was taken to keep the column wet at all times. After addition of the sample to the resin the column was washed with water (25ml) which was allowed to drain under gravity. The column was then eluted with methanol (30ml). Water (5ml) was added to the methanol eluent and the solution reduced to a volume of approximately 2ml under reduced pressure. The final volume was made up to 5ml with water. MECC analysis of the samples (i) and (ii) showed evidence of degradation in both samples which was much reduced in the latter. This indicated that the problem was being caused by the solid phase packing material possibly due to pH fluctuations.

To investigate this possibility samples were introduced in and washed with phosphate buffer, pH 7.0, instead of water. A 100mg l^{-1} penicillin standard was prepared in a 0.05M, pH 7.0, phosphate buffer and an aliquot (5ml) placed on the Amberchrom column. The resin was rinsed with 0.05M buffer, pH 7.0, (25ml) and the column eluted with methanol (30ml). A volume of water (5ml) was added to the methanol eluent and the solvent removed by rotary evaporation. The final volume of the solution was adjusted to 5ml by the addition of water and MECC injections made. The resultant electropherograms showed no evidence of degradation of the penicillin. However, the penicillin peak was split on both the extracted sample and on the trace produced by an injection of the buffered 100mg l^{-1} stock solution. The peak splitting was considered to be a consequence of the high buffer concentration and the experiment was repeated using a lower concentration of phosphate in the buffer.

A sample of fresh 100mg l^{-1} penicillin (9ml) prepared in a 0.01M phosphate buffer, pH 7.0, was allowed to drain through a 1g Amberchrom column by gravity. The resin was washed with buffer (25ml) and eluted with methanol (30ml). The methanol eluent was collected,

water (3ml) added, and the volume reduced to ca. 2ml under reduced pressure. Water was used to adjust the final volume of the sample to 3ml and MECC injections made. The results, shown in fig. 3.9, indicated that degradation of penicillin was kept to a minimum and recovery of the antibiotic was 94±4% (LOD 0.4mg l^{-1}). From the work conducted in this section it was apparent that the penicillin required a stable pH environment whilst absorbed on the Amberchrom resin and samples needed to be kept wet at all times.

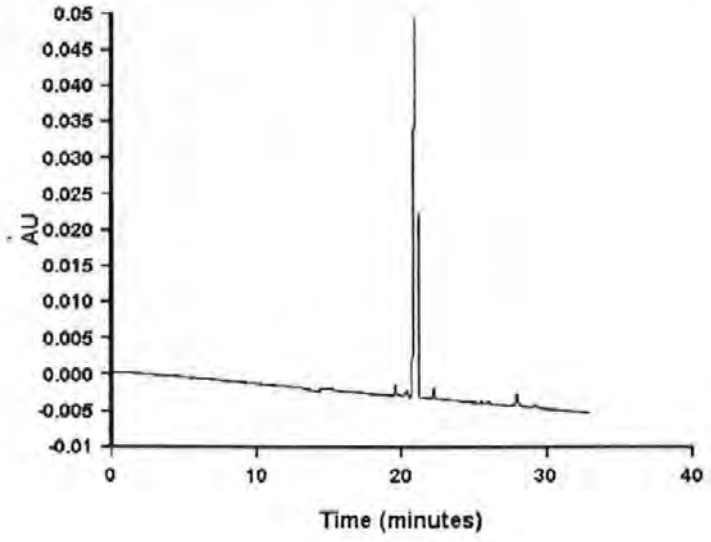


Fig. 3.10 Electropherogram showing the reduced amount of degradation using an extraction technique when phosphate buffer, pH 7.0, was used to wash the Amberchrom column. Benzylpenicillin migrated at t_m 21.20(min).

3.3.6 Milk extraction including solid phase clean up.

The procedure detailed in section 3.3.4 was modified so that the compounds of interest were buffered at pH 7.0 whilst in contact with the Amberchrom resin. Milk samples were spiked with (i) fresh 100mg l^{-1} penicillin and (ii) 100mg l^{-1} penicillin incubated at 37°C for 4 days and was therefore completely degraded.

To a volume of each sample (9ml), acetonitrile (18ml) was added dropwise with vigorous shaking. The solution was then centrifuged at 2500rpm for 20 minutes and the supernatant filtered through a plug of defatted cotton wool. The filtrate was placed in a 50ml round bottomed flask and the volume reduced to approximately 0.5ml by rotary evaporation. To

the oily green residue 0.01M pH 7.0 phosphate buffer (9ml) was added and the solution pipetted onto a 1g Amberchrom column. The column was wetted as described in section 3.2.2 and preconditioned with methanol (30ml), water (30ml) followed by buffer, pH 7.0, (30ml). The resin was rinsed with 0.01M buffer, pH 7.0, (25ml) and eluted with methanol (30ml). The eluent was reduced in volume to <1ml by rotary evaporation at 25°C and water added to adjust the final volume to 3ml. The procedure used is summarised in fig. 3.11.

The resultant electropherograms indicated that using buffer instead of water to wash the resin had no effect on the degree of clean up achieved. The small matrix peak still interfered with the resolution of the breakdown compound labelled as B in fig 3.8. The benzylpenicillin migrated after 23.95(min), with 90±4% recovery (LOD 1.2mg l^{-1}). The recoveries of the degradation compounds labelled A, B and C in fig. 3.8 were 81±2, 80±4 and 80±3% respectively. The relative success of this method allowed its application to all subsequent extractions of milk samples, including the *in-vivo* work described in chapter 5.

3.3.7 Attempts to improve the resolution of benzylpenicillin degradation compounds from the milk matrix by modifying the operating electrolyte.

In an attempt to resolve the breakdown compound co-migrating with a milk component, a milk sample inoculated with 100mg l^{-1} of completely degraded penicillin (incubated at 37°C for 4 days) was extracted as described in the previous section. MECC injections were made using carrier electrolytes, shown in Table 3b, in which the pH was lowered and/or various concentrations of acetonitrile modifier were used.

Table 3c. Electrolytes prepared to improve the resolution of penicillin degradation compounds from milk matrix interference following extraction and purification.

Electrolyte number	pH	% Organic Modifier	Current (μA)
1	8.5	0% Acetonitrile	17-18
2	9.0	5% Acetonitrile	28
3	9.0	15% Acetonitrile	26
4	9.0	0% Acetonitrile	32

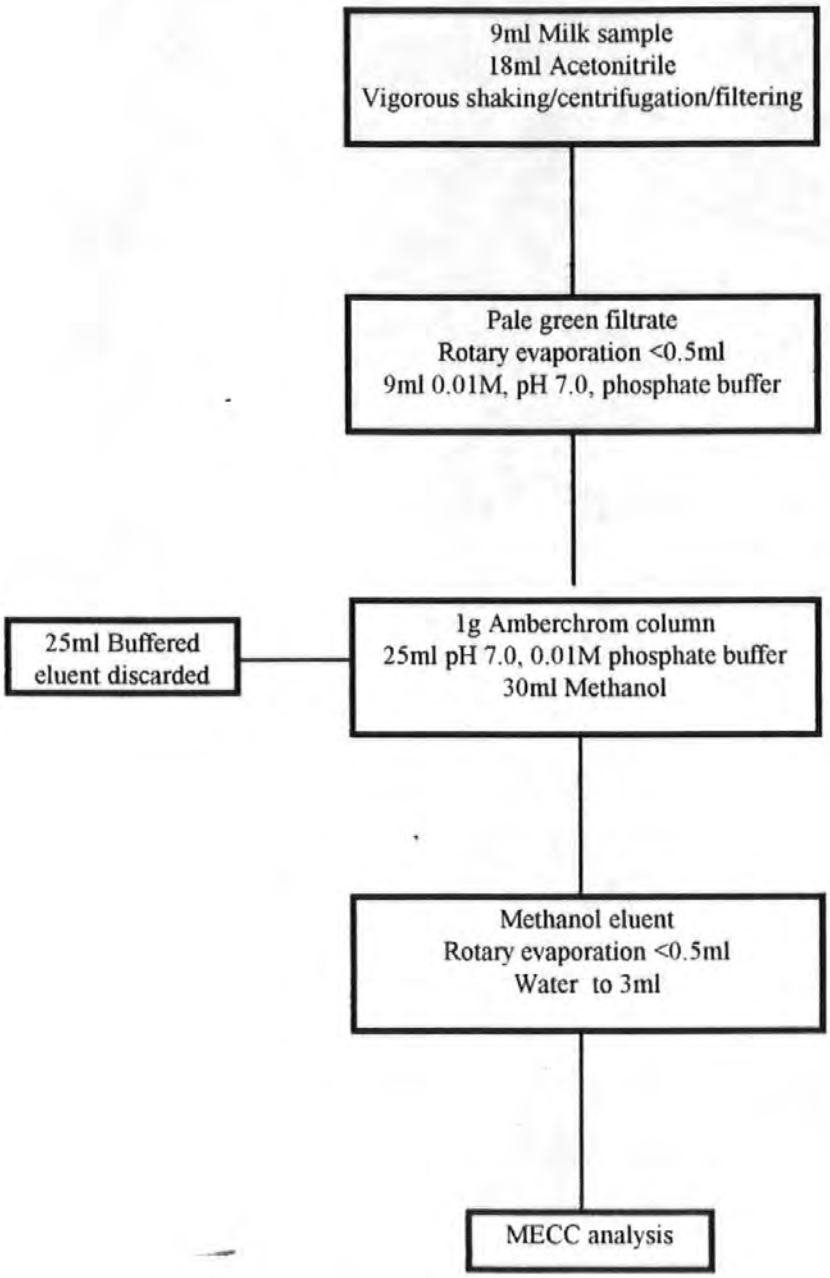


Fig. 3.11 Summary of the procedure used in section 3.3.6. Milk samples extracted with acetonitrile and buffered at pH 7.0 whilst subjected to solid phase purification on Amberchrom 161-G resin.

All solutions were prepared by titrating the required volume of 0.06M boric acid with 0.06M sodium tetraborate and adding 0.06M SDS to the final solution. Electrolyte 1. provided a poorer separation of the degradation compounds than the usual system (4). This was because at lower pH electrophoretic movement occurs towards the negative (cathode) detector end of the capillary and the EOF is consequently reduced. Solutes, therefore, migrate faster and in a narrower band. In high pH systems, such as the usual electrolyte (solution 4), the electrophoretic movement is towards the anodic (positive) end of the capillary, however, the overriding force is the EOF which carries the analytes to the detector at a retarded velocity because of the electrostatic forces.

As mentioned previously the addition of organic modifiers such as methanol, acetonitrile and IPA to the background electrolyte can result in increased resolution of components. This is a result of the electrophoretic migration of the solutes being strongly towards the anode in high pH systems and the EOF reduced because of the presence of the solvent. The net velocity of the solutes to the detector is consequently slower and thus a better separation is achieved. Addition of 5% and 15% acetonitrile to the electrolyte lengthened migration times and caused a general broadening of peaks with increasing solvent content. Addition of the organic modifier failed to provide the complete resolution of the degradation compound labelled B in fig. 3.8. Complete resolution of all of the major degradation residues of penicillin was not achieved using the existing extraction procedure and MECC system. However, because of restrictions imposed by time and the need for measurement of *in-vivo* samples the existing methods were used in subsequent studies.

3.3.8 Simultaneous determination of penicillin-G, penicillin-V, ampicillin, cloxacillin and novobiocin by MECC.

Penicillin-G, penicillin-V, cloxacillin, ampicillin and novobiocin are commonly occurring antibiotics used in bovine medicine. Mastitis treatments contain a variety of such antibiotics in conjunction with penicillin and consequently it was necessary to determine whether the presence of these compounds affected the resolution of the penicillin and its degradation compounds. Fig. 3.12 shows the separation of an aqueous mixture of the five antibiotics using the usual MECC conditions of 0.06M SDS, 0.06M borate buffer (pH 9.0). Identification of each antibiotic was

made by co-injection of 50mg l^{-1} standards of each compound. Co-injection of the antibiotic mixture with a sample of degraded penicillin (100mg l^{-1} maintained at 37°C for 4 days) showed that resolution of the breakdown compounds was unaffected by the presence of the other antibiotics.

Aqueous 100mg l^{-1} standards of ampicillin, penicillin-V, penicillin-G, cloxacillin and novobiocin were extracted using the usual procedure described in section 3.3.6. Recoveries of the residues are shown in Table 3d.

Table 3d. Recoveries of extracted aqueous standards of five antibiotics.

Antibiotic	Recovery from aqueous standard
Ampicillin	92±3%
Penicillin-G	90±4%
Penicillin-V	94±2%
Novobiocin	91±3%
Cloxacillin	74±9%

A milk sample was spiked to a concentration of 10mg l^{-1} with each of the five antibiotics and extracted using the procedure described in section 3.3.6. From the results, shown in fig. 3.13, all five residues were detected, however, the resolution of novobiocin and cloxacillin were affected by milk matrix interference.

The importance of this experiment was to show that the antibiotics used in conjunction with penicillin in mastitis treatments do not interfere with the detection of the penicillin breakdown compounds. The results also show that if the chromatography was adjusted the extraction and clean up procedure may be used for a wide range of residues used in veterinary medicine.

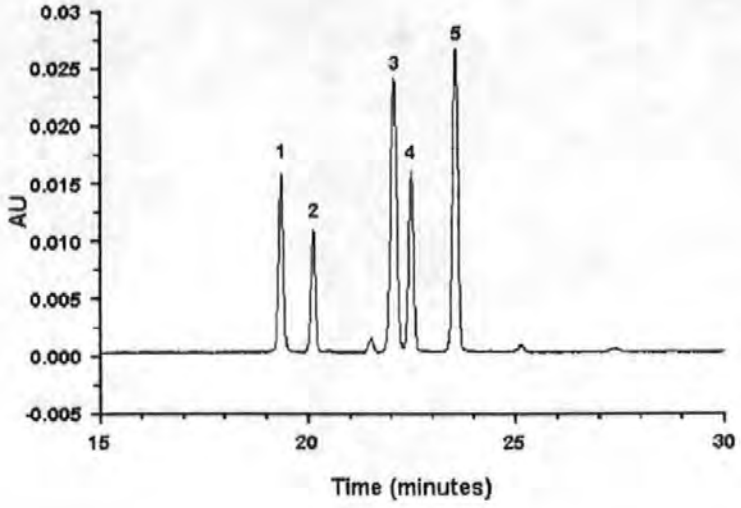


Fig. 3.12 Electropherogram obtained for the separation of five different antibiotic residues using a carrier electrolyte of 0.06M borate buffer (pH 9.0), 0.06M SDS. The aqueous standard contained 50mg l^{-1} of: 1. ampicillin 2, penicillin-G, 3 penicillin-V, 4 novobiocin and 5 cloxacillin.

