THE USE OF ^{1}H-NMR ANALYSIS OF URINE TO DISCRIMINATE BETWEEN CALCIUM OXALATE KIDNEY STONE PATIENTS AND HEALTHY CONTROLS

by

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ABSTRACT

Calcium oxalate kidney stone formation is a complex process, which is dependent on many variables. The exact combination of physicochemical circumstances that causes stones to form has not yet been completely unraveled. There is still a need for a simple method for distinguishing kidney stone patient urine from healthy subjects and a reliable measure of the overall risk of stone formation.

NMR has been used to study the metabolites found in body fluids, like urine, in the past three decades. It has more recently been used to study a large range of diseases and the toxicological effect of drugs on animals, by looking at metabolite patterns in the urine. The major advantages of using NMR are that it is fast, non-invasive, non-destructive and non-equilibrium perturbing technique, allowing the detection of a diverse range of compounds in a single experiment. The complexity of the resulting spectra has led to the application of pattern recognition methods to aid in spectral interpretation and sample classification according to the physiological or pathological state of the donor.

This thesis describes the development of a method of urine analysis using ^{1}H-NMR spectroscopy, with the aid of pattern recognition, to distinguish between urine from healthy subjects and calcium oxalate kidney stone patients.

^{1}H-NMR spectra were obtained of the urine of a number of calcium oxalate kidney stone patients and healthy control subjects. These were then integrated to produce

numerical spectral descriptors, which were analyzed using principal component analysis and canonical discriminant analysis. This approach enabled calcium oxalate kidney stone patients to be distinguished from healthy control subjects and allowed identification of the spectral descriptors that caused this separation. This model can be used to classify unknown samples as calcium oxalate kidney stone patients or healthy subjects or alternatively to assess the risk of stone formation.

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CHAPTER 1

KIDNEY STONE DISEASE

1.1 INTRODUCTION

Stone disease occurs in many organs in the human body, including the kidney, urinary bladder, prostate, gallbladder, salivary gland, and pancreas (Rodgers and Spector, 1981). Kidney stones, also known as renal calculi, are the most clinically important. High incidence figures occur world wide (greater than 10% in Western countries) (Baumann, 1998). Although it is not usually life threatening, stone disease is debilitating. It affects the functioning of the kidneys and causes the loss of a kidney in a small percentage of patients. It causes considerable pain and has a recurrence rate of 60 to 75% (Baumann, 1998). The occurrence of urinary calculi in the white population of South Africa is between 10 and 15 percent. It is interesting that the South African black population seems to be immune to this disease (Rodgers and Spector, 1981; Blacklock, 1982).

1.2 COMPOSITION OF STONES

Renal calculi consist of crystal aggregates embedded in a macromolecular matrix. They are about 97.5 percent polycrystalline aggregate and 2.5 percent glycoprotein or mucoprotein (Finlayson, 1974). There are five main types of renal calculi classified on the basis of their composition: uric acid, cystine, magnesium ammonium phosphate (struvite), calcium phosphate, and calcium oxalate. 70 to 80

percent of renal calculi contain calcium oxalate or a mixture of calcium oxalate and calcium phosphate crystals (Klugmann and Favus, 1995).

Most research is concentrated on calcium oxalate stones, because they are the most common and are difficult to manage as many are idiopathic. These are calculi for which no definitive cause can be identified on the basis of commonly accepted screening procedures. Such stones comprise approximately 75% of all renal stones (Ryall and Marshall, 1990). Underlying metabolic or genetic disorders only cause about 10 to 15% of all calcium stones (Robertson and Peacock, 1983). The study presented in this thesis focuses on calcium oxalate stone formation only.

1.3 EPIDEMIOLOGY

Since the late 19th century there has been a change in urolithiasis epidemiology, with a decrease in the incidence of vesical (bladder) stones and an increase in the incidence of upper urinary tract (renal and uretric) stones in technically advanced communities (Blacklock, 1982).

Vesical stones occurred frequently in the past and are currently prevalent in poor socio-economic communities. These are known as endemic stone regions. In these cases, stone formation takes place in the early years of life and is usually non-recurrent (Blacklock, 1982).

Upper urinary tract stones are more frequently found in technically developed regions including Europe, North America and Japan (Blacklock, 1982). It has been suggested that this may be linked to the higher dietary consumption of refined carbohydrate (in the form of sugar products) (Blacklock, 1982) or the high dietary protein intake in these communities (Rodgers and Spector, 1981). Stone formation has been linked to diet content for many years. Urinary oxalate has been identified as important in stone formation (Robertson and Nordin, 1982). A high intake of animal protein has been shown to increase urinary calcium, oxalate and uric acid thereby increasing the risk of stone formation (Rodgers and Spector, 1981).

Studies show correlations between stone incidence and epidemiological factors such as age, sex, climate, socio-economic conditions, hereditary factors, ethnic factors and dietary factors (Blacklock, 1982; Robertson and Peacock, 1983). Each of these can adversely influence one or more of the urinary risk factors, described later in Section 1.5.1, thereby increasing the incidence of stone formation. Figure 1.1 shows the epidemiological risk factors and their influence on urinary risk factors.

Sex and age linked incidence rates can be linked to differences in calcium and oxalate excretion levels in these groups (Hesse et al., 1986). The disease is predominant in males, who have double the incidence rate of females (Hesse et al., 1986). The recurrence rate in both sexes is high.

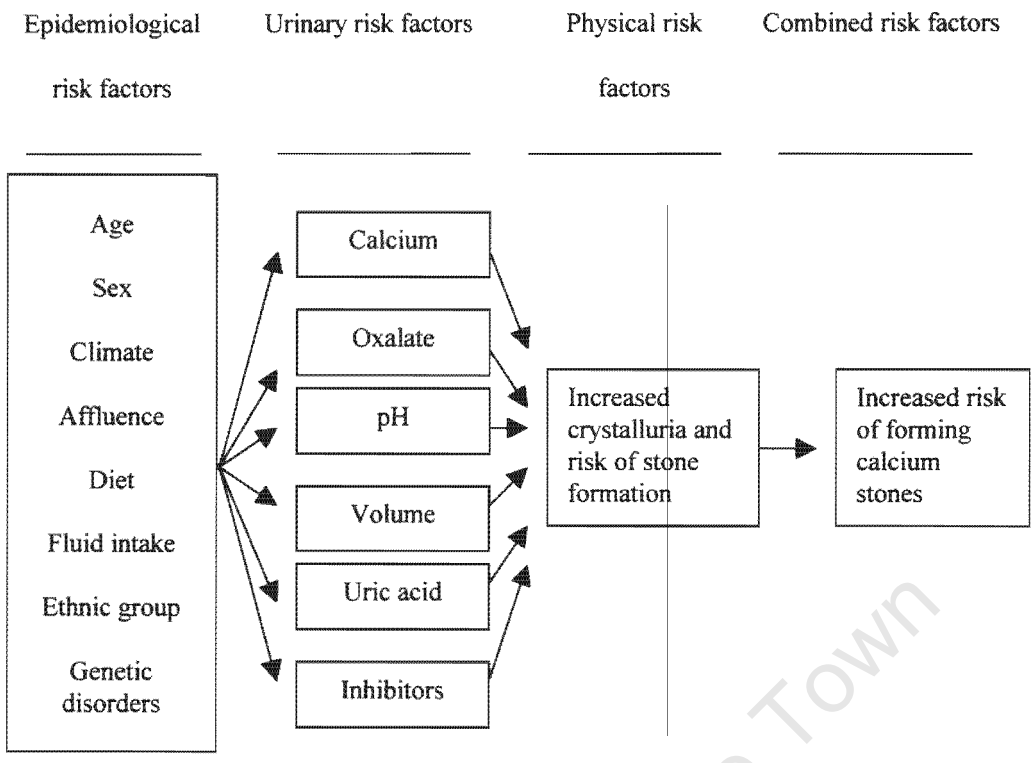


Figure 1.1 A model illustrating the effect of epidemiological risk factors on the physical risk of stone formation. Adapted from (Robertson and Peacock, 1983)

The geographical prevalence of urolithiasis in stone belts can be correlated to socio-economic conditions, climate and drinking water (Blacklock, 1982). Affluent socio-economic communities have higher dietary protein levels (Blacklock, 1982). This can lead to an excess excretion of stone salts and thereby an increased risk of stone formation. High atmospheric temperatures in a region cause dehydration and a resultant decrease in urine flow rate and an increase in the concentration of solutes in the urine. Drinking water composition may also increase the risk of stone formation in a specific region (Rodgers and Spector, 1981). Although several investigations into stone incidence and drinking water composition have been conducted, contradictory results have been reported (Churchill et al., 1978; Juuti and Heinonen,

1980). More recent studies have shown that mineral waters containing calcium and magnesium have prophylactic and therapeutic capability (Rodgers, 1997).