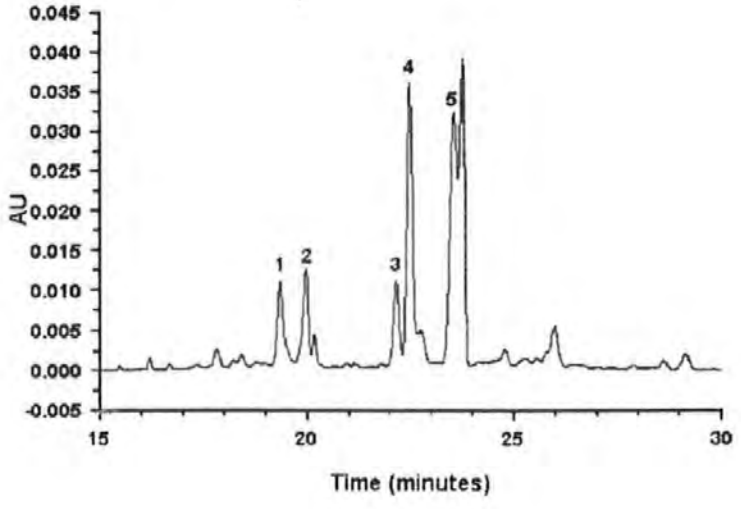


Fig. 3.13 Electropherogram obtained from the extraction of an *in-vitro* milk sample containing 10mg l^{-1} of each antibiotic. 1 ampicillin, 2. penicillin-G, 3 penicillin-V, 4 novobiocin and 5 cloxacillin. Samples were extracted as indicated in section 3.3.6 and analysed using a carrier electrolyte of 0.06M borate buffer (pH 9.0), 0.06M SDS.

3.3.9 Quantification of results.

Ideally, if degradation compounds of benzylpenicillin are present in milk following treatments for mastitis both qualitative and quantitative data is required. However, since no previous work had been performed in this field, the initial focus of this research work concentrated on acquiring qualitative information and the characterisation of any such residues which may be present in *in-vivo* milk samples. A rigorous approach to quantification was therefore not taken, as this would have necessitated developing an internal standardisation procedure requiring time to perform analyses to ensure that the standard survived the extraction procedure and that the resolution of the compounds of interest were not affected.

The quantitative data for benzylpenicillin to be reported in chapter 5 was calculated from the calibration graphs shown in fig 3.14 and 3.15, constructed from MECC analysis of aqueous standards. Greater precision in the quantification of results would have been obtained if the calibration graphs had been constructed from milk samples spiked with benzylpenicillin and extracted as standard (section 3.3.6). This would have allowed for losses of antibiotic incurred during the extraction of samples. However, as has been seen in previous sections, the work up of milk samples is a lengthy process. The additional time which would be required to obtain the calibration graph based upon an internal standard in milk was felt to be more than was available at this stage of the research work. It was realised that a calibration based on aqueous solution would be inferior to that obtained from extracted milk samples. However, as the focus of the research was on qualitative information the approximation obtained using this method was considered sufficient.

Calibration Graph for benzylpenicillin.

Aqueous standards of concentrations 100, 50, 10, 5, 1 and 0.5mg l^{-1} were freshly prepared from potassium and procaine benzylpenicillin 1000mg l^{-1} stock solutions and were electrophoresed using the standard carrier electrolyte of 0.06M Borate buffer, pH 9.0, 0.06M SDS. The calibration graphs shown in fig 3.14 and 3.15 were produced from the average area under the penicillin peak, obtained from three analyses of each aqueous standard.

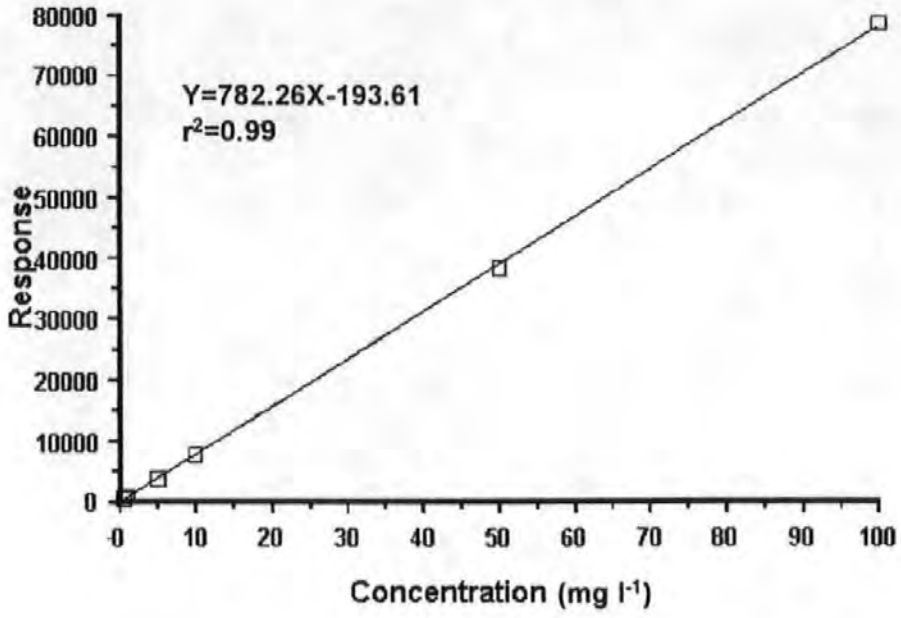


Fig. 3.14 Calibration graph obtained from aqueous standards of freshly prepared potassium benzylpenicillin and electrophoresed using the standard carrier electrolyte of 0.06M borate buffer, pH 9.0, 0.06M SDS.

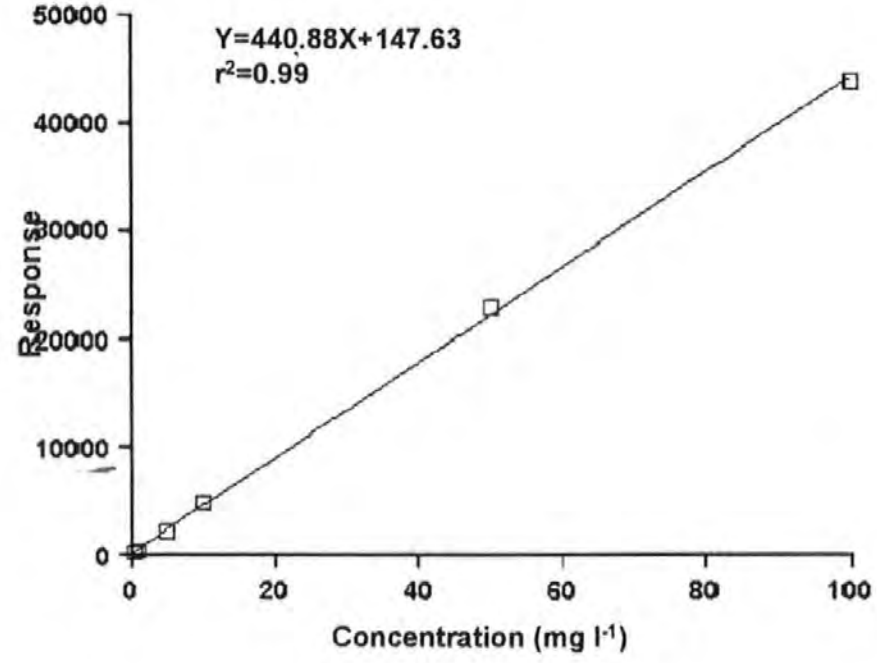


Fig. 3.15 Calibration graph obtained from aqueous standards of freshly prepared procaine benzylpenicillin and electrophoresed using the standard carrier electrolyte of 0.06M borate buffer, pH 9.0, 0.06M SDS.

Chapter 4

Preparation and Identification of Benzylpenicillin Degradation Compounds

4.0 Preparation and identification of benzylpenicillin degradation compounds.

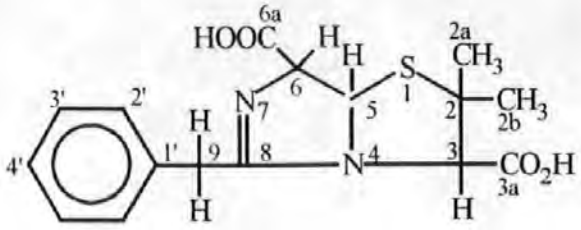
Following its discovery in 1929 the degradation of penicillin in acidic media has been extensively studied (Kessler *et al.*, 1981 and 1983). The driving force behind these studies was two fold: first the search for orally active penicillins which would not be degraded in the acidic environment of the human stomach, secondly it was thought that such studies might lead to the understanding of allergic responses that may have been related to complexation of breakdown compounds with proteins (Longridge and Timms, 1971[a]).

Although 8 penicillin degradation compounds were separated from aqueous solution using HPLC and MECC, published work on the degradation of penicillin indicated the 5 major degradation compounds shown in fig 4.1 were present when a solution of penicillin was incubated at pH 2.5 and 37°C (Degelaen *et al.*, 1978 and Kessler *et al.*, 1981). However, one of these residues, benzylpenicillenic acid, is a reactive intermediate formed at an early stage in the hydrolysis and reaching maximum concentrations after several minutes. Consequently it will not result in the formation of a peak on chromatographic analysis. From work reported in the literature it was concluded that benzylpenillic acid, benzylpenicilloic acid, benzylpenilloic acid and benzylpenamaldic acid were the penicillin degradation compounds most likely present in incubated aqueous samples. Synthetic work therefore focused on the preparation and characterisation of these compounds using FT-IR and FT-NMR. To determine which peak corresponded to which degradation compound in the aqueous standard, co-injection analysis was performed using MECC. Peaks were also identified using this technique, by calculating the ratio of the peak area at two fixed wavelengths in comparison with that of prepared standards.

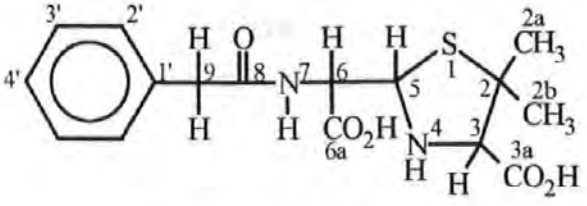
4.1 Experimental.

4.1.1 General solvents and Reagents.

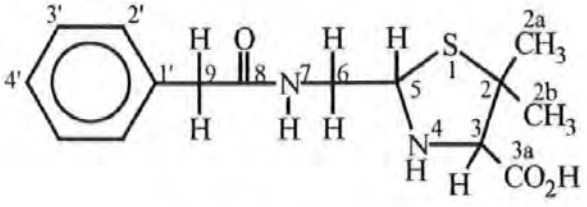
Solvents and reagents obtained from commercial sources were used as received. Melting points (mp) were determined in capillary tubes using an 'Electro-thermal' apparatus, and quoted values are uncorrected.



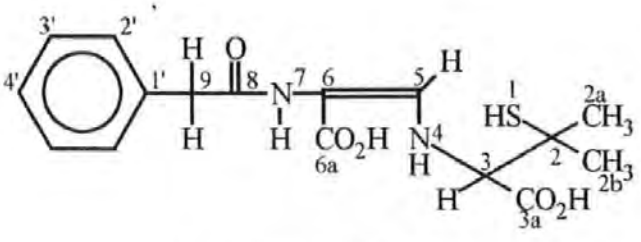
Benzylpenillic Acid



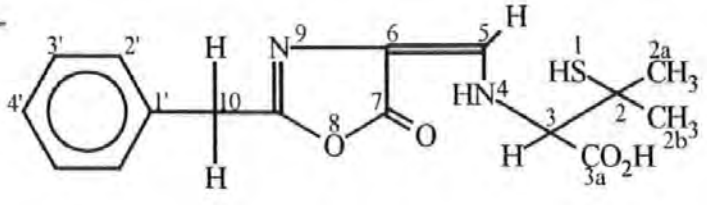
Benzylpenicilloic Acid



Benzylpenilloic Acid



Benzylpenamaldic Acid



Benzylpenicillenic Acid

Fig. 4.1 Diagram showing the structures of the major benzylpenicillin degradation compounds: Benzylpenillic acid, benzylpenicilloic acid, benzylpenilloic acid, benzylpenamaldic acid and benzylpenicillenic acid.

4.1.2 Infrared Spectroscopy.

Spectra were recorded using a Bruker IFS 66 Fourier Transform spectrometer, using a potassium bromide disc prepared according to standard procedures (Williams and Flemming, 1989).

4.1.3 Nuclear Magnetic Resonance Spectroscopy (NMR).

^{1}H, ^{13}C and ^{13}C-DEPT spectra were recorded using a 270 MHz Joel spectrophotometer. Data for interpreted spectra are shown in Tables 4a-g. The techniques used to interpret the spectra are discussed below.

Proton (^{1}H) NMR Spectroscopy.

Proton NMR spectra were used to assign observed chemical shifts to protons in the compounds studied using the following procedures:-

(i) Proton signals were assigned to certain areas of the spectra using additivity data from chemical shift tables (Silverstein *et al.*, 1991).

(ii) Integration of proton signal peaks was used to find the ratio of protons in each environment.

(iii) Multiplicity of peaks was used to determine the number of protons in neighbouring groups.

(iv) The magnitude of coupling constants was also used to indicate coupled protons in certain spectra.

Carbon-13 (^{13}C) NMR Spectroscopy.

All carbon-13 spectra were obtained using broad-band proton-decoupling. Chemical shifts were assigned using the same principles of additivity used in the interpretation of proton spectra.

DEPT Experiments.

DEPT experiments were used to obtain ^{13}C-^{1}H multiplicities of carbon-13 atoms. Spectra were acquired using a $3\pi/4°\theta$ probe. Signals from CH and CH_3 groups appeared in-phase whereas CH_2 groups appeared as anti-phase peaks.

4.2 Preparation and identification of the degradation compounds of benzylpenicillin in aqueous standards used to inoculate milk samples.