Metabolic or genetic disorders may cause stone disease e.g. renal tubular acidosis and hyperparathyroidism (Scheinman, 2000). These defects usually manifest as the loss of enzyme function, which leads to stone forming conditions. Therefore, hereditary factors will have an influence on stone formation.

Certain ethnic groups appear to be immune to urinary stones. These include the Indians of Mexico, Peru, Ecuador and Bolivia, the South African black population, Eskimos and Aborigines (Rodgers and Spector, 1981). The reason for this apparent immunity is unknown, but is likely to be linked to dietary custom and physiological characteristics, which cause higher concentrations of crystallization inhibitors in the urine (Rodgers and Spector, 1981).

1.4 KIDNEY STONE FORMATION

1.4.1 Physical chemistry of calcium oxalate stone formation

Stone formation is a crystallization process. It begins with supersaturation of the urine with respect to calcium and oxalate, favouring the formation of a crystal nidus (nucleation), crystal growth, and aggregation of calcium oxalate crystals. Crystallization in urine comprises two physicochemical aspects: a thermodynamic one, which includes supersaturation and results in nucleation of micro-crystals and a

kinetic one comprising rates of crystal nucleation, growth, aggregation and phase transformation (Hess and Kok, 1996). The rates of these processes will determine the phase, shape, size and number of crystals formed.

1.4.1.1 Supersaturation

The free energy required to drive these crystallization processes is associated with the supersaturation of urine with stone forming minerals (Finlayson, 1974). The greater the supersaturation the greater the free energy to drive stone formation. Free energy of supersaturation can be expressed as (Hess and Kok, 1996):

$$\Delta G = RT \ln\left(\frac{A_i}{A_0}\right)$$

where R is the gas constant, T is the temperature, and A_i and A_0 are the activity products of the unionised salt species in solution at any given condition (A_i) and at equilibrium (A_0) respectively. The ratio A_i/A_0 is referred to as supersaturation ratio (S). At S values >1, the urine is supersaturated.

Most urine is supersaturated with calcium oxalate. It is a result of the excretion of poorly soluble minerals, such as calcium oxalate, in a small urinary volume. Urine becomes supersaturated with the salt when the relative supersaturation rises above the solubility product (where the salt is at equilibrium with its bathing salt). The solution will remain stable until its formation product (metastable limit) is reached. This is the so-called metastable region (Robertson and Nordin, 1982). This is

illustrated in Figure 1.2. When the formation product is reached, the solution becomes unstable and new crystals will form spontaneously. Crystals will not spontaneously precipitate in metastably supersaturated solutions; in this region seed crystals must be added before crystallization will occur. A high degree of supersaturation is needed for spontaneous nucleation to occur. After nucleation, a metastable supersaturation level drives crystal growth and aggregation.

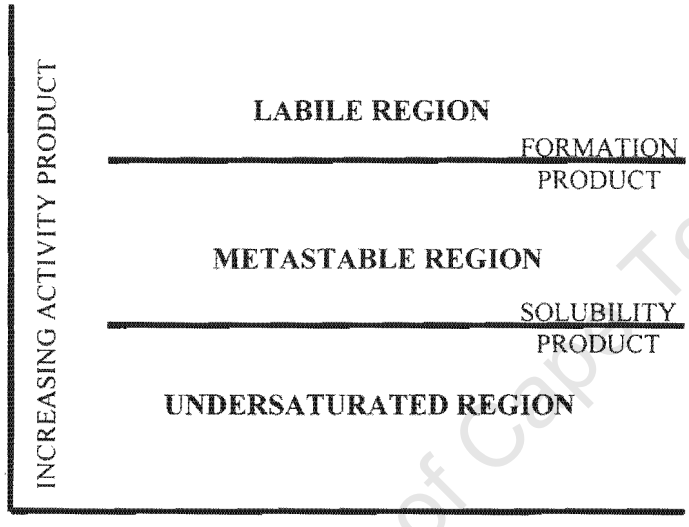


Figure 1.2 The saturation zones of urine relative to the solubility and formation products of the stone forming salts. Reproduced from (Robertson and Nordin, 1982)

Supersaturation of the urine with calcium oxalate is expressed as the ion activity product (Robertson and Nordin, 1982). This can be written as:

$$K_{CaOx} = [Ca^{2+}]f_{Ca}[Ox^{2-}]f_{Ox}$$

Where [ ] represents the free, ionised concentration of enclosed ion and f is the corresponding activity coefficient. Calculation of the ion activity product for calcium oxalate essentially involves measuring the total concentrations of all the main ionisable species in urine and calculating their free, ionized concentrations from the knowledge of the extent with which they interact to form soluble complexes and ion

pairs. This has been done using an iterative computer procedure (Robertson, 1969). This is a laborious process and therefore simplified methods for estimating the ion activity product have been derived and are discussed in Section 1.5.2. Relative supersaturation is now routinely calculated using the EQUIL software program (Werness et al., 1985; Brown et al., 1994).

As mentioned earlier, three different mechanisms of crystallisation have been identified as being of importance in kidney stone formation. These are nucleation, growth and aggregation.

1.4.1.2 Nucleation

Nucleation is defined as the initiation of precipitation of crystals from a solution (Rose, 1987). There are two main types of nucleation: homogeneous and heterogeneous. Homogeneous nucleation results from supersaturation only. It occurs spontaneously when there is high degree of supersaturation (above the formation product) and involves the binding of a number of ions to form an elementary crystal lattice. It requires energy and only the solute undergoing crystal formation is involved (Rose, 1987).

Heterogeneous nucleation occurs with metastable supersaturation and is induced by a foreign insoluble material in solution (Rose, 1987). The solute precipitates onto particles such as cellular debris or macromolecules in the urine, thereby lowering the energy required for crystal formation.

There are two phenomena related to heterogeneous nucleation (Hess and Kok, 1996): secondary nucleation and epitaxy. Secondary nucleation is the nucleation of new crystals on pre-existing surfaces of their own species. Epitaxy is nucleation on the surface of another crystal type whose lattice is similar.

There is debate about the type of nucleation that takes place in stone formation. It is most likely heterogeneous, because urine supersaturation levels are not usually high enough to induce homogeneous nucleation and urine contains many endogenous particles that may induce the heterogeneous mechanism.

1.4.1.3 Growth

Growth is defined as the addition of salt ions to an existing crystal lattice (Hess and Kok, 1996). When the nucleus has reached critical size and the relative supersaturation remains above one, then the free energy is decreased by adding new crystal components to the nucleus. The crystal growth rate is related to the supersaturation, temperature and concentration of the urine. It determines the size, shape, purity and strength of the resultant crystals.

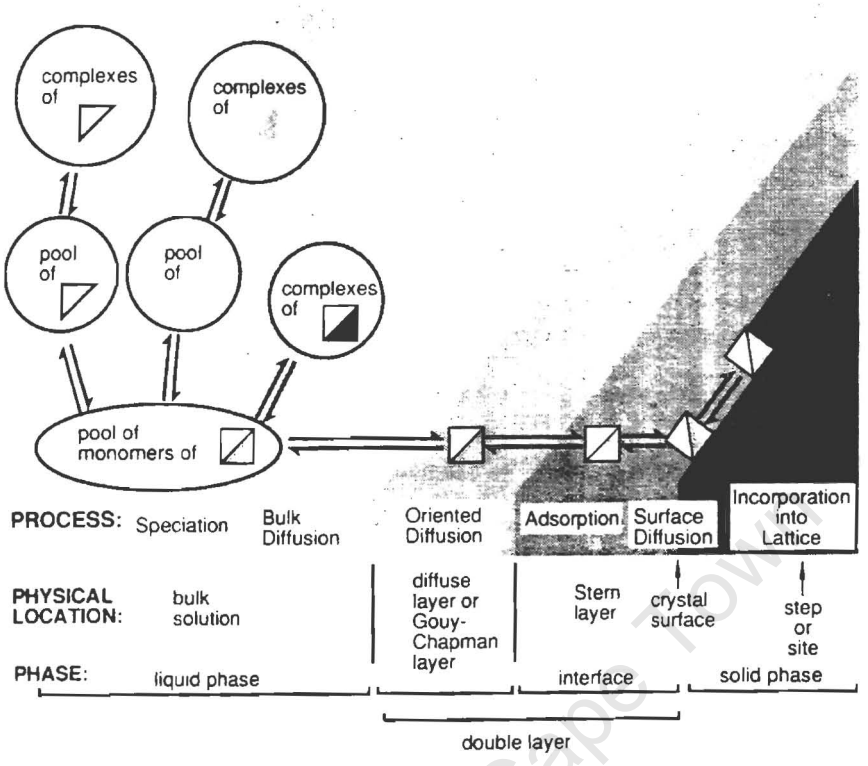


Figure 1.3 Summary of the processes involved in crystal growth. Reproduced from (Hess and Kok, 1996; Brown et al., 1994)

Figure 1.3 depicts how the particles diffuse onto and become attached to the crystal surface before being incorporated into the crystal. Out of a pool of precursors in solution, calcium and oxalate form soluble complexes with other ions or combine to form monomers. This is called speciation. The monomers are subject to bulk diffusion forces. In the vicinity of the crystal surface, monomers experience the charge gradient field created by the surface. This causes orientated diffusion. A combination of the local electrostatic charge and chemical forces cause the attachment of monomers to the crystal surface by a process called adsorption.

Adsorbed molecules diffuse along the surface until a favourable lattice interaction leads to incorporation into the growing crystal.

This growth process is slow and the transient time of urine in the kidney may not be long enough to allow crystals to become large enough to lodge inside the kidney's tubules. Only if crystals attach to the epithelium or aggregate will they reach pathological size (Hess and Kok, 1996).

1.4.1.4 Aggregation

Aggregation is defined as the binding together of multiple crystals, governed by the balance of forces. to form large polycrystalline clusters (Baumann, 1998). Calcium oxalate stones consist of polycrystalline aggregates of calcium oxalate monohydrate and/or calcium oxalate dihydrate crystals in a macromolecular matrix. Forces involved in aggregation are depicted in Figure 1.4. When inter-particle distances are small, attractive van der Waals forces favour particle aggregation. This process is also promoted by binding of crystals by foreign compounds with multiple binding sites. The main force of disaggregation is repulsive electrostatic surface charge. Finally shear forces due to solvent currents in the tubular fluid can promote or disrupt aggregation.

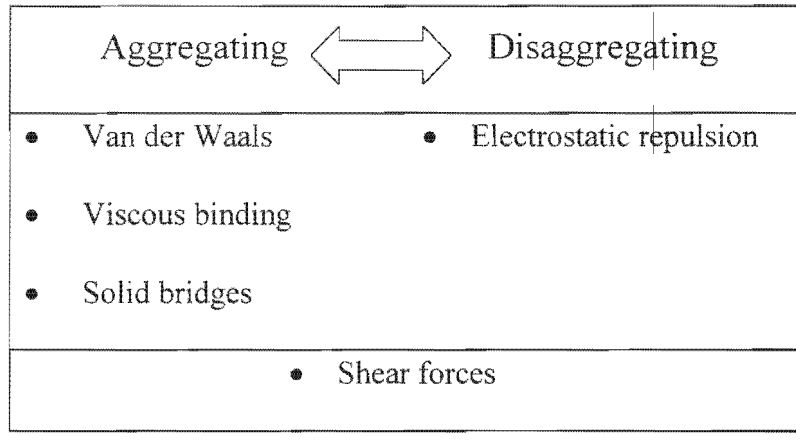


Figure 1.4 Forces of aggregation and disaggregation. Adapted from (Hess and Kok, 1996)

The crystal surface consists of a double layer: the outer Gouy-Chapman layer and the inner Stern layer. Species in solution alter the surface charge characteristics at the solution-crystal interface thereby influencing crystal growth and aggregation (Brown et al., 1994). Solid surfaces, including those of crystals, adsorb chemical substances from solution at solid–liquid interfaces as a result of physisorption or chemisorption. This is because these surfaces have a net charge and ions in solution will be attracted or repelled from this charge. Crystals in the urine are coated with substances adsorbed from the urine. This modifies the charge of the crystal surface. Calcium oxalate monohydrate crystals have a negative surface charge that forms by interaction of the surface with the calcium and oxalate ions in solution. The Stern charge is the effective charge of the surface and represents the combined charge due to adsorption of any solute molecules in solution (Brown et al., 1994). This Stern surface will then attract ions of opposite charge in the surrounding solution. This is called the Gouy charge (Brown et al., 1994). It is equal to the sum of the Stern charge and the surface charge.

Knowledge of surface charge characteristics of crystals helps in the understanding of crystal growth, inhibition of growth by inhibitors binding to the crystal surface and stability of crystals towards aggregation.

1.4.2 Crystalluria

Calcium oxalate may exist in vitro as mono-, di- or trihydrate. In urine however, the trihydrate is seldom, if ever, present. Most urine contains calcium oxalate monohydrate crystals in varying amounts. An important difference between calcium oxalate recurrent stone patients and normal subjects is that the former pass more crystals in their urine than normal subjects (Robertson and Nordin, 1982). These grow much larger in size and aggregate, increasing the risk that the particle will become lodged in the urinary tract and form a stone. Normal subjects pass a unimodal distribution of small calcium oxalate monohydrate crystals of about 5μm in diameter, while urine of stone patients contain a second distribution peak of larger crystals between 20-50μm in diameter. This is depicted in Figure 1.5. Stone patients often have calcium oxalate dihydrate crystals in their urine. These are often fused into polycrystalline aggregates. These aggregates can be retained in the kidneys and present as stone disease.

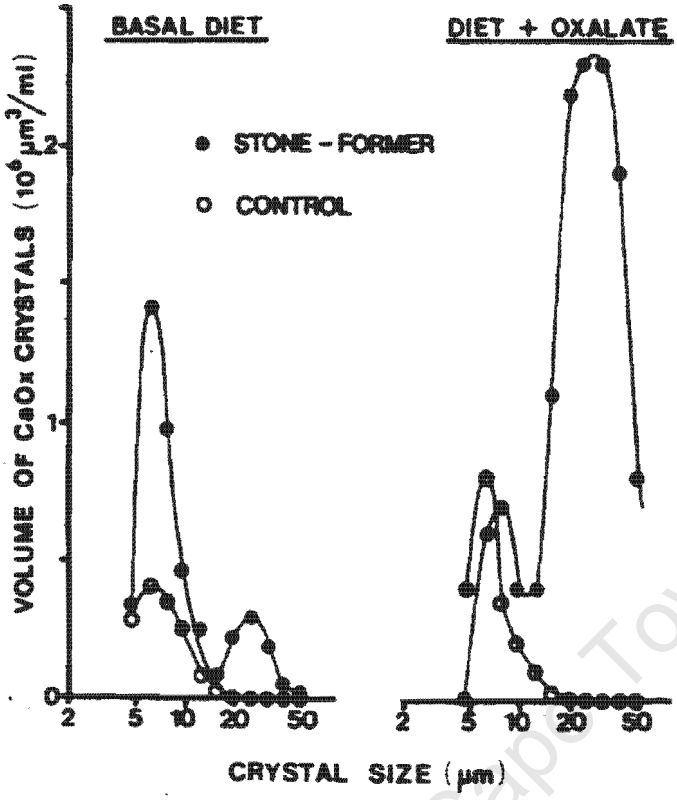


Figure 1.5 Particle size distribution of calcium oxalate crystals in the urine of a recurrent stone former and a control. Reproduced from (Robertson and Nordin, 1982)

Higher calcium oxalate supersaturation in stone formers causes increased crystalluria. Larger crystals form as a result of a faster rate of crystal growth, which is thought to be caused by a lack of urinary inhibitors. Urinary inhibitors are naturally occurring components in the urine that have been shown to prevent or retard nucleation, growth or aggregation of calcium oxalate crystals. Lower concentrations or absence of these inhibitors in stone formers urine allows the crystal to grow larger and aggregate.