4.2.1 Preparation and identification of benzylpenillic acid.

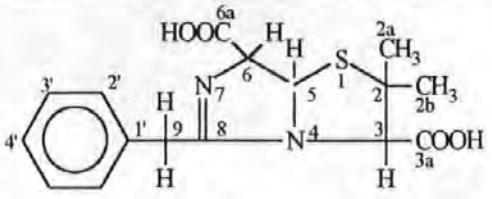
Benzylpenillic acid has the molecular formula $C_{16}H_{18}N_2O_4S$ and crystallises as either fine needles, hexagonal plates or long hexagons. The structure is shown in fig. 4.1.

Benzylpenillic acid was prepared following the method of Clarke *et al.* (1949). Potassium benzylpenicillin (213mg) was dissolved in water (45ml) and the pH adjusted to 2 with 0.1M HCl thus liberating the free acid. The solution was allowed to stand for seven days and the fine bunches of needles were collected by filtration with a yield of 54mg (25.4%, mp 178-180°C, Lit mp 181-183°C).

Benzylpenillic acid (50mg) was sonicated for 10 minutes in $(CD_3)_2SO$ (deuterated DMSO) (1ml). The solution was filtered through a plug of glass wool to remove any undissolved material and ^{13}C, ^{13}C-DEPT. and ^{1}H NMR measurements made. The spectra obtained are interpreted in Table 4a.

On first inspection of the ^{1}H NMR spectrum there appeared to be one peak missing. However, using integration it was determined that H-3 and H-5 signals were unresolved. C-3, C-5 and C-6 correspond to peaks at δ 70.9, 72.5 and 74.0 (ppm) but could not be fully resolved without a C-H correlation which was considered unnecessary for this work. C-3, C-3α and C-8 corresponded to peaks at δ 166.1, 169.8 and 171.7 (ppm) which would have required an INADEQUATE experiment to resolve them. The INADEQUATE experiment detects the connectivities between two adjacent ^{13}C atoms but its expense prevents routine use (Silverstein *et al.*, 1991).

Table 4a. NMR Spectroscopic data for Benzylpenillic acid. Key: * see text in section 4.2.1. Where (m)=multiplicity, (d)=doublet and (s)=singlet.

BENZYLPENILLIC ACID	
SOLVENT:- $(CD_3)SO$	SPECTROPHOTOMETER:- JOEL 270 MHz

PROTON (^1H) CHEMICAL SHIFTS

	4'	3'	2'	1'	9	8
δ(ppm)	7.22(m)	7.22(m)	7.22(m)	-	3.82(s)	-

	7	6α	6	5	4	3α
δ(ppm)	-	-	5.68(d)	4.64(d)	-	-

	3	2β	2α	2	1
δ(ppm)	4.64(s)	1.48(s)	1.43(s)	-	-

CARBON (^{13}C) CHEMICAL SHIFTS

	4'	3'	2'	1'	9	8
δ(ppm)	126.8	128.9	128.4	135.5	33.7	*

	7	6α	6	5	4	3α
δ(ppm)	-	*	*	*	-	*

	3	2β	2α	2	1
δ(ppm)	*	32.5	25.9	56.2	-

Interpretation of the data obtained from FTIR analysis of the prepared compound is shown in Table 4b. No literature values were found for a comparison.

Table 4b. FTIR data obtained from the analysis of benzylpenillic acid.

Functional Group	Wavenumber (cm^{-1})	Width	Intensity
O-H Stretching	3325.3	Broad	Strong
C-H Stretching (CH_3)	2982.9	Sharp	Medium
C-H Stretching (CH_3)	2942.2	Sharp	Medium
C-H Stretching (C-H)	2890.9	Sharp	Medium
C=O Stretching (CO_2H)	1745.8	Sharp	Strong
C=O Stretching (CO_2H)	1682.0	Sharp	Strong
C-N Stretching	1584.1	Sharp	Strong
C=N Stretching	1538.7	Sharp	Strong
C-O Stretch	1311.2	Sharp	Strong
C-O Stretch	1262.2	Sharp	Strong
C=O Bend	1233.3	Sharp	Strong
C-S Stretching	1126.2	Sharp	Medium
Monosubstituted Benzene	763.1	Sharp	Strong
Monosubstituted Benzene	736.0	Sharp	Strong

From the interpretation of both NMR and FTIR data, coupled with the close agreement between the literature melting point and that of the prepared benzylpenillic acid, it was concluded that the target compound had been synthesised.

The benzylpenillic acid was identified on the MECC electropherogram by co-injection. A $10mgl^{-1}$ solution of benzylpenillic acid was prepared and an aliquot (1ml) added to a solution of $100mg\ l^{-1}$

benzylpenicillin that had been incubated at 37°C for 4 days. The benzylpenillic peak in the degraded sample was identified by the increase in area of the peak at t_m 28.75(min). Fig. 4.2 shows the trace obtained from the aqueous degraded penicillin sample with the penillic acid peak indicated.

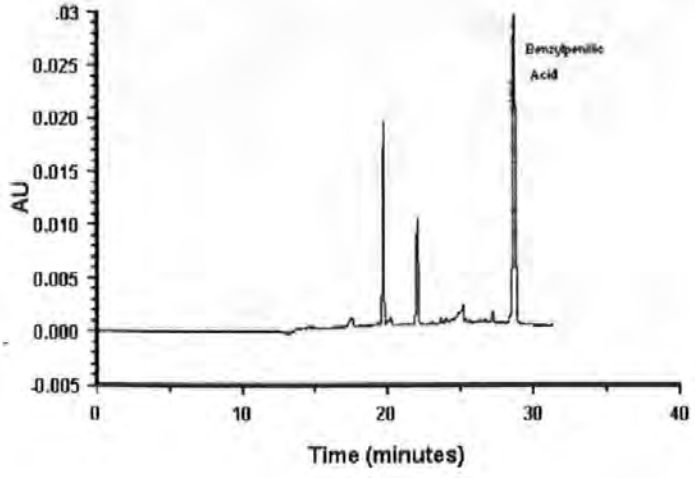


Fig. 4.2 Electropherogram obtained when benzylpenillic acid was co-injected with an aqueous solution of benzylpenicillin that had been incubated at 37°C for 96 hours. Using the standard carrier electrolyte of 0.06M borate buffer, pH 9.0, 0.06M SDS.

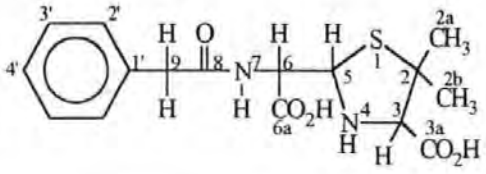
4.2.2 Preparation and identification of benzylpenicilloic acid.

Benzylpenicilloic acid has the molecular formula $C_{16}H_{20}N_2O_5S$ and the structure shown in fig. 4.1. It is a major breakdown product of penicillin (Ghebre-Sellassie *et al.*, 1984). Although it possesses no bactericidal properties it is a minor antigenic compound exerting its activity by reacting with disulphide linkages of proteins. It has been suggested that penicilloic acid is an important intermediate in the formation of certain antigenic compounds and may play an important role in penicillin hypersensitization. Benzylpenicilloic acid is extremely stable in mildly alkaline conditions where it exists as a mixture of isomers (5R,6R and 5S,6R) and can remain unchanged for substantial periods of time (Ghebre-Sellassie *et al.*, 1984).

5R,6R Benzylpenicilloic acid was prepared according to the method described by Ghebre-Sellassie *et al.* (1984). Benzylpenicillin (4.32g) was dissolved in water (7.5ml) and cooled to 0°C

in an ice bath. Cold 10M sodium hydroxide (2.32ml) was added in one increment and after 15 minutes the pH was adjusted to the equivalence point of the potassium salt of benzylpenicilloic acid (pH 8.7) using 1M HCl. The mixture was immediately frozen in liquid nitrogen and lyophilized (Ghebre-Sellassie *et al.*, 1984). The resultant pale yellow solid was obtained in a yield of 5.13g (56.4%) and decomposed at 210-215°C (no lit. values available). The solid product was dissolved in D_2O and ^{13}C and ^{13}C-DEPT NMR analyses were made, interpretation of the spectra obtained is shown in Table 4c. Proton measurements were also made and are interpreted in Table 4e.

Table 4c. ^{13}C NMR spectroscopic data for benzylpenicilloic acid.

BENZYLPENICILLOIC ACID (5R,6R)	
SOLVENT:- D_2O	SPECTROPHOTOMETER:- JOEL 270 MHz

CARBON (^{13}C) CHEMICAL SHIFTS						
	4'	3'	2'	1'	9	8
δ(ppm)	127.4	128.9	128.4	134.3	41.8	175.5
	7	6α	6	5	4	3α
δ(ppm)	-	174.9	59.4	65.4	-	173.6
	3	2β	2α	2	1	
δ(ppm)	74.6	26.1	25.7	56.2	-	

From the spectroscopic data complete resolution of C-3, C-5 and C-6 was not possible without C-H correlation. C-3α, C-6α and C-8 also could not be identified unambiguously without the INADEQUATE experiment. Both techniques were considered unnecessary in this work. The peaks C-3, C-5, C-6, C-3α, C-6α and C-8 were assigned to their chemical shifts by comparison with data reported by Ghebre-Sellassie *et al.* (1984).

Interpretation of the data obtained from FTIR analysis of the prepared compound is shown in Table 4d. No literature values were found for a comparison.

Table 4d. FTIR data obtained from the analysis of benzylpenicilloic acid.

Functional Group	Wavenumber (cm^{-1})	Width	Intensity
O-H Stretching	3415.9	Broad	Strong
N-H Stretching	3303.6	Sharp	Strong
C-H Stretching (CH_3)	2966.6	Sharp	Weak
C-H Stretching (CH_3)	2927.2	Sharp	Weak
C-H Stretching (C-H)	2871.5	Sharp	Weak
C=O Stretching (CO_2H)	1628.6	Sharp	Strong
C=O Stretching (CO_2H)	1594.7	Sharp	Strong
C=O Stretching (C=O)	1539.6	Sharp	Medium
C-N Stretching	1396.6	Sharp	Strong
C-S Stretching	1133.0	Sharp	Medium
Monosubstituted Benzene	726.8	Sharp	Medium
Monosubstituted Benzene	701.4	Sharp	Medium

From the interpretation of both NMR and FTIR data it was concluded that the intended compound, benzylpenicilloic acid, had been synthesised.

Identification of benzylpenicilloic acid in an aqueous solution of degraded benzylpenicillin.

To identify the benzylpenicilloic acid peak in an aqueous solution of degraded penicillin, a 50mg l^{-1} solution of benzylpenicilloic acid (1ml) containing both isomers was co-injected with a 50mg l^{-1} solution of penicillin (1ml) that had been incubated at 37°C for 3 days. From the results it was apparent that penicilloic acid was not present when aqueous standards of the antibiotic were incubated at 37°C over a period of several days.

Resolution of benzylpenicilloic acid from in-vitro milk samples.

To investigate whether the penicilloic acid residue could be resolved from the milk matrix a milk sample was spiked to a concentration of 100mg l^{-1} with benzylpenicilloic acid and extracted as described in section 3.3.6. The results, fig. 4.3, showed benzylpenicilloic acid migrating at t_m 30.03 (min) with a recovery of 83±3% and a LOD of 1.0mg l^{-1}.

Calibration Graph for benzylpenicilloic acid.

Aqueous standards of concentrations 100, 50, 10, 1 and 0.5mg l^{-1} were freshly prepared from potassium and procaine 5R,6R benzylpenicilloic acid 1000mg l^{-1} stock solutions and were electrophoresed using the standard carrier electrolyte. The calibration graphs shown in fig 4.4 and 4.5 were produced from the average area under the benzylpenilloic acid peak from three analyses of each aqueous standard.

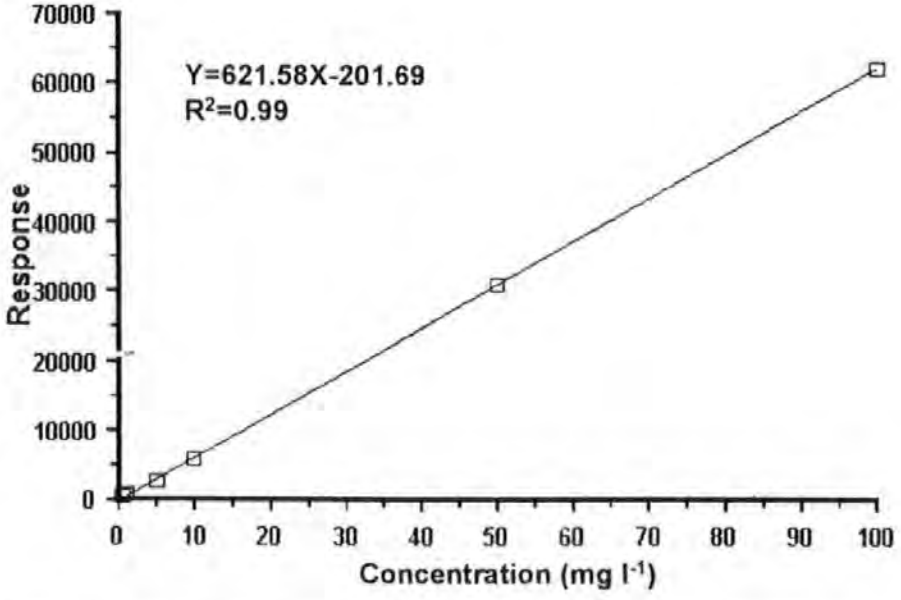
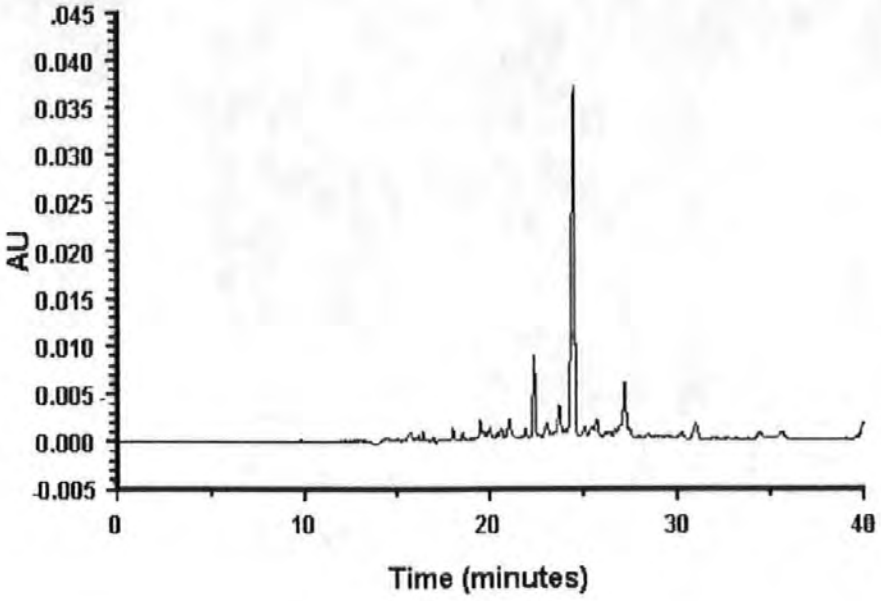


Fig. 4.4 Calibration graph obtained from aqueous standards of freshly prepared potassium 5R,6R benzylpenicilloic acid and electrophoresed using the standard carrier electrolyte of 0.06M borate buffer, pH 9.0, 0.06M SDS.