Although there is general agreement that stone patients pass crystals more frequently and that these crystals are larger, the above study may be overstating the case. Other

Although there is general agreement that stone patients pass crystals more frequently and that these crystals are larger, the above study may be overstating the case. Other studies (Bader et al., 1994; Kavanagh, 2001) give a more critical appraisal of this hypothesis.

1.4.3 Theories of stone formation

There are many hypotheses for the formation of urinary stones, but no single one offers an adequate explanation. It is generally agreed that the criteria for stone formation are a persistent period of oversaturation of urine with stone forming minerals, during which crystals are formed and retained in the urinary tract, followed by a subsequent period of metastable oversaturation during which crystals grow and aggregate to form stones (Robertson and Nordin, 1982). These conditions arise due to abnormal urine composition, urine flow or function and morphology of the renal tubule cells.

There are two groups of theories of the mechanism of stone formation. These are described by Robertson and Nordin, 1982. The first (fixed particle theory) describes the formation and retention of the stone nucleus as an intracellular process. It involves fixation of a crystal to lesions of the renal papilla (called Randall's plaques). Subsequent growth and aggregation to attached crystals produces calculi.

The second group of theories involves extracellular stone formation (free particle theory). Nucleation occurs in the lumen of the urinary tract. Crystalluric

particles become trapped because of their size and grow in the urinary tract. There are three schools of thought. The 'matrix hypothesis' regards the matrix as the cause of stone disease. It postulates that the matrix (made up of mucopolysaccharides and mucoproteins) is precipitated first and then the deposition of the inorganic salts occurs as a secondary phenomenon. The matrix initiates precipitation and provides a structural framework for crystal growth.

The 'supersaturation hypothesis' regards supersaturation of the urine with stone constituents as the driving force for precipitation. Calcium oxalate stone formation results from prolonged oversaturation of the urine with calcium and/or oxalate ions. Spontaneous precipitation is postulated, and is independent of the matrix. Here, the matrix is physically adsorbed onto the growing stone surface in a secondary process and is a result of crystal growth in a medium containing various proteins.

Thirdly the 'crystallization inhibitor hypothesis' proposes that normal urine contains endogenous inhibitors which slow down or prevent the various processes of stone formation, such as nucleation or aggregation. Stone formers lack or have low concentrations of these inhibitors and therefore have a higher risk of stone formation.

1.5 EVALUATION AND ASSESSMENT OF STONE PATIENTS

1.5.1 Urinary risk factors

From the above discussion, it is obvious that calcium oxalate renal stone formation is a combined result of a number of chemical determinants called risk factors. These are urinary parameters that have the theoretical potential of increasing the likelihood of stone formation.

Primary chemical factors governing stone formation are those that determine the degree of saturation of the urine with calcium oxalate and hence govern crystal nucleation, growth and aggregation. The most important of these factors are urinary pH, volume, and excretion of calcium, oxalate, uric acid, citrate and magnesium (Ryall and Marshall, 1990). Other components such as sodium, potassium, phosphate, chloride, sulphate and bicarbonate contribute synergistically to the overall supersaturation.

Secondary risk factors are those that are either empirically determined in crystallization experiments on test urine (e.g. metastable limit) or are those that use one or more of the primary factors in physicochemical calculations (e.g. relative supersaturation).

1.5.1.1 Calcium

Over-excretion of stone constituents leads to high concentrations of either calcium (hypercalciuria) or oxalate (hyperoxaluria) in the urine. This leads to a high degree of supersaturation and consequent calcium oxalate crystal formation. Hypercalciuria has been linked to recurrent idiopathic calcium oxalate stone formation for many years. Recently it's importance as a determinant in diagnosis has been questioned (Ryall and Marshall, 1990). This is due to conflicting findings of many research groups. Some have reported no significant differences in the urinary calcium concentrations in stone forming patients and normal subjects, while others have found higher calcium excretion in patients. Many researchers have failed to compare patients to local normal subjects, and instead use daily calcium output of normal subjects reported in the literature. This is impractical as daily calcium excretion has been shown to vary widely from one geographical location to another. Therefore, there is no conclusive evidence for or against hypercalciuria being useful as a determinant of idiopathic calcium oxalate stone formation.

Increasing calcium intake as a treatment method has in fact been proposed. Curhan et al, 1993, demonstrated that a high dietary intake of calcium actually decreases the risk of stones by causing increased binding to oxalate in the gastrointestinal tract, leading to a decrease in oxalate absorption and hence, decreased urinary oxalate excretion. This causes a lower risk of stone formation because oxalate (and not calcium) has been shown to be the critical limiting factor in calcium oxalate crystal formation (Finlayson, 1974; Robertson and Nordin, 1982).

1.5.1.2 Oxalate

As mentioned in the previous paragraph, oxalate has been shown to have a more powerful effect on the degree of calcium oxalate supersaturation of urine than calcium (Robertson and Nordin, 1982). Therefore urinary over-excretion of oxalate is more likely to cause spontaneous crystalluria than calcium. Persistent hypercalciuria can raise the saturation of urine with calcium oxalate to a plateau near the formation product. Small oxalate concentration fluctuations then cause supersaturation of the urine above the metastable limit. As discussed by Ryall and Marshall, 1990 some researchers have reported significantly higher oxalate concentration in patients than normal subjects, while others have not. This is probably because oxalate concentration is difficult to quantify, due to its low concentration in the urine and because the techniques used to measure oxalate concentration are imprecise or non-specific. Measurements are also compromised because ascorbate interferes with its determination (Ryall and Marshall, 1990). Thus although oxalate is one of the most important indicators of urolithiasis, reliable quantification thereof remains difficult and challenging.

1.5.1.3 Urine volume

Persistently low urine volume will raise the supersaturation of urine with respect to all solutes, including calcium oxalate, thereby increasing the risk of crystalluria. Although no significant differences in urine volumes between stone

formers and controls have been proven, it has been shown that dilution of the urine by increasing fluid intake lowers urinary saturation with respect to calcium oxalate.

1.5.1.4 Urinary pH

A persistent imbalance of urinary pH will cause increased precipitation of calcium oxalate crystals, as the solubility decreases with increasing pH (Ryall and Marshall, 1990). A high pH will therefore cause higher urinary supersaturation of calcium oxalate and hence increase the probability of crystal (and stone) formation.

1.5.1.5 Renal morphology

The potential to form stones is a function of urine supersaturation and crystal residence time in the urinary tract lumen. Obstructions or abnormal renal morphology may cause attachment of crystals to the renal epithelia. This allows calcium oxalate crystals to become lodged and retained in the urinary tract, which will facilitate crystal growth and aggregation. Abnormal renal morphology is therefore a physical risk factor, rather than a chemical one.

1.5.1.6 Urinary inhibitors

Urinary inhibitors are naturally occurring components of the urine which have been shown in model crystallisation systems to prevent or reduce nucleation, growth

or aggregation of the crystals (Ryall and Marshall, 1990). Low levels of these inhibitors are therefore considered risk factors.