(a)



(b)

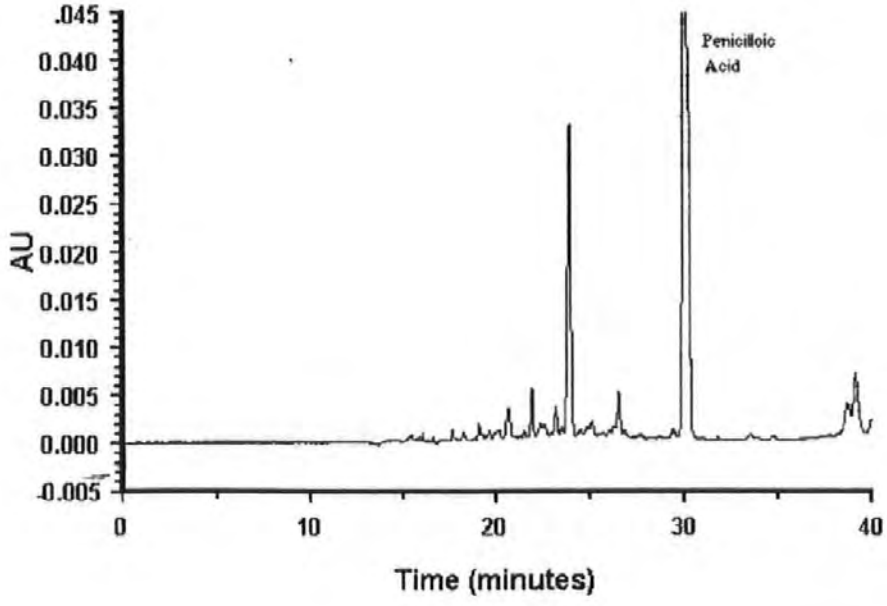


Fig. 4.3 Electropherograms obtained from milk samples extracted using the procedure indicated in section 3.3.6. (a) Milk blank, (b) Milk sample spiked with 100mgl^{-1} penicilloic acid. The penicilloic acid is resolved at t_m 30.03(min) with a recovery of 83±3%.

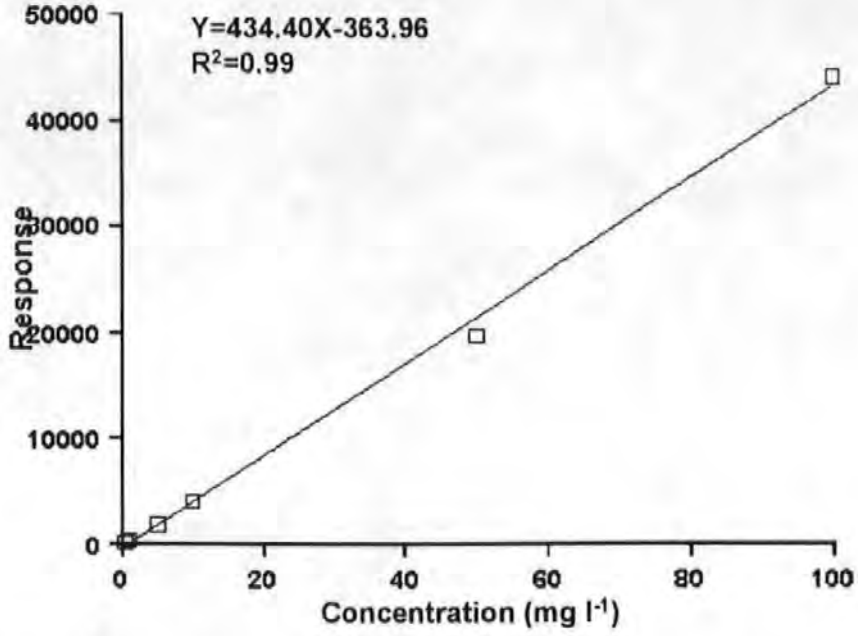


Fig. 4.5 Calibration graph obtained from aqueous standards of freshly prepared procaine 5R,6R benzylpenicilloic acid and electrophoresed using the standard carrier electrolyte of 0.06M borate buffer, pH 9.0, 0.06M SDS.

Epimerization of Benzylpenicilloic acid.

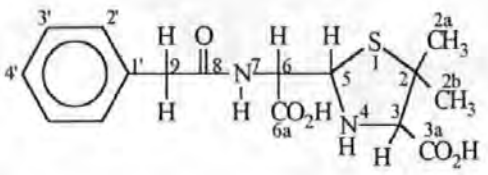
In aqueous solution benzylpenicilloic acid exists as a mixture of isomers. The 5R,6R benzylpenicilloic acid gradually epimerizes at room temperature to the 5S,6R analogue until equilibrium is established, Fig. 4.6.

(a) (b)

Fig. 4.6 (a) 5R,6R (b) 5S,6R isomers of benzylpenicilloic acid (where R=$C_6H_5CH_2CONH$-).

Epimerization was followed using proton NMR. The first measurement was made immediately following dissolution of the prepared 5R,6R analogue (50mg) in D_2O (2ml) and further spectra obtained after 20 and 48 hours with the solution maintained at room temperature. The spectra obtained after 0 and 48 hours are interpreted in Table 4e.

Table 4e. ^1H NMR spectroscopic data for the epimerisation of 5R,6R benzylpenicilloic acid to 5S,6R benzylpenicilloic acid. Key: * = peaks lost due to exchange with D_2O.

EPIMERISATION OF BENZYLPENICILLOIC ACID	
SOLVENT:- D_2O	SPECTROPHOTOMETER:- JOEL 270 MHz

PROTON (^1H) CHEMICAL SHIFTS OBTAINED AT ANALYSIS AFTER O HOURS

	4'	3'	2'	1'	9	8
δ(ppm)	7.34(m)	7.34(m)	7.34(m)	-	3.63(d)	-
	7	6α	6	5	4	3α
δ(ppm)	*	-	4.15(d)	4.97(d)	*	-
	3	2β	2α	2	1	
δ(ppm)	3.34(s)	1.42(s)	1.14(s)	-	-	

PROTON (^1H) CHEMICAL SHIFTS OBTAINED AT ANALYSIS AFTER 48 HOURS

	4'	3'	2'	1'	9	8
δ(ppm)	7.38(m)	7.38(m)	7.38(m)	-	3.76(s)	-
	7	6α	6	5	4	3α
δ(ppm)	-	-	4.73(d)	5.02(d)	-	-
	3	2β	2α	2	1	
δ(ppm)	3.37(s)	1.53(s)	1.00(s)	-	-	

Proton signals were identified using methods described in section 4.1.3 and with reference to the work reported by Ghebre-Sellassie *et al.* (1984). The results indicated that the benzylpenicilloic acid had epimerized from the 5R,6R diastereoisomer to the more thermodynamically favoured 5S,6R epimer. The spectra obtained from each diastereoisomer clearly showed the structural differences, with the signals for the methyl groups on C-2 and the chemical shifts of the proton on C-6 being the main changes.

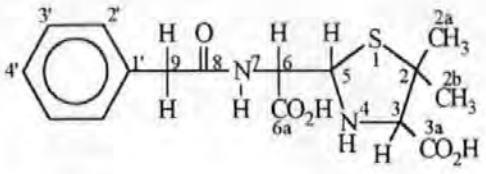
Epimerisation was also followed using the usual MECC system. An aqueous 50mg l^{-1} solution of the 5R,6R benzylpenicilloic acid was prepared and MECC analyses made at times 0, 8, and 17 hours. Resulting electropherograms showed the gradual epimerisation of the kinetically favoured 5R,6R analogue which migrated at t_m 27.88(min) to the thermodynamically favoured 5S,6R epimer which migrated at the slightly later time of t_m 28.05(min).

Investigation into the action of β-lactamase on penicillin.

β-lactamases, discovered by Abraham and Chain in 1940, are bacterial enzymes capable of catalyzing the rapid degradation of penicillins to penicilloic acids (Kishore *et al.*, 1994). The β-lactamases produced by Gram-positive microorganisms are extracellular enzymes and are formed in response to the presence of antibiotic substrate. Gram-negative β-lactamases are endocellular and can not be induced. An experiment was carried out to predict the effect of β-lactamases on penicillin.

The action of β-lactamase (penicillinase), isolated from bacillus cereus (Sigma Chemicals Ltd.), on penicillin was studied using proton NMR. Benzylpenicillin (50mg) was dissolved in D_2O (1ml) and the ^1H NMR spectrum measured. To the penicillin solution, penicillinase (1000 units) in D_2O (1ml) was added and proton measurements made after 5 minutes. Since one unit of the enzyme will hydrolyze 1μmole of substrate per minute at pH 7.0 and 25°C, 5 minutes was considered sufficient time for complete conversion of the antibiotic material. The resultant proton NMR spectrum, interpreted in Table 4f, showed chemical shifts that agreed with those obtained for the 5R,6R benzylpenicilloic acid in section 4.2.2. The results indicated that in mastitis infections caused by a β-lactamase producing micro-organism, the presence of 5R,6R penicilloic acid may be expected in the milk if treated with penicillin.

Table 4f. ^{1}H NMR spectroscopic data obtained for 5R,6R benzylpenicilloic acid produced by the action of β-lactamase on benzylpenicillin

5R,6R BENZYLPENICILLOIC ACID		
SOLVENT:- D_2O	SPECTROPHOTOMETER:- JOEL 270 MHz	
PROTON (^{1}H) CHEMICAL SHIFTS		
	4' 3' 2' 1' 9 8	
δ(ppm)	7.29(m) 7.29(m) 7.29(m) - 3.60(d) -	
	7 6α 6 5 4 3α	
δ(ppm)	- - 4.11(d) 4.90(d) - -	
	3 2β 2α 2 1	
δ(ppm)	3.29(s) 1.37(s) 1.10(s) - -	

4.2.3 Preparation and identification of benzylpenilloic acid.

Benzylpenilloic acid, an acidic degradation compound of penicillin, has the molecular formula $C_{15}H_{20}O_3N_2S$ and the structure shown in fig. 4.1. 5R,6R Benzylpenilloic acid is relatively stable as a salt in alkaline solution, but in acid decarboxylation occurs readily.

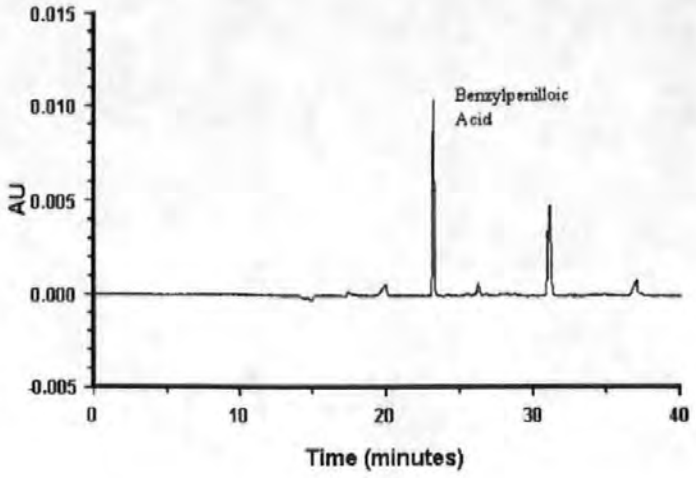
Preparation of benzylpenilloic acid from benzylpenicillin.

The procedure reported by Clarke *et al.* (1949) was repeated as follows. Sodium benzylpenicillin (0.5g) was added to 0.1M sulphuric acid (100ml) and the mixture refluxed for 2 hours leading to the liberation of one mole of carbon dioxide and addition of one mole of water. The result was the formation of an acidic product which was isolated by a continuous extraction with ether. Clarke *et al.* (1949) failed at this stage to obtain the material in a solid form, however, its analytical composition $C_{15}H_{20}O_3N_2S$ indicated that it was essentially a homogeneous solution of benzylpenilloic acid.

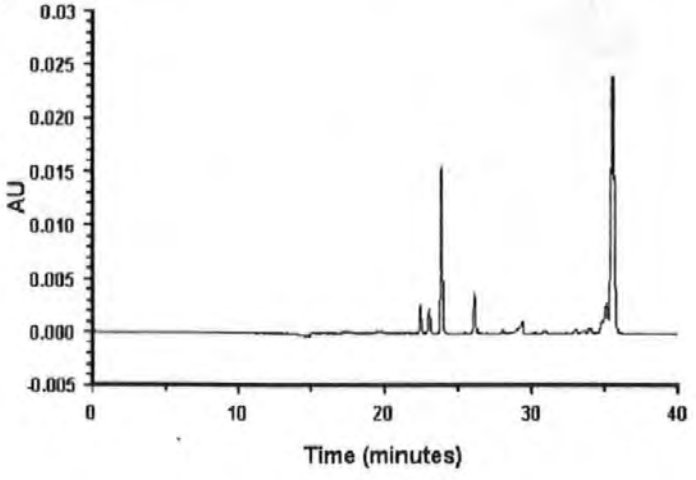
To investigate this, the ether was removed under reduced pressure at 20°C leaving an oily brown residue on the surface of the round bottomed flask. Phosphate buffer 0.01M, pH 9.0, (100ml) was added and the solution stirred for 2 hours. A ×100 dilution of the solution was made with water and injections into the standard MECC system made. The solution (1ml) was co-injected with an aqueous standard of 50mg l^{-1} benzylpenicillin (1ml) that had been incubated at 37°C for 36 hours. The results obtained, shown in fig. 4.7, indicate that the ether extract contained 2 products at migration times of 23.22(min) and 31.73(min). The major product was considered to be benzylpenilloic acid and co-migrated with the first degradation compound resolved in an aqueous degraded sample of penicillin. The identification of benzylpenilloic acid was only tentative since no spectral data was obtained for the sample. The preparation of benzylpenilloic acid was therefore repeated using a different procedure.