Both small molecules and macromolecules have been reported to have inhibitory effects. The former include citrate, magnesium, pyrophosphate, adenosine triphosphate (ATP), adenosine diphosphate (ADP) and certain trace metals while the latter include macromolecules such as glycosaminoglygans (GAGs), RNA, acidic glycoproteins and non polymerised Tamm-Horsfall mucoprotein (THM) or uromucoid (Angell and Resnick, 1987; Ryall and Marshall, 1990). More recently, urinary proteins have become the focus of inhibitor studies. Nephrocalcin, prothrombin fragment 1, bikunin and uropontin have been identified as playing inhibitory roles (Ryall, 1997).

There have been many studies on the role, mode of action and concentrations of these inhibitors, but little consensus has been reached. Most inhibitors are thought to act by either of two mechanisms or both (depicted in Figure 1.6). Firstly, they act by adsorbing to the surfaces of preformed crystals. These adsorbents alter the chemical, physical and electrical properties of the crystal surfaces thereby altering rates of growth and aggregation (Hess and Kok, 1996). This is thought to be the mode of action of macromolecules such as GAGs, glycoproteins and THM. They bind irreversibly to crystal growth sites. This inhibits crystal growth or alters the crystal surface potential and inhibits crystal aggregation.

The concentration of GAG's in urine is too low to decrease calcium supersaturation significantly by chelating it (see second mechanism of inhibition

below). GAG's have also been shown to prevent adhesion to renal cells (Dussol and Berland, 1998; Fleisch, 1978).

Glycoproteins, such as nephrocalcin, can bind calcium ions, but have also been shown to inhibit nucleation of calcium oxalate, growth and aggregation of crystals and adhesion to renal cells (Dussol and Berland, 1998; Worcester, 1994).

THM has been shown to act as a promoter or an inhibitor, depending on the solution conditions. It weakly inhibits calcium oxalate nucleation and growth and is a powerful inhibitor of aggregation in solutions with high pH and low ionic strength (Dussol and Berland, 1998; Worcester, 1994).

Secondly, inhibitory substances can decrease the level of supersaturation by acting as chelators i.e. they form soluble complexes with calcium or oxalate (Hess and Kok, 1996). For example citrate and pyrophosphate bind calcium, while magnesium binds oxalate. Citrate forms a complex ion with calcium, thereby decreasing the calcium oxalate supersaturation, but can also act by binding to the growing crystal surface, inhibiting crystal growth (Ryall and Marshall, 1990). Magnesium competes with calcium for oxalate complexation, leading to the formation of a more soluble magnesium oxalate complex (Ryall and Marshall, 1990). Due to their low concentrations the inhibitory role of small ions such as citrate, magnesium and pyrophosphate is not as significant as that of macromolecular polyanions (Ryall and Marshall, 1990).

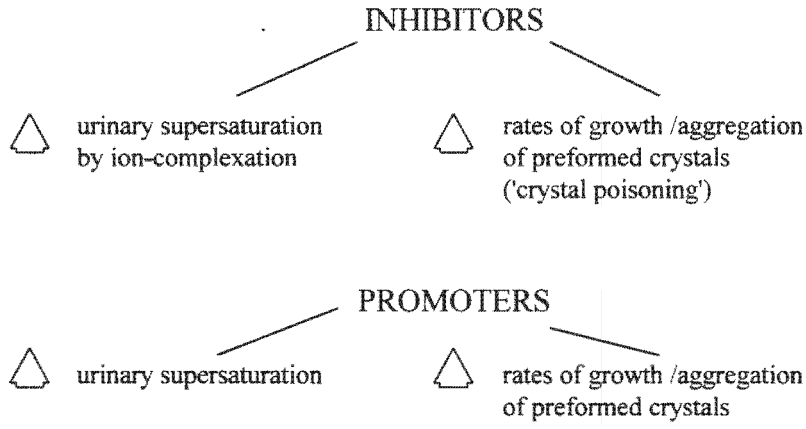


Figure 1.6 Mode of action of inhibitors and promoters. Adapted from (Hess and Kok, 1996)

1.5.1.7 Urinary promoters

Promoters of crystallisation have also been reported. These substances act by increasing the urinary supersaturation or by providing foreign surfaces for heterogeneous nucleation (Hess and Kok, 1996). For example, increased amounts of sodium urate in the urine (hyperuricosuria) are thought to increase calcium oxalate precipitation by acting as a heterogeneous nucleator or by interfering with the action of glycosaminoglycan inhibitors (Robertson and Peacock, 1983; Dussol and Berland, 1998).

THM is thought to change from an inhibitor to a promoter of aggregation when it polymerises. It acts by presenting a preformed surface for crystallization. Polymerisation is favoured by highly concentrated urine, high urinary calcium and low pH (Baumann, 1998; Dussol and Berland, 1998).

1.5.1.8 Factors preventing accurate determination of risk factors

Factors preventing accurate determination of risk factors are presented by Ryall and Marshall, 1990 and are summarised as:

- The episodic nature of stone disease. Stone disease occurs spasmodically. By the time the patient suffers symptoms, urine chemistry may bear no resemblance to that prevailing at the time the stone was formed. This is important for metabolites whose excretion is affected by diet.
- Common unilateral occurrence of the disease. Urine collected is derived from both kidneys, whereas lithiasis is frequently confined to one kidney only. Mixing of the urine in the bladder may cause fluctuations in urinary metabolite concentrations to be lost.
- Day to day variation. Urinary metabolites may vary from day to day and will therefore depend on when the sample is taken.

1.5.2 Diagnosis and management of stone disease

At present, patients are diagnosed when they present with their first stone. First time stone patients require only a basic evaluation, whereas patients with multiple or recurrent stones require a more complete metabolic evaluation (Preminger and Harvey, 1987; Tiselius, 1997). This is because many patients will only have one stone episode and modern surgical procedures allow for quick, simple removal of stones. Full metabolic work-ups only become viable when stones reoccur.

The initial laboratory evaluation should include the following analyses (Klugmann and Favus, 1995):

- Stone analysis is carried out to identify the crystalline composition of the stone (stone type). This can be done by analysis of the passed stone using polarisation microscopy, x-ray diffraction or infrared spectroscopy. Identification of stone type can give an indication of its causes.
- Blood chemistry profile with specific attention to serum calcium, phosphate, electrolytes, uric acid and creatinine is carried out. Abnormalities will indicate underlying metabolic causes such as hyperthyroidism, hyperuricemia and renal tubular acidosis. Creatinine levels are taken for baseline purposes.
- Urinalysis and urine culture provides information on the concentrations of risk factors and the presence or absence of infection.

Full metabolic evaluation is essential for recurrent stone formers and helps in disease management and to monitor the efficacy of treatment (Rous, 1987). Tests will also include at least three 24-hour urine collections.

Table 1.1 PRIMARY URINE RISK FACTORS	
calcium	sodium
oxalate	potassium
citrate	chloride
urate	sulphate
magnesium	phosphate
pH	bicarbonate
volume	creatinine

Primary urine chemistries listed in Table 1.1 are used to calculate secondary risk factors. These are computed quantitative diagnostic indices of urine supersaturation, crystal formation, the inhibitory capacity of the urine, or the overall risk of stone formation. The most important of these is the relative supersaturation. Others include the calcium oxalate ion activity product (discussed in Section 1.4.1.1), relative supersaturation of calcium oxalate, AP(calcium oxalate)-index (Tiselius, 1982; Tiselius, 1989; Tiselius, 1991), Tiselius risk index (Tiselius, 1982) and the calcium-citrate index (Parks and Coe, 1986).

As mentioned above, the most important is the relative supersaturation. This physicochemical property provides an estimation of the energy available in the solution to drive nucleation and crystal growth (Brown et al., 1994). It is the ratio of the amount of stone salt in solution to the amount present at equilibrium in that

solution. Relative supersaturation is routinely calculated using the EQUIL software program (Werness et al., 1985; Brown et al., 1994).

Two indices have been developed by Tiselius as simplified estimates of the ion activity product of calcium oxalate (Tiselius, 1982; Tiselius, 1989; Tiselius, 1991). These are the AP(calcium oxalate)-index and the calcium oxalate risk index (also known as the Tiselius risk index). The AP(calcium oxalate)-index is a useful measure of supersaturation. It uses calcium, oxalate, citrate and magnesium concentrations and the urine volume. Sodium, potassium, sulphate, phosphate, ammonium, and chloride concentrations have a small effect on AP(calcium oxalate)-index and therefore are not considered in the simplified estimate. A modified version of this index has been formulated for use with variable volumes i.e. collection periods (Tiselius, 1991).