In a second method described by Clarke *et al.* (1949), exactly 1 molar equivalent of HCl (50ml) was added to potassium penicillin (422mg) in water (120ml) and the solution refluxed for 1 hour. The resultant mixture was frozen in liquid nitrogen and lyophilized. Using the white crystalline solid product, a 100mg l^{-1} solution was prepared and MECC analyses made. The resultant electropherogram indicated the presence of 3 major compounds with the largest co-migrating with the peak previously tentatively identified as benzylpenilloic acid. Proton NMR data was complicated by the presence of the other degradation compounds making a positive identification impossible.

(a)



(b)



(c)

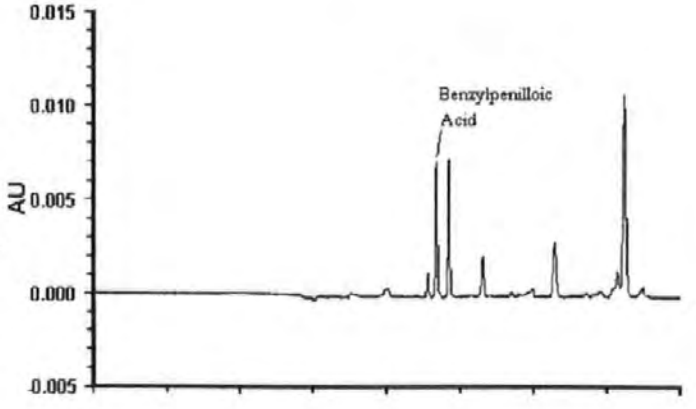


Fig. 4.7 Electropherograms of (a) Solution containing benzylpenilloic acid prepared as indicated in section 4.2.3. (b) penicillin 100mg l^{-1} incubated at 37°C for 36 hours, (c) Co-injection of a 50:50 solution of (a) and (b).

Preparation of 5R,6R benzylpenilloic acid from benzylpenicillamine.

In an attempt to obtain pure benzylpenilloic acid in solid form for NMR analysis, a third preparation of benzylpenilloic acid described by Clarke *et al.* (1949) was attempted. 2,2-Diethoxyethylamine (20g) was added to ice cold 5% aqueous sodium hydroxide solution (200ml). Maintaining the temperature at 0°C, phenylacetylchloride (23g) was gradually added with constant stirring. Clarke *et al.* (1949) indicated that the product phenylacetamidoacetaldehyde ethylacetal, was formed with a yield of 21.7g. However, no solid product precipitated and the solution was cooled at 3°C over night. After 12 hours long colourless crystals (1.38g) were formed and identified as pure phenylacetamidoacetaldehyde ethylacetal by ^{1}H and ^{13}C NMR studies. The phenylacetamidoacetaldehyde ethylacetal (1.35g) was added to D-penicillamine (1g), ethanol (5ml), 1M HCl (2.5ml), water (7.5ml) and potassium acetate (0.8g). The mixture was heated on a steam bath for 5 minutes. The reaction pathway is shown in fig. 4.8.

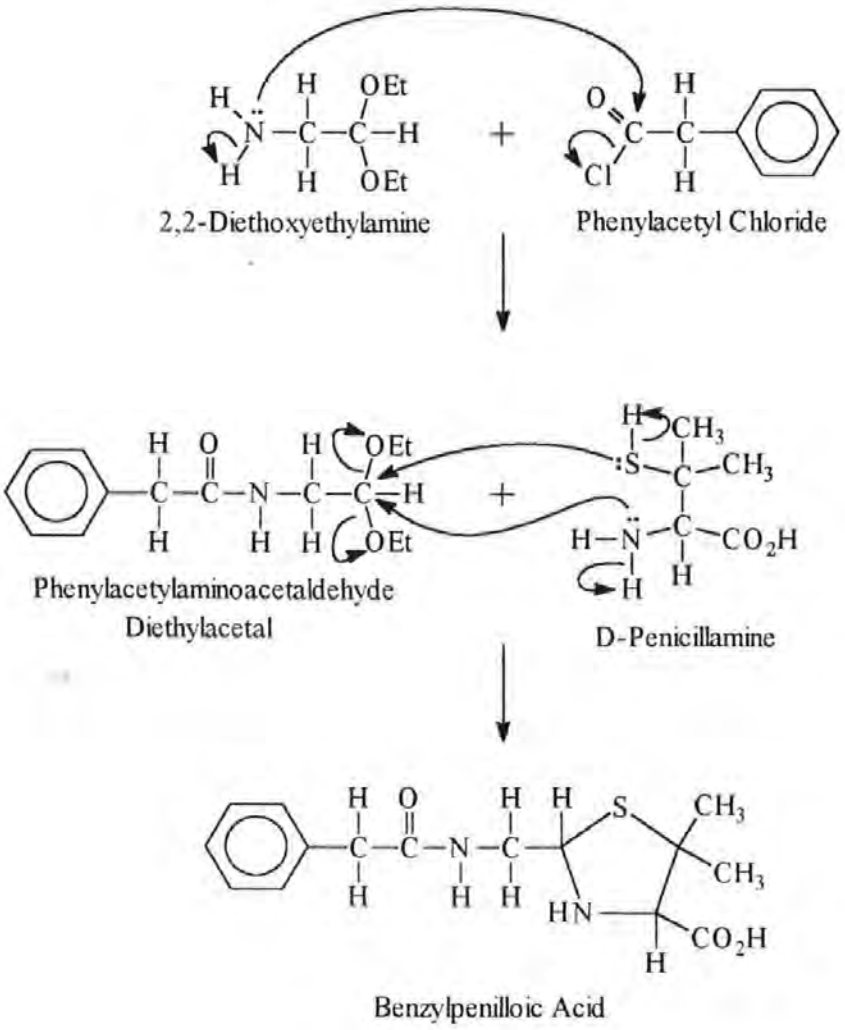


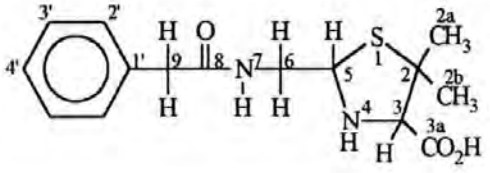
Fig 4.8 Reaction pathway for the preparation of benzylpenilloic acid from D-penicillamine.

No crystals were formed as reported by Clarke *et al.* (1949) even when the volume of the solution was reduced by rotary evaporation.

Preparation of 5R,6R benzylpenilloic acid from benzylpenicilloic acid.

An alternative approach (Clarke *et al.*, 1949) to the preparation of benzylpenilloic acid was repeated. To benzylpenicilloic acid (50mg), dissolved in D_2O (2ml), HCl was added to pH 2.5 and incubated at 27°C for 120 hours. Analysis by proton NMR produced a spectrum which is interpreted in Table 4g, and indicated the presence of 5R,6R benzylpenilloic acid.

Table 4g. ^1H NMR spectroscopic data for 5R,6R benzylpenilloic acid. Key * see text below (page 103).

5R,6R BENZYLPENILLOIC ACID	
SOLVENT:- D_2O	SPECTROPHOTOMETER:- JOEL 270 MHz

PROTON (^1H) CHEMICAL SHIFTS

	4'	3'	2'	1'	9	8
δ(ppm)	7.42(m)	7.42(m)	7.42(m)	-	3.55(s)	-

	7	6	5	4	3α	3
δ(ppm)	-	*	4.89(t)	-	-	3.96(s)

	2β	2α	2	1
δ(ppm)	1.32(s)	1.28(s)	-	-

From the interpretation of the proton data it is apparent that there was no signal for the proton on C-6. This was considered to have been hidden by the large D_2O signal. From the results obtained it was concluded that relatively pure 5R,6R benzylpenilloic acid had been prepared.

To determine the position of the benzylpenilloic acid peak in an aqueous degraded penicillin sample, the NMR sample was used to prepare a 100mgl^{-1} solution which was analysed under the normal MECC conditions. A single peak was obtained at t_m 22.99(min), which when co-injected with an aqueous sample of degraded penicillin (100mg l^{-1}, incubated at 37°C for 36 hours) supported the result reported in fig. 4.7.

4.2.4 Preparation of benzylpenamaldic acid.

Although many breakdown pathways have been postulated for penicillin degradation in acidic media, all agree that benzylpenamaldic acid (of the molecular formula $C_{16}H_{20}O_5N_2S$ and structure shown in fig. 4.1) plays an integral role in the degradation pathway.

Preparation of benzylpenamaldic acid from benzylpenicillenic acid.

Longridge and Timms (1971[b]) described the preparation of benzylpenamaldic acid by the acid hydrolysis of a methanolic solution of benzylpenicillenic acid. However, they were unable to obtain the product in the crystalline or solid form. Benzylpenicillenic acid (50mg) was dissolved in methanol (5ml) and the volume of the solution adjusted to 50ml with dilute HCl with a pH of <1.0. The solution was subjected to the normal MECC conditions which resulted in the recording of a very broad peak at t_m 23.95(min) making it impossible to identify which peak in the degraded aqueous solution it corresponded to. The identification was also complicated by the presence of methanol in the final solution which altered the elution range of the analytes. The experiment was repeated with similarly disappointing results. NMR analysis of the penicillenic acid indicated that it was impure or had decomposed in the storage vessel. Time constraints prevented further attempts at preparation of benzylpenamaldic acid using purer starting materials.

4.3 Summary of the identification of the major peaks obtained by the aqueous degradation of benzylpenicillin separated by MECC.

In an aqueous sample of penicillin incubated at 37°C for a period of several days 3 major degradation compounds are evident as shown in fig. 4.9.

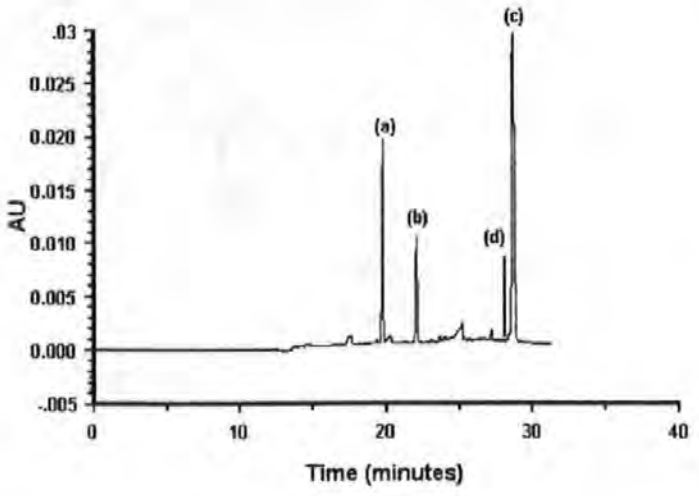


Fig. 4.9 Diagram to summarize the identification of penicillin degradation compounds. (a) benzylpenilloic acid, (b) benzylpenamaldic acid, (c) benzylpenillic acid and (d) benzylpenicilloic acid.

The peak resolved at t_m 19.68(min) and labelled (a) was identified as benzylpenilloic acid. Peak (b) at t_m 24.12(min) was not unambiguously recognised but may be tentatively identified as benzylpenamaldic acid since it was the only major penicillin degradation compound not to be successfully prepared and identified. The final and largest peak (c) at t_m 27.97(min) was identified as benzylpenillic acid. The position of benzylpenicilloic acid, not present in an aqueous degraded sample, is marked in fig. 4.9 as (d).

4.4 An investigation into the role of microorganisms in penicillin breakdown.

Two 100mg l^{-1} solutions of fresh potassium penicillin were prepared: The first under normal laboratory conditions using milli-Q water and the second under aseptic conditions. Both samples were incubated at 37°C for 48 hours and the resulting solution analysed under the usual MECC conditions. The results showed extensive penicillin degradation in the laboratory prepared

sample, but in the aseptic sample penicillin breakdown was greatly reduced. From these observations it was possible to conclude that micro-organisms played an essential role in the penicillin breakdown observed in aqueous solution.

Chapter 5

In-vivo Milk Studies

5.0 *In-vivo* Mastitis Case Studies.

5.1 Sample collection, extraction and analysis.

Sample collection.

Three mastitis case studies were undertaken. Cows were selected that had one clinically infected quarter, from which samples were collected by hand. The first two squirts from the infected teat were discarded to prevent contamination of the sample with dirt from the teat canal. Samples were taken at each milking time, before the cow was milked and placed in a 20ml plastic container with a screw top. Milk samples were frozen immediately, until required for analysis.

Standard Extraction method used for in-vivo samples.

The following procedure was used for the extraction and purification of all *in-vivo* milk samples and is discussed in more detail in section 3.3.6. Acetonitrile (18ml) was added to each sample (9ml). Some samples, especially those collected prior to and during the early part of the treatment, required a pre-centrifugation step because of the increased viscosity common with mastitic milk, which makes accurate measurement of the volume difficult. The solution was shaken vigorously, centrifuged at 2500rpm for 20 minutes and the supernatant layer was filtered through a plug of defatted cotton wool in the stem of a Pasteur pipette. The volume of the pale green filtrate was reduced to 0.5ml by rotary evaporation, 0.01M, pH 7.0, phosphate buffer (9ml) was added, and the resultant mixture introduced to a pre-wetted Amberchrom column (1g). The column was then washed with 0.01M, pH 7.0, buffer (25ml) and methanol (30ml). The methanol eluent was collected in a 50ml round bottomed flask and the volume reduced to 0.5ml by rotary evaporation. The volume of this extract was adjusted to 3ml with milli-Q water, filtered through defatted cotton wool in a Pasteur pipette and electrophoresed under the standard MECC conditions.

The appearance of the final extract of each sample depended upon the time of collection during treatment. Extracts of milk taken early on in the course of treatment were generally turbid and white in colour, whereas those taken from later milkings were the typical clear pale green solutions as seen for *in-vitro* samples.

Quantification of results.

The concentrations of analytes in *in-vivo* samples were calculated using the calibration graphs shown in sections 3.3.9 and 4.2.2. Each value took into account the 3 times concentration that was incurred during extraction and purification of the samples. However, the actual concentrations in the milk may have been greater than those reported in Tables 5a-c due to possible loss of analyte during work up.

Pathogen identification.

Identification of the pathogen involved in the three *in-vivo* mastitis cases was conducted at the Seale Hayne Faculty of Agriculture and Land Use, of the University of Plymouth using standard procedures. Tests for *Staphylococcal* strains were performed on Blood agar whereas *Streptococci* and Coliforms were grown on a Blood Edwards plate. All plates were streaked with sample milk using a 0.5mm loop cleaned with a surfactant and flame followed by incubation at 37°C for twenty four hours.

5.2 *In-vivo* Mastitis case study one.

Farm details.

The first of the three *in-vivo* mastitis case studies was carried out on a Derbyshire farm owned by Mr Alan Bowler. A family run concern, the 78 Friesian-Holstien herd is milked in a 6-12 herringbone milking parlour where 6 cows are milked at once. Herringbone milking parlours, shown in fig. 5.1, are by far the most common in the UK where the cows are brought into angled cells either side of a central pit. While the first batch is being milked the next is brought into the standings on the other side of the pit. Once milking of the first batch is completed and the teat cups have been transferred to the second batch they are allowed to leave the parlour. This system is used when there is only one set of collector units for both sides of the parlour as is the case on Mr Bowler's farm.

Routine sanitation.

The herd calves mainly in the autumn with 33% spring calving. Dry cow therapy is applied using Leo Red intramammary preparation. The cows teats are dry wiped before milking but if really dirty they are washed with Chlorhexidine udder wash and then dried before milking. After

milking the cows teats are dipped using proprietary iodophor post milking teat dip. The milking parlour machinery is cleaned in the mornings with a hot caustic wash and in the evenings with a cold acid wash.

Mastitis detection.

The average cell count of 195,000 cells/ml of milk occasionally rises to >250,000 because of out-breaks of mastitis. The majority of the mastitis, typically 4-5 cases, occurs soon after calving, with "a couple of cases during lactation and the odd one towards drying off". The mastitis pathogen most often detected in the herd is *Staph. aureus* with the occasional coliform infection. Mr Bowler is concerned about *Staph. aureus* resistance to penicillin used in intramammary treatments and dry cow therapy. In the past Mylipen QR and Streptopen QR, both penicillin containing treatments, were extensively used as the "first line of defence" against clinical mastitis but both are now ineffective against most infections. This meant that a broad spectrum antibiotic treatment needed to be employed, whereas penicillin alone (Mylipen QR) would have been more convenient for this work. Penicillin resistance is a major concern and it was, therefore, not possible to use a penicillin only product for the *in-vivo* studies.

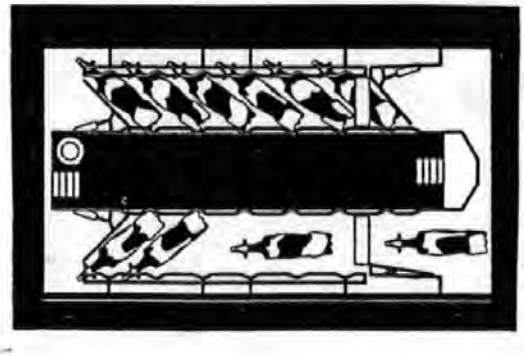


Fig. 5.1 Diagram of a Herringbone milking parlour commonly used throughout the UK.

5.2.1 Establishment of routine procedure.-Mastitis case 1.

Intramammary infusions of Tetradelta, the contents of which are reported in section 1.5.3, were used to treat a *Staph. aureus* infection in a seven year old cow listed as "Donna". She had suffered from mastitis infections for most of her milk producing life, typically two out of three lactation cycles. Clinical mastitis was detected at the morning milking of 19/04/95 and the first intramammary treatment was administered after milking, with samples taken prior to milking. A second treatment was made after twenty four hours at the morning milking of 20/04/95 and milk samples collected from the next seven consecutive milkings, finishing at the evening milking on 23/04/95.

5.2.2 Results from mastitis case1.

The presence of penicillin and/or any of its breakdown residues was confirmed by co-injection of extracted samples with aqueous standards of fresh procaine benzylpenicillin and solutions containing degradation compounds. Only the more relevant electropherograms are shown in this section to avoid unnecessary repetition of similar results. Observations made from the analysis of samples collected at each milking are summarised in Table 5a and are discussed in more detail as follows:-

19/04/95 (am)

The sample was collected pre-treatment and consequently showed no evidence of penicillin or any of its breakdown residues. The milk was visibly more viscous than normal un-infected milk, with large aggregates of cellular material. The sample required a centrifugation step before extraction with acetonitrile to facilitate accurate volume measurements of the sample. The electropherogram produced, fig. 5.2, from the extracted sample showed evidence of greater amounts of milk matrix interference than seen in *in-vitro* samples, probably as a result of the increase in concentrations of low molecular weight fatty acids and whey proteins.

19/04/95 (pm)

The sample was taken ten hours after the initial intramammary infusion. A large penicillin peak was detected at t_m 19.20(min). A peak at t_m 28.85(min), which was not present in the previous sample, was found to co-migrate with the 5R,6R benzylpenicilloic acid diastereoisomer. Milk quality was poor due to the severity of mastitis and a pre-centrifugation step was again required.

20/04/95 (am)

Sample collection was twenty four hours after the first infusion of Tetradelta. As shown in fig. 5.3 penicillin was detected at t_m 20.53(min) in a concentration of 14mg l^{-1}, a reduction of 54% from that detected in the previous sample. This reduction was calculated using the relative areas under the penicillin peak. A small peak again co-migrated with the 5R,6R penicilloic acid diastereoisomer at t_m 29.65(min). A second tetradelta infusion was made following sample collection. The quality of the quarter milk was much improved from earlier samples, being less viscous but still requiring a pre-centrifugation step. This indicated that the treatment was having a beneficial effect.

20/04/95 (pm)

Ten hours following the last infusion, the penicillin levels had apparently increased because of the second Tetradelta infusion at the morning milking 20/04/95. Penicilloic acid was again detected at t_m 29.02(min). The milk was virtually free of solid material which indicated inflammatory response and centrifugation of the sample was unnecessary.

21/04/95 (am)

Sample spoilt in transit. No analysis was possible.

21/04/95 (pm)

The sample was collected approximately thirty six hours following the last infusion of Tetradelta. The penicillin was present as a small peak at t_m 23.02(min) and the 5R,6R penicilloic acid was detected at t_m 33.21(min).

22/049/5 (am)

Forty eight hours after the last Tetradelta infusion, the penicillin was below the levels of detection, however, a small peak still co-migrated with the 5R,6R penicilloic acid diastereoisomer.

22/04/5 (pm)

Sample spoilt in transit. No analysis was possible.

23/04/95 (am)

Penicillin was not detected in the last sample of the 72 hour withdrawal period. Benzylpenicilloic acid, however, was still evident at t_m 32.15(min) as shown in fig. 5.4.

5.2.3 Discussion.

From the milk samples collected it was possible to detect a visual change in the composition of the milk, as inflammation decreases and the secretion becomes less viscous. Electropherograms obtained from MECC analysis of samples collected early in the treatment period show a much greater degree of milk matrix interference than those towards the end of the withdrawal period. As mentioned previously it was concluded that this was due to the greater concentration of low molecular weight proteins and fatty acids caused by the on-set of a mastitis infection (Heeschen *et al*, 1985. See Table 1e on page 34).

From the results, summarised in Table 5a, it can be seen that penicillin reaches a maximum concentration of 34.1mg l^{-1} at the first milking following the second infusion of Tetradelta. A gradual decrease in the concentration of penicillin can be seen following treatments, as the antibiotic is excreted in subsequent milkings.

Evidence from the electropherograms suggests that only one major benzylpenicillin degradation compound is present in milk following intramammary infusion of Tetradelta for the treatment of mastitis. The results suggest that benzylpenicilloic acid is produced as the more kinetically stable 5R,6R epimer (t_m 29.65min fig 5.3) and is present in gradually falling concentrations in the milk up to the 72 hour withdrawal period. It reached a maximum concentration of 2.9 mg l^{-1} 24 hours

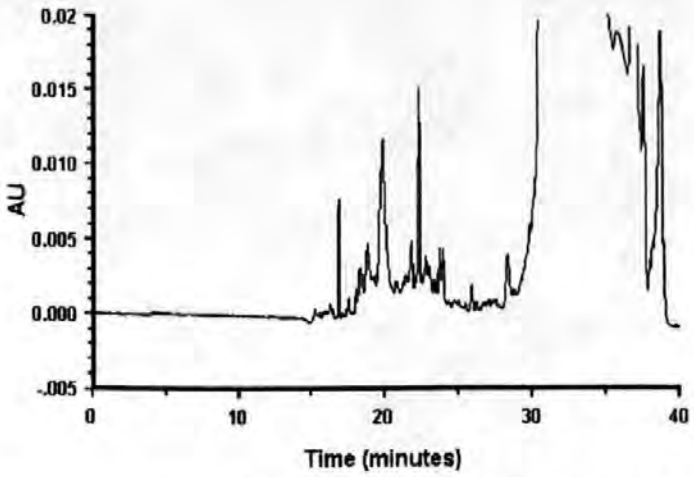


Fig. 5.2 Electropherogram obtained from the extraction and analysis of the milk sample taken at 19/04/95(am) from the cow listed as 'Donna' when mastitis was first detected. Following sample collection the first intramammary infusion of Tetradelta was made.

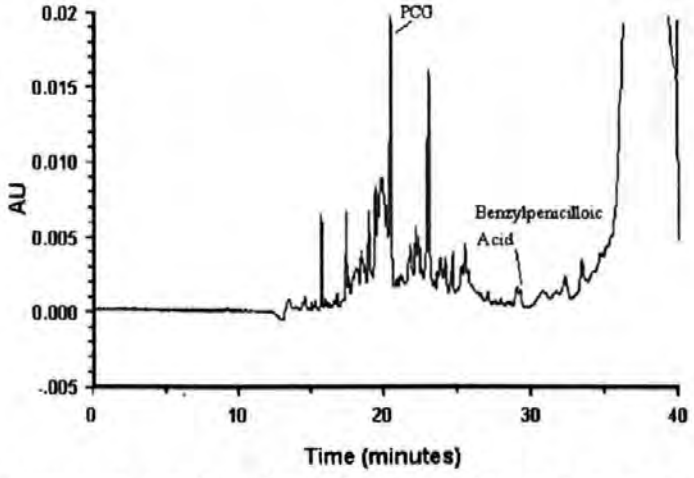


Fig. 5.3 Electropherogram obtained from the extraction and analysis of the milk sample collected 20/04/9(am) from 'Donna', 24 hours after the initial treatment of Tetradelta. Penicillin is resolved at t_m 20.53(min) and benzylpenicilloic acid at t_m 29.65(min).

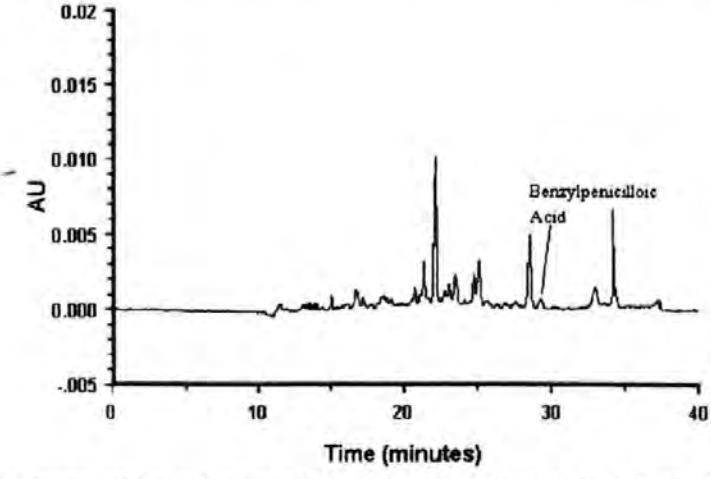


Fig. 5.4 Electropherogram of extracted milk sample collected at 23/04/95(am) from 'Donna', 96 hours after the initial Tetradelta treatment. No evidence of the presence of penicillin was detected, however, benzylpenicilloic acid was resolved at t_m 28.32(min).

Table 5a. Summary of the results obtained from a single analysis of samples taken at each milking from *in-vivo* case one.

Infusion Made	Date	Time	Benzylpenicillin concentration ($mg\ l^{-1}$)	Benzylpenicilloic acid concentration ($mg\ l^{-1}$)	Aesthetic quality of milk
•	19/04/95	am	—	—	Milk viscous with large amounts of inflammation.
	19/04/95	pm	30.7	2.7	"
•	20/04/95	am	14.0	2.9	Milk less viscous with reduced amounts of inflammation.
	20/04/95	pm	34.1	2.1	Milk virtually free of inflammation indicators.
	21/04/95	am	—	—	—
	21/04/95	pm	0.5	2.0	Milk had normal appearance
	22/04/95	am	—	1.6	"
	22/04/95	pm	—	—	—
	23/04/95	am	—	1.6	"

after the first infusion of Tetradelta, when high concentrations of antibiotic had been available for degradation by β-lactamase produced by the infecting *Staph. aureus* micro-organisms. From this hypothesis it may be predicted that the concentration of penicilloic acid would have reached a maximum in the sample collected at the morning milking of 21/04/95, had the sample not been spoiled in transit.

Two intramammary infusions of Tetradelta proved sufficient for a clinical recovery of the cow from the *Staph. aureus* infection and the milk was re-admitted to the bulk tank after the 72 hour withdrawal period.

5.3 *In-vivo* mastitis case study two and three.

Farm details and mastitis detection.

The second and third *in-vivo* experiments were conducted on Oakerthorpe farm, near Alfreton, Derbyshire owned by Mr David Easom. This is a Diversy Ltd. trial site and "flagship" farm with 295 Friesian-Holstien cows. Milking is performed in a 20-20 herringbone milking parlour (i.e. maximum of twenty cows milked at once). The herd calves all year round and consequently mastitis is an ever present problem with an average of two cases per week. The average cell count is 212,000 cells/ml of milk which is currently falling slowly. Infections are mainly *Staph. aureus* but some coliforms and *Str. uberis*, approximately 10% of all cases, do occur. Recently there have been small outbreaks of *Str. agalactiae* infections. The herd is divided into three with the high yielders kept in the cow shed all the time except in the dry period; the medium yielders which are kept in the cow shed except between June and September and the low yielders which are put out to grass when the conditions are appropriate.

Routine sanitation.