The Tiselius risk index is used to express the biochemical risk of calcium oxalate stone formation (Tiselius, 1982). It is based on the 24 hour urinary excretion of calcium, oxalate, magnesium and citrate. It also includes the inhibition of crystal growth using the inhibition index (I), with all variables related to urinary creatinine. It attempts to distinguish between stone formers and normal subjects. This index is independent of volume and is relative to urinary creatinine excretion (Tiselius, 1982).

The calcium citrate index is a score derived from the discriminant analysis of patients' and healthy subjects' urinary concentrations of calcium, and citrate. According to the authors it successfully distinguishes between the two groups (Parks and Coe, 1986).

The metastable limit of urine is an empirically determined property of the urine. It is a measure of the propensity of urine to induce crystal formation (Ryall et al., 1985). Specifically it provides a measure of the urine's capacity to tolerate increasing amounts of oxalate before the formation product of calcium oxalate is exceeded. The practical limit of metastability with respect to calcium oxalate is determined by titrating urine with increasing amounts of sodium oxalate and estimating the minimum amount of oxalate that is required to induce precipitation. It will depend to varying degrees on the urinary ionic concentrations of calcium, oxalate and inhibitors and promoters (Ryall et al., 1985).

1.5.3 Comment

Although primary and secondary factors provide a measure of the risk of stone formation, none of them fully utilises all the complex interactions in urine. There is still a need for a simple measurement of some, as yet unidentified property of whole urine which can follow the progress of a particular treatment regime and verify it's efficacy and which can ultimately distinguish between stone formers and healthy subjects by quantifying stone risk.

CHAPTER 2

NMR OF BIOFLUIDS

2.1 INTRODUCTION

The body is made up of approximately 60 % fluids. Cells contain intracellular fluid and are surrounded by extracellular fluid, mainly water, which acts as a solvent for the transportation of chemicals in the body and as a mechanism of heat distribution in the body. Extracellular fluid is made up of interstitial fluid, between the cells and transcellular fluid which includes blood plasma, lymphatic fluid, cerebrospinal fluid, digestive fluid and urine etc. These biological fluids perform a variety of physiological and biomechanical functions in the body, including transportation, excretion and homeostasis.

In order to maintain metabolic functioning, all animals depend on the many chemical reactions taking place in their cells. The rates of these reactions depend on the concentration of the reactants and the temperature of the internal environment. These factors are affected by the external environment of the cells. For most cells the internal environment is maintained by maintaining the composition and temperature of the extracellular fluids. This is known as homeostasis and is carried out by the kidneys and the circulatory system (Harper, 1977).

The relationships between biochemical pathways operating in fluid secreting tissues and the fluids they secrete, are diverse and complex. Body fluid metabolites are in dynamic equilibrium with those inside the cells and tissues which produce them and

therefore their composition varies according to whole body homeostatic demands. Dietary variations and pathological processes in the body also influence the composition of these physiological fluids. Therefore understanding the biochemistry of body fluids, such as urine and plasma, is of great clinical and diagnostic importance as their chemical characteristics reflect abnormal metabolic processes in the whole body (Nicholson et al., 1995).

Biofluid NMR is the use of nuclear magnetic resonance spectroscopy as an analytical tool to determine the composition of biofluids, thereby giving an insight into the metabolic status of the donor.

2.2 DEVELOPMENT OF BIOFLUID NMR

^{1}H-NMR spectroscopy was first performed on urine in 1957 (Odeblad and Westin, 1958). Although the spectra were not very useful in determining its composition, the potential of NMR for use on intact biological samples was identified. In the early 1970's NMR was used to detect and identify the chemical species in biological systems. This became possible after the development of Fourier Transform NMR and hardware advances (such as field strength and coil design improvements, more sophisticated pulse sequences and better computing capability), giving NMR the necessary sensitivity, resolution, and dynamic range to look at biological samples. Since then NMR has become an important tool for investigation of complex biological matrices, including tissues and biological fluids of several animal species.

Early studies used ^{1}H-NMR to determine metabolite concentrations in whole fluids or to identify novel metabolites. In 1979 the first attempt was made to use high-resolution NMR data of blood plasma to diagnose cancer (Ohsaka et al., 1979). In the early 1980's researchers (Matsushita et al., 1982; Bales et al., 1984) carried out NMR studies on urine samples from subjects with diabetes and renal damage. Marked changes in the concentration of a number of endogenous metabolites were observed. Following these investigations, it became clear that high resolution NMR could aid in the diagnosis of diseases. More recently the bias of biofluid NMR has moved towards measuring perturbations in metabolite patterns and ratios that are NMR detectable (Nicholson and Wilson, 1989). This has lead to applications in drug metabolism, toxicology studies, metabolic monitoring and disease screening. NMR has been carried out on a range of body fluids (Lindon et al., 2000) such as urine, blood plasma or serum, bile, seminal fluid, amniotic fluid, cerebrospinal fluid (Lindon et al., 2000) and fluid from polycystic kidney disease (Foxall et al., 1992). The ^{1}H-NMR spectrum of each biofluid has a characteristic 'fingerprint' pattern of endogenous metabolites. Figure 2.1 shows a ^{1}H-NMR spectrum of urine, blood plasma and bile, each showing a characteristic biochemical pattern of metabolites.

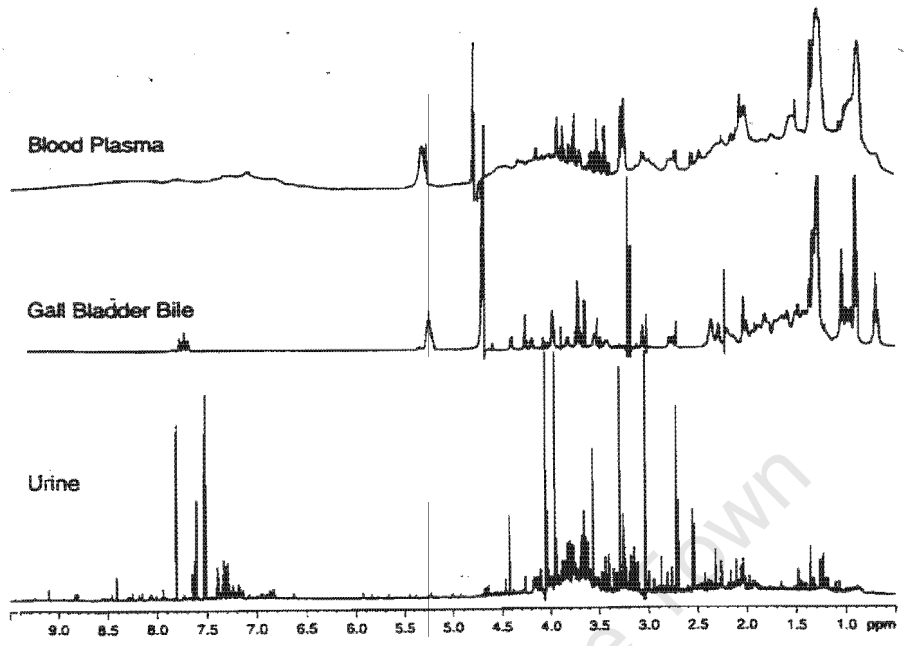


Figure 2.1 Typical 800 MHz ^{1}H-NMR spectra of human biofluids (reproduced from Neild et al., 1997).

2.3 APPLICATIONS OF NMR OF BIOFLUIDS

The main areas of application of NMR of biological fluids are drug metabolism and excretion studies, toxicology investigations and clinical applications, such as disease screening and metabolic monitoring (Nicholson and Wilson, 1989).

2.3.1 Disease screening

Inborn errors of metabolism are congenital defects that arise from genetically determined deficiencies of enzymes that affect specific biochemical pathways

(Nicholson and Wilson, 1989). These errors present as the accumulation of millimolar concentrations of unusual metabolites in the body fluids. The presence of unusual metabolites is often difficult to detect using clinical laboratory procedures, because tests for the specific metabolite may not be included in routine testing. These metabolites are often detectable in the ^{1}H-NMR spectrum of body fluids.

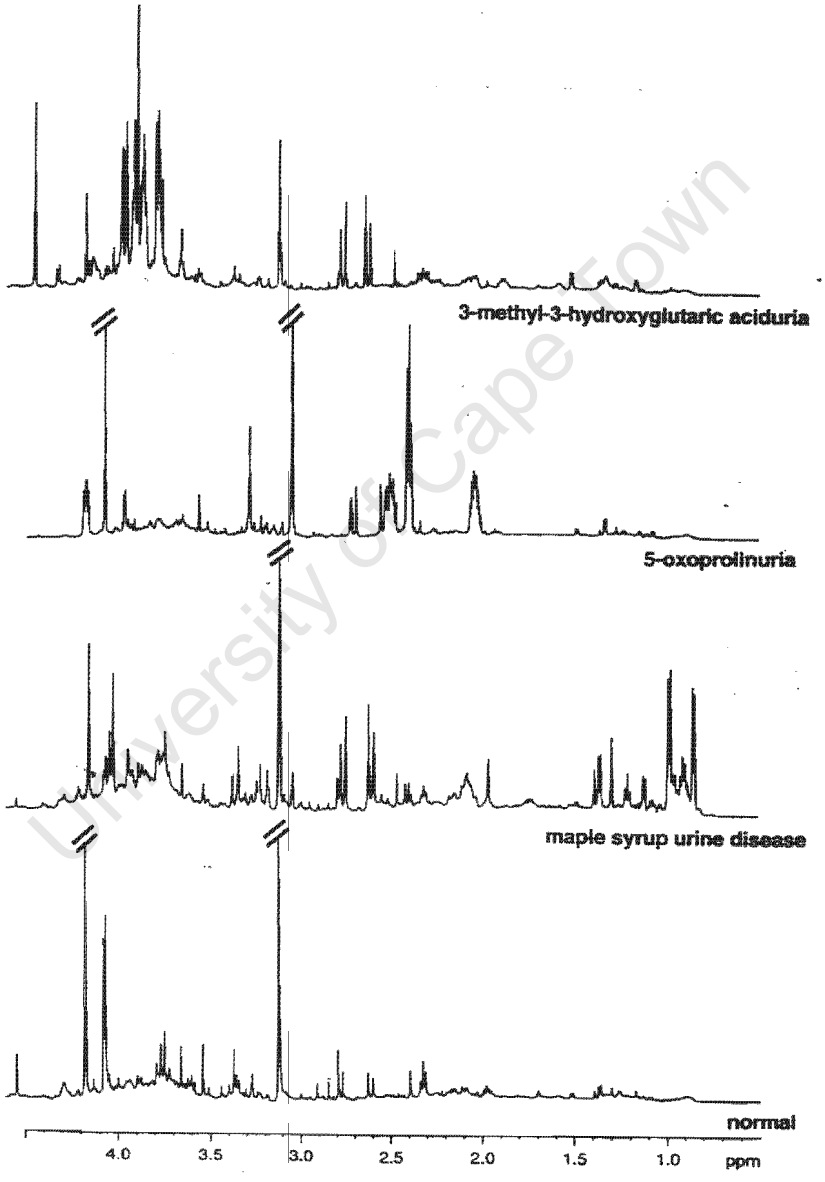


Figure 2.2 600 MHz ^{1}H-NMR spectrum of urine from a healthy subject and three patients with different inborn errors of metabolism (reproduced from Neild et al., 1997).

Figure 2.2 shows the spectrum of urine from a healthy human subject and patients with different inborn errors in metabolism. Each shows the presence of an abnormal metabolite, indicative of an abnormal metabolic pathway. ^{1}H-NMR allows identification of the resultant metabolite; thereby aiding in the accurate diagnosis of different metabolic disorders, with the added advantage that novel metabolites related to a given disease can be detected. A large number of inherited metabolic disorders, including those listed in Table 2.1, have been studied by NMR spectroscopy.

Table 2.1 Inborn errors of metabolism studied by NMR

Inborn errors of metabolism	**Reference**
2-Hydroxyglutaric aciduria	Holmes *et al.*, 1997
Maple syrup urine disease	Holmes *et al.*, 1997
Isovaleric aciduria	Bell *et al.* 1988
Dicarboxylic aciduria	Bell *et al.* 1988
Tyrosinemia	Bell *et al.* 1988
Purine and pyrimidine metabolism	Wevers *et al.*, 1999
Oxalic aciduria	Holmes *et al.*, 1994
Fanconi syndrome	Holmes *et al.*, 1994
Porphyria	Holmes *et al.*, 1994
5-Oxoprolinuria	Holmes *et al.*, 1994
Alkaptonuria	Bamforth *et al.*, 1999
Cystinuria	Bamforth *et al.*, 1999
Glycerol kinase deficiency	Bamforth *et al.*, 1999
Defect in dimethylglycine	Moolenaar *et al.*, 1999

^{1}H-NMR of blood plasma has been used to study the lipoprotein content of blood and its link to coronary heart disease (Bathen et al., 1999) and to the diagnosis of cancer (de Certaines et al., 1996). Lipoprotein particles in blood plasma contain millimolar levels of triacylglycerols, phospholipids, cholesterol and proteins. These particles control transport and delivery of fats and cholesterol. The aliphatic region of the ^{1}H-NMR spectrum of blood plasma contains methyl and methylene resonances from the protein, triglycerol and cholesterol of lipoprotein particles. These can be used to

determine the relative amounts of very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) in blood plasma. This technique can be used for the classification of the risk of coronary heart disease (Bathen et al., 1999).

2.3.2 Metabolic Monitoring

In many diseased states, biochemical changes cause loss of homeostatic control, resulting in abnormal levels of endogenous metabolites in the body fluids. This gives rise to an ^{1}H-NMR spectral fingerprint. Once these disease makers have been identified, quantitative measurements can be performed to follow the progress of the diseased state or to monitor therapeutic changes. An example of this is diabetes, where plasma levels of 3-D-hydroxybutyrate, acetoacetone, acetone and glucose become elevated in diabetes mellitus patients when insulin is withdrawn (Nicholson et al., 1984; Bales et al., 1984). All of these metabolite changes can be simultaneously tracked using NMR.

Altered profiles of naturally occurring low molecular weight metabolites in urine and plasma, may also indicate organ dysfunction, such as renal damage. Several novel markers of renal dysfunction have been identified using ^{1}H-NMR, including trimethylamine oxide (TMAO), dimethylamine (DMA), and dimethylglycine (DMG) (Lindon et al., 2000). NMR can monitor these as an indication of renal function. An example of this is given by Neild et al., 1997, where urine of renal transplant patients was studied for 14 days. ^{1}H-NMR was used to follow renal damage and recovery after transplantation, and to distinguish between graft dysfunction and rejection episodes.

Monitoring of small molecules in plasma can be used in kidney dialysis patients (Neild et al., 1997) to monitor liver functioning. Liver failure gives rise to a characteristic pattern of histidine, tyrosine and phenylalanine in the ^{1}H-NMR of plasma. NMR methods have been used to map changes in the plasma composition during dialysis and thus provide insights into the biochemical consequences of dialysis, as well as a novel way of monitoring dialysis efficiency.

Other effects on metabolism that have been studied using NMR of plasma or urine are the effects of exercise, fluid deprivation and diet (Bales et al., 1984; Holmes et al., 1994).

2.3.3 Clinical toxicology studies

Diagnosis and evaluation of the metabolic situation of accidental or deliberate drug overdoses can be aided by NMR analysis of biofluids, such as blood and urine. A drug or poison will alter the metabolism and/or cause organ dysfunction (i.e. liver or renal failure). Markers of these can be seen in the ^{1}H-NMR of the biofluids, along with the metabolites of the drug. Figure 2.3 shows the urine spectrum of a subject with phenol induced renal failure. It shows phenol metabolites and altered metabolite profiles, indicating renal damage. This can aid in diagnosis of the toxin and prognosis of the patient's metabolic state.