The parlour machinery is cleaned after the morning milking with cold acid wash and after the evening milking with cold hyporchlorite. Consequently two hot caustic washes are made every week to prevent any build-up of milk protein in the plant. Mr Easom uses dry cow therapy with Leo Red although he is using Auremycin, a non-penicillin formulation, sporadically because of concerns over penicillin resistance. The cows teats are carefully sprayed after milking with Deosan Superxcel, an iodophor based teat spray.

5.3.1 Establishment of routine procedure.-Mastitis case two.

In-vivo case two was a *Staph. aureus* infection of a 5 year old listed as 4/80. Two intramammary infusions of Leo Yellow, a broad spectrum antibiotic treatment (contents reported in section 1.5.3), were made at the morning milking on 19/04/95 and the second twenty four hours later. Samples were collected at each of the next six milkings until the end of the 72 hour withdrawal period. Two extra samples were collected after the withdrawal period at twenty four hour intervals to investigate the persistence of any penicillin degradation compounds in the milk.

5.3.2 Results from mastitis case 2.

As in the previous *in-vivo* experiment, the presence of the compounds of interest was confirmed by co-injection of aqueous standards. Again only the more relevant electropherograms are shown in this section to avoid unnecessary repetition of similar results. The results obtained were as follows:-

19/04/95 (am)

The sample was collected pre-treatment and showed far more milk-matrix interference than in the previous case study (i.e. the mastitis infection was more severe). The sample required an initial centrifugation to remove solid gel-like material before accurate volumetric measurement could be made. The resultant electropherogram is shown in fig. 5.5.

19/04/95 (pm)

As in the previous sample, the milk collected approximately ten hours following the first treatment was extremely viscous and required centrifugation before extraction. As shown in fig. 5.6 a large penicillin peak was resolved at t_m 22.37(min). Also evident on the resultant trace was a large peak at t_m 31.59(min) which co-migrated with 5R,6R benzylpenicilloic acid.

20/04/95 (am)

Twenty four hours after the initial infusion, the milk retained its viscous consistency with large aggregates of solid material contributing to the generally poor quality. Again the milk sample needed to be centrifuged before extraction. The area under the penicillin peak, at t_m 22.54(min), had reduced by 88%. 5R,6R benzylpenicilloic acid was again resolved at t_m 30.97(min). A second Leo Yellow infusion was made after collection of this sample.

20/04/95 (pm)

The sample was collected approximately ten hours after the last Leo Yellow infusion. Indicators of inflammation were still apparent in the secretion, which again needed to be centrifuged before extraction with acetonitrile. The penicillin peak, at t_m 22.29(min), had apparently increased in size because of the repeated treatment as had the 5R,6R benzylpenicilloic acid occurring at t_m 32.19(min).

21/04/95 (am)

Twenty four hours after the second infusion penicillin could still be detected at t_m 22.13(min), fig. 5.7, although the area under the peak had reduced by 92% from the previous milking. Milk quality was considerably improved and volumes of milk could be accurately measured without the necessity of pre-centrifugation. 5R,6R benzylpenicilloic acid appeared at t_m 32.33(min) with levels 56% less than recovered at the previous milking.

21/04/95 (pm)

Penicillin was resolved at t_m 22.15(min) with a recovery 73% lower than in the sample taken at the 21/04/95(am) milking. The concentration of penicilloic acid was reduced by 69% from that detected in the sample 21/04/95 (am).

22/04/95 (am)

Neither penicillin nor penicilloic acid were recovered in the extracted sample. The milk had a normal appearance.

22/04/95 (pm), 23/04/95 (am), 24/04/95 (am)

None of these milk samples showed evidence of penicillin or penicilloic acid. The milk retained its normal appearance without evidence of inflammation.

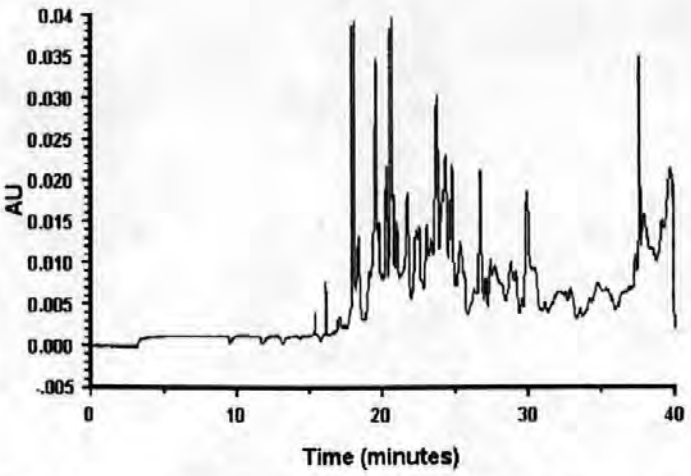


Fig. 5.5 Electropherogram from the extraction and analysis of the milk sample collected at 19/04/95(am) from cow 4/80 when mastitis was first detected. After sample collection the first intramammary infusion of Leo Yellow was made.

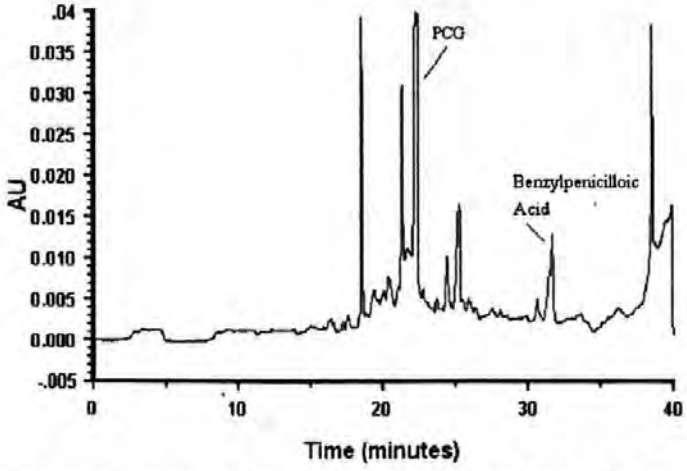


Fig. 5.6 Electropherogram from the extraction and analysis of the sample collected 19/04/95(pm) from cow 4/80, 10 hours after the initial treatment of Leo Yellow. Penicillin was resolved at t_m 22.37(min) and benzylpenicilloic acid at t_m 31.59(min).

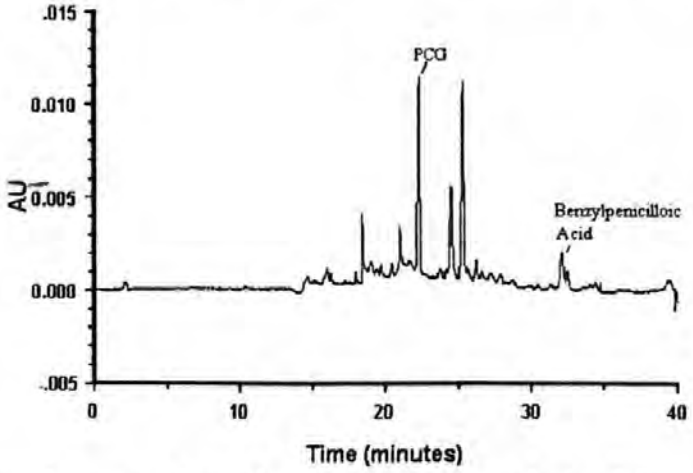


Fig. 5.7 Electropherogram from the extraction and analysis of the sample collected 21/04/95(am) from cow 4/80, 24 hours after the second treatment of Leo Yellow. Penicillin was detected at t_m 22.13(min) and benzylpenicilloic acid at t_m 32.13(min).

5.3.3 Discussion.

As in the previous case study a general improvement in the aesthetic quality of the milk was observed as the treatment proceeded. Initial samples required a pre-centrifugation step to allow accurate measurement of sample volumes.

The results, summarised in table 5b., show that two infusions of Leo Yellow containing penicillin were made at the morning milkings of 19/04/95 and 20/04/95. The penicillin concentration reached a maximum of 51.3mg l^{-1} at the evening milking of 20/04/95. High levels of up to 91.2% of the antibiotic were excreted at subsequent milkings but persisted in the milk at detectable levels thirty six hours after the last infusion. Again only one major benzylpenicillin degradation compound was detected in the samples collected. 5R,6R benzylpenicilloic acid was evident at the highest concentration (10.6mg l^{-1}) in the sample taken following the initial treatment, when *Staph. aureus* numbers and consequently β-lactamase concentration would also be at their highest. After the second infusion the numbers of *Staph. aureus* would be expected to be reduced because of the action of the antibiotics, and therefore, the concentration of β-lactamase would also be lower resulting in less breakdown of the penicillin to penicilloic acid. Levels of the penicilloic acid residue gradually decreased as the penicillin was excreted from the milk reaching a minimum of 1.1mgl^{-1} at the evening milking of 21/04/95.

The electropherograms obtained from the MECC analysis of extracted samples showed far less milk matrix interference in samples collected closer to the withdrawal period, as the mastitis infection was treated. The *Staph. aureus* infection of the cow 4/80 was cured by the two Leo yellow infusions and the milk was re-admitted to the bulk tank 72 hours after the last treatment.

5.3.4 Establishment of routine procedure.-Mastitis case three.

The third *in-vivo* study was a chronic case of *Staph. aureus* clinical mastitis. The three year old cow, listed as 8/4, was treated initially with Synulox (a broad spectrum antibiotic infusion containing 50 mg Clavulanic acid and 200mg amoxycillin) but without success. This was

Table 5b. Summary of the results obtained from a single analysis of samples taken at each milking from *in-vivo* case two.

Infusion Made	Date	Time	Benzylpenicillin concentration ($mg\ l^{-1}$)	Benzylpenicilloic Acid concentration ($mg\ l^{-1}$)	Aesthetic quality of milk
•	19/04/95	am	—	—	Milk viscous with large amounts of inflammation.
	19/04/95	pm	41.2	10.6	"
•	20/04/95	am	5.3	2.1	Milk less viscous but still containing large clumps of cellular material.
	20/04/95	pm	54.3	5.7	Secretion still showed signs of inflammation but was more fluid than previous samples.
	21/04/95	am	4.8	2.7	Milk more fluid with only small signs of inflammation.
	21/04/95	pm	1.5	1.1	Milk free from signs of any mastitis infection.
	22/04/95	am	—	—	"
	22/04/95	pm	—	—	"
	23/04/95	am	—	—	"
	24/04/95	am	—	—	"

followed by four Leo Yellow infusions and samples taken at subsequent milkings. All of the milk samples contained signs of inflammation and needed to be centrifuged before any volumetric measurements were possible.

5.3.5 Results from mastitis case 3.

As in the previous *in-vivo* case studies, identification of penicillin and any of its metabolites was confirmed by co-injection with the relevant aqueous standards.

21/04/95 (am)

The milk taken before the initial infusion of Leo Yellow was, as expected, free from contamination of penicillin and consequently its degradation residues. The milk was extremely viscous having an almost gel-like appearance. The electropherogram produced from the sample extract is shown in fig. 5.8. As can be seen, the trace is very complex indicating the severity of the infection.

21/04/95 (pm)

Penicillin was resolved at t_m 19.36(min) and 5R,6R benzylpenicilloic acid at t_m 27.63(min).

22/04/95 (am)

Twenty four hours after the initial treatment, the penicillin persisted in the milk at t_m 19.12(min) in a high concentration with levels only reduced by 11.6% from the previous milking. This persistence of high levels of the antibiotic was probably a result of reduced secretion, commonly observed in mastitis infections. Consequently, high concentrations of antibiotic would be expected to be subjected to β-lactamase enzymes produced by the penicillin resistant *Staph. aureus* during the twelve hour period between milkings. As shown in fig. 5.9 this resulted in the detection of a large penicilloic acid peak at t_m 27.05(min); 35.8% larger than that recorded in the extract from the previous sample. The quality of the milk was still affected by the mastitis and following sample collection, another Leo Yellow infusion was made.

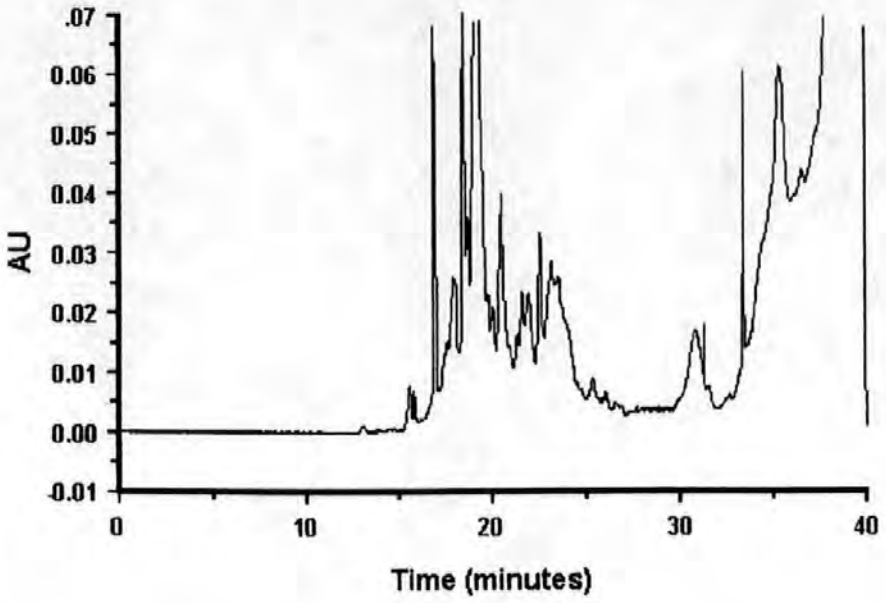


Fig. 5.8 Electropherogram obtained from extraction and analysis of the milk sample collected at 21/04/95(am) from cow 8/4 when mastitis was first detected. Following sample collection the initial intramammary infusion of Leo Yellow was made.

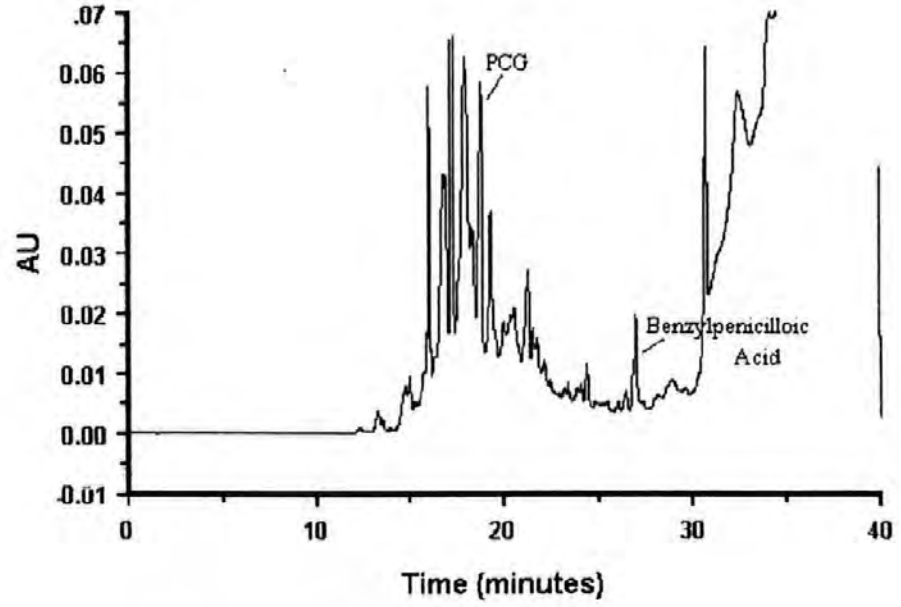


Fig. 5.9 Electropherogram obtained from the extraction and analysis of the milk sample collected at 22/04/95(am) from cow 8/4, 24 hours after the initial treatment of Leo Yellow. Penicillin was detected at t_m 19.12(min) and benzylpenicilloic acid at t_m 27.05(min).

22/04/95 (pm)

Sample spoilt in transit. No analysis was possible.

23/04/95 (am)

The concentration of penicillin was higher than previously recorded by 35%. The concentration of penicilloic acid, however, was 73% lower than that in the secretion of 22/04/95(am), possibly as a result of reduced numbers of β-lactamase producing bacteria due to the bactericidal action of the antibiotics. The milk was slightly less viscous than that taken in earlier milkings but the quality was still poor with large clumps of cellular material. A third Leo Yellow infusion was made after milking.

23/04/95 (pm)

The penicillin concentration reached a maximum in this secretion with levels 31% greater than in the previous sample. Penicilloic acid concentration had also increased by 50%. This would indicate that the bacterial population had increased again, hence the observation of no cure being effected.

24/04/95 (am)

Concentrations of both penicillin and penicilloic acid had decreased (34% and 59.5% respectively) from the levels extracted in the previous milking. The milk quality was still very poor although the sample was visibly more fluid than that collected before the first treatment.

24/04/95 (pm)

A reduction of 56% of the penicillin was evident and penicilloic acid was not detected.

25/04/95 (am)

The only contaminant detected was a small penicillin peak resolved at t_m 20.79(min). The concentration had decreased by 75% from that detected in the previous sample taken 24/04/95 pm. A fourth and final infusion was made after milking.

Table 5c. Summary of the results obtained from a single analysis of samples taken at each milking from *in-vivo* case three.

Infusion Made	Date	Time	Benzylpenicillin concentration ($mg\ l^{-1}$)	Benzylpenicilloic Acid concentration ($mg\ l^{-1}$)	Aesthetic quality of milk
•	21/04/95	am	—	—	Milk extremely viscous with a gel-like consistency
	21/04/95	pm	30.1	3.8	"
•	22/04/95	am	26.6	10.2	"
	22/04/95	pm	—	—	—
•	23/04/95	am	40.7	2.9	"
	23/04/95	pm	60.0	5.5	"
	24/04/95	am	38.9	2.4	Milk slightly less viscous although still with considerable evidence of inflammation
	24/04/95	pm	17.2	—	"

Table 5c. (continued)

Infusion Made	Date	Time	Benzylpenicillin concentration (mgl^{-1})	Benzylpenicilloic Acid concentration (mgl^{-1})	Aesthetic quality of milk
•	25/04/95	am	2.1	—	Milk less viscous although inflammation still evident
	25/04/95	pm	43.5	2.7	Milk more fluid with inflammation still present
	26/04/95	am	24.5	—	"
	26/04/95	pm	12.6	—	"
	27/04/95	am	2.3	—	Milk less flowing with increased amounts of cellular material
	27/04/95	pm	—	—	"
	28/04/95	am	—	—	"
	28/04/95	pm	—	—	Secretion essentially the same as the initial sample

25/04/95 (pm)

A large penicillin peak was evident at t_m 21.03(min) with penicilloic acid also present at t_m 30.03(min). Inflammation in the milk was still obvious despite the four infusions of Leo Yellow, and the earlier Synulox treatment.

26/04/95 (am)

A 38% reduction in the extracted penicillin was obtained in the sample, however, benzylpenicilloic acid was not detected. The milk retained a poor aesthetic quality but was more fluid than in previous samples. The reduced viscosity of the milk suggested that the yield had increased and this could explain the greater rate at which the antibiotic was excreted from the mammary gland.

26/04/95 (pm), 27/04/95 (am), 27/04/95(pm), 28/04/95 (am) and 28/04/95 (pm).

The penicillin was gradually excreted from the milk, the concentration being below the limit of detection 48 hours following the fourth and final treatment (i.e. 25/04/95(am). During the treatment period there were only minor changes in the aesthetic quality of the secretion. In the final sample collected at the evening milking on 28/04/95 indicators of inflammation were still clearly evident.

5.3.6 Discussion.

The results obtained in the third *in-vivo* experiment are quantified and summarised in Table 5c. An increase in the concentration of penicillin was evident at each milking following treatment, reaching a maximum of 60mg l^{-1} 12 hours after the third infusion, at the evening milking of 23/04/95. The persistence of penicillin after and during the first three infusions is noteworthy, with a build-up of high levels of the antibiotic in the milk persisting for twenty four hours after treatment had ceased. Excretion rate of penicillin at this stage in the treatment period was 11.6% of the detected load (calculated from the area under the penicillin peak on the electropherograms obtained). This indicates that the yield of milk obtained at each milking was considerably reduced by the mastitis infection, as is often observed. The antibiotic did not persist in the milk at such high levels after the fourth infusion possibly due to an increase in the yield as the milk became

less viscous and showed aesthetic improvement. This indicated that the treatment was having some benefit at this stage. The excretion rate at this stage was approximately 45% of the penicillin load. The concentration of 5R,6R penicilloic acid reached a maximum of 10.2mg l^{-1} in the sample collected 22/04/95 (am), twenty four hours after the initial Leo Yellow infusion, when the penicillin concentration was high. Presumably at this stage in the treatment the *Staph aureus* population was also relatively large and therefore the concentration of β-lactamase would also be considerable. Despite the penicillin persisting at high concentrations in samples prior to that collected at 22/04/95 (am), penicilloic acid levels remained low. This probably occurred due to conditions approaching biostasis resulting from elevated amounts of antibiotic in the milk.

The quality of the milk at the time of the first treatment was very poor with large amounts of cellular material giving the secretion an almost gel-like consistency. The milk gradually became more fluid after three infusions of the Leo yellow preparation and the aesthetic quality improved. This resulted in the excretion of penicillin in greater concentrations because of a greater milk yield. However, forty eight hours after the fourth treatment the quality of the milk began to deteriorate with the return of indicators of inflammation. Electropherograms obtained from MECC analysis of extracted samples showed a large degree of matrix interference similar to that evident in figs. 5.8 and 5.9. The mastitis infection of cow 8/4 was not cured by the intramammary infusions of Leo Yellow and the animal had subsequently to be destroyed.

5.4 General conclusions from *in-vivo* case studies one, two and three.

Each of the three *in-vivo* mastitis case studies was an example of clinical mastitis caused by the micro-organism *Staph. aureus*. Two different types of intramammary infusions were used to treat the infections, however, the concentrations of benzylpenicillin found in the milk samples of each case were comparable. The relative rates at which the penicillin was excreted from the milk however, were different. This may be explained by different milk yields which are affected by the severity of the infection. One intramammary infusion of 150mg of benzylpenicillin, irrespective of the type of preparation administered, was found to cause contamination of milk collected at the next milking at a concentration of 30-40mg l^{-1}. Contaminated milk of this kind may have detrimental effects on the health of penicillin hypersensitive individuals, despite being diluted by

the milk in the bulk tank. Hence the milk withdrawal period for these products is typically at least 72 hours.

The *in-vivo* work indicates that of the 4 major benzylpenicillin degradation compounds investigated, only one is present in milk following treatments for clinical mastitis caused by *Staph. aureus*. The results obtained suggest that 5R,6R penicilloic acid is present in milk samples at concentrations approaching $10mgl^{-1}$, when intramammary infusions of Leo Yellow are made.

Benzylpenicilloic acid may persist in the milk throughout the seventy two hour withdrawal period following infusion with Tetradelta. One explanation for this persistence is that the penicillin is present as the procaine salt in Tetradelta, and is more stable than in other forms. Consequently it is more likely to be absorbed into the tissues, and if retained for longer it will available for degradation by β-lactamase which is produced by *Staph. aureus* that may reside in scar tissue.

The concentrations of penicilloic acid were found to be greater in milk collected after Leo Yellow infusions than in Tetradelta samples. This was probably due to the greater stability of the procaine penicillin administered in Tetradelta compared to the free base present in Leo Yellow.

Chapter 6

General Conclusions, Future Work and References

6.1 General Conclusions.

HPLC was found to be unsuitable for the analysis of benzylpenicillin degradation compounds from milk. The irreversible adsorption of hydrophobic compounds from milk samples necessitated regular replacement of column guard cartridges which proved expensive. Initial extraction techniques used were adaptations of existing methods reported in the literature for the detection of penicillin residues. However, the degradation compounds of benzylpenicillin were more polar than the antibiotic residue itself, being insoluble in organic solvents such as DCM. Consequently the only method available for the separation of these compounds from the water soluble milk matrix components was solid phase extraction. Despite extensive sample purification which took into consideration the presence of proteins, lipids and carbohydrates, the principle constituents of milk, HPLC failed to provide the required resolution for the detection of the benzylpenicillin degradation compounds from *in-vitro* milk samples.

Micellar Electokinetic Capillary Chromatography (MECC) was used as an alternative chromatographic approach. It was successfully applied to the separation and detection of four of the eight benzylpenicillin degradation compounds, detected in aqueous solution, from *in-vitro* milk samples. Resolution of one degradation compound was affected by the presence of minor milk matrix interference and changes in chromatographic conditions, including varying the pH and concentration of organic modifier, failed to obtain complete resolution. The extraction of benzylpenicillin and its breakdown residues from milk was complicated by the rapid degradation of the antibiotic residue when introduced to the Amberchrom extraction column in aqueous solution. This problem was avoided by stabilizing the pH environment on the extraction column using phosphate buffer, pH 7.0. The automation and adaptability of MECC was found to suit this type of analysis.

Once the optimum MECC system and extraction technique had been developed, they were applied to the analysis of *in-vivo* samples collected from mastitis cases treated with two different intramammary infusions. In all cases the pathogen causing the infection was *Staph. aureus*; a gram positive bacteria, of which certain strains are penicillin resistant thus producing β-lactamase which catalyses the rapid conversion of benzylpenicillin to benzylpenicilloic acid.

Benzylpenicilloic acid was the only penicillin degradation compound found in the three *in-vivo* cases, and when the penicillin was administered as the more stable procaine salt, as in Tetradelta intramammary infusions, the penicilloic acid persisted in samples up to the 72 hour withdrawal period. This may have been due to the benzylpenicillin being absorbed into the epithelial tissues of the quarter, and when slowly released degraded by β-lactamase produced by *Staph. aureus* bacteria residing in scar tissue. When benzylpenicillin was administered as the free base, as in Leo Yellow infusions, a rapid degradation was found to occur, with higher concentrations of penicilloic acid being detected. This was a consequence of the free base penicillin residue being more labile than the procaine salt.

The degree of matrix interference increased in milk samples showing signs of mastitis, reflecting the severity of the infection. This may in future with further research be used as a test for sub-clinical mastitis, for example if indicative compounds can be detected in the milk at an early stage of an infection.

The majority of practical work in this thesis focused on development of the extraction procedure, which was required to provide adequate removal of interfering components from the milk matrix for the resolution of the compounds of interest. Once this was achieved and the maximum number of degradation compounds could be simultaneously resolved from *in-vitro* samples, work on the identification of these residues began. Identification and preparation of one major benzylpenicillin degradation compound, found in aqueous solution, was not completed due to impurity of purchased starting materials which resulted in an impure synthetic product. The instability of the starting material hampered attempts to purify it before synthesis. However, the peak was identified theoretically as benzylpenamaldic acid, as this was the only known major acid/base degradation residue of penicillin not to be successfully prepared.

6.2 Future work.

Possible future work arising from this research includes the following:-

1. The derivatization of benzylpenicilloic acid following sample extraction may enhance the limit of detection (1.0 mg l^{-1} found with the existing procedure) and allow determination of trace concentrations in secretions approaching the 72 hour withdrawal period. This may lead to different procedures of work up and analysis.

2. Using a quarter milker, which was not available for use on any of the existing trial sites, the volume of milk from mastitis infected quarters could be measured. This would allow the accurate quantification of penicillin and any degradation compounds.

3. In mastitis cases treated with Tetradelta or other infusions containing procaine benzylpenicillin, milk samples should be taken at subsequent milkings after the 72 hour withdrawal period. This would examine the theory that if the penicillin is absorbed into the epithelial tissues and slowly released it becomes available for degradation by β-lactamase in the milk produced by bacteria residing in scar tissue. It would also indicate if benzylpenicilloic acid is present in milk taken after the withdrawal period which is made available to the consumer. For this study, work on reducing the detection limits of the existing approach would be required.

4. Further *in-vivo* cases should be treated with other benzylpenicillin containing intramammary infusions. Mylipen 300QR contains 300,000 iu. (approximately 200mg) which would increase the concentration of penicillin in the secretion and consequently the concentration of degradation compounds for detection. Such a study, however, would not be practical on commercial farms because of penicillin resistance, which would endanger the health of the test animal.

5. *In-vivo* cases from mastitis infections caused by bacteria other than *Staph. aureus* should be studied. Common mastitis pathogens such as *E. Coli*, *Str. agalactiae* and *Str. uberis* may cause changes in the rate or direction of penicillin degradation.

6. Investigation into the possibility of degradation of other antibiotics commonly used in mastitis treatments such as novobiocin, penicillin-V ampicillin and amoxacillin using similar methodology; and in particular the persistence of any degradation residues beyond the withdrawal period.

6.3 References.

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