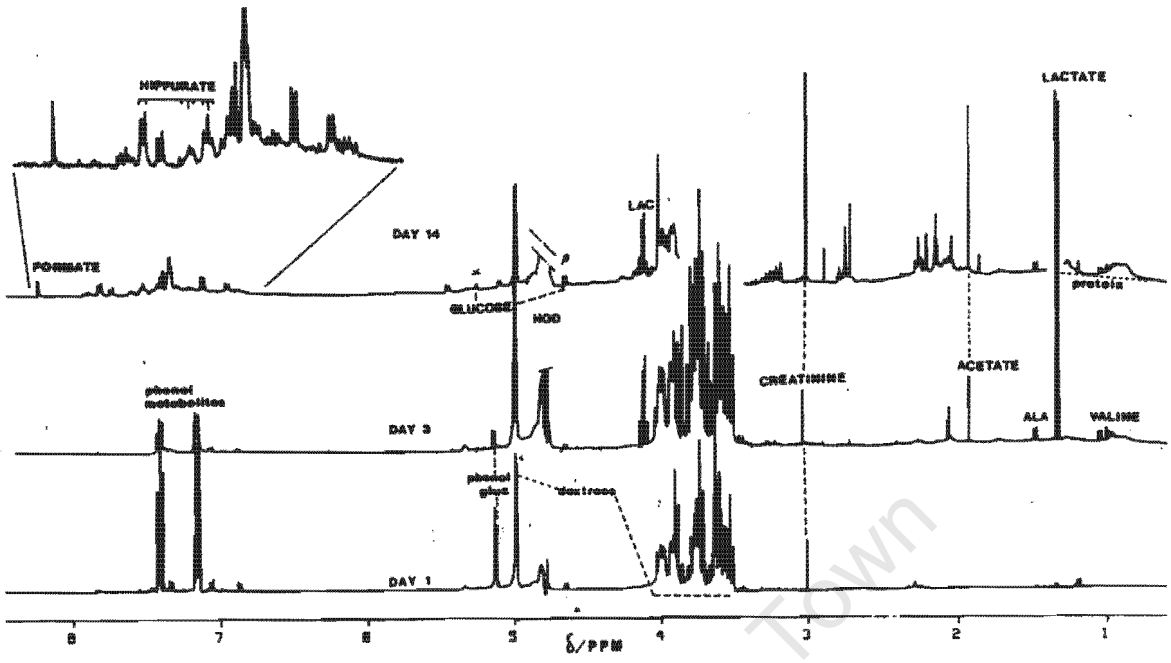


Figure2.3 400 MHz ^{1}H-NMR spectrum of urine from a subject collected 1, 3 and 14 days after phenol poisoning (reproduced from Nicholson and Wilson, 1989).

An example of a clinical diagnosis is contained in a study by Meshitsuka *et al.*, 1999. In the course of the routine screening of patient's urine by NMR, high levels of polypropylene glycol were seen in an infant patient. This was found to be due to the ingestion of polypropylene glycol (usually broken down in the liver) contained in patient's prescribed medication. Due to an inherent metabolic disorder affecting liver functioning, the patient was unable to metabolise polypropylene glycol resulting in toxic levels being reached. This diagnosis illustrates the usefulness of NMR in clinical toxicology, where conventional methods for the diagnosis were inconclusive.

Dietary consumption also affects the urinary metabolite profiles, and it is important to distinguish these from disease related processes in clinical and toxicology studies.

2.3.4 Drug metabolism and excretion

The metabolism of xenobiotics is conventionally carried out by one of two methods. Firstly, the metabolic products of radioactively labelled drugs can be traced. This is time consuming, expensive and presents safety hazards. Secondly, drug metabolites can be extracted from physiological fluids using HPLC linked to a suitable detector. This requires the prediction of the chromatographic properties of the metabolites. In this situation, NMR has advantages over conventional techniques as it can be run directly on the biofluid of choice. From a dosed subject the drug metabolites or excretion products can be identified without pre-selection of conditions. The multi component spectrum produced enables the identification of novel metabolites and quantitation of the parent drug and all its metabolites in a single experiment. This technique is not suitable for all xenobiotics. For NMR to be successful in the identification of drug metabolites the compounds must contain NMR detectable nuclei, drug dose levels have to be high (as NMR is relatively insensitive) and the metabolite resonances must not overlap with endogenous metabolite signals if they are to be quantified (Nicholson and Wilson, 1989). Paracetamol was one of the first drugs whose metabolism was studied by ^{1}H-NMR. Figure 2.4 shows the ^{1}H-NMR urine spectrum of a patient dosed with paracetamol, showing its metabolites.

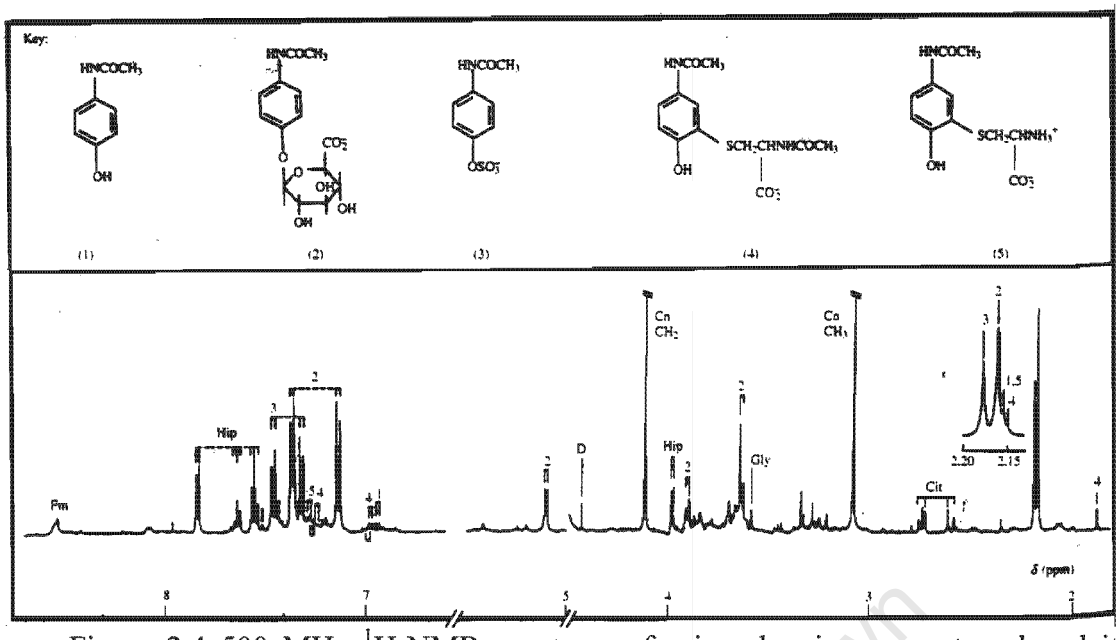


Figure 2.4 500 MHz ^{1}H-NMR spectrum of urine showing paracetamol and its metabolites (reproduced from Nicholson and Wilson, 1989).

NMR has now been successfully applied to the study of many xenobiotics including 5-fluorouridine, ifosfamide, ibuprofen, penicillamine, flucloxacillin, ampicillin, hydrazine, N-methylformamide, and carboplatin (Nicholson and Wilson, 1989; Bell et al., 1988). Nicholson and Wilson, 1989 lists a number of these studies and their references.

Several magnetic nuclei are used in NMR studies of xenobiotics (Malet-Martino and Martino, 1991), though ^{1}H and ^{19}F are usually the nuclei of choice. The ^{1}H nucleus is present in most drugs and has the highest NMR sensitivity of any stable nucleus. It is also the most abundant nucleus (99.98% natural abundance). The small chemical shift range and the multiplicity of signals (due to homonuclear J-coupling) makes observation and quantitation of metabolites difficult due to severe overlapping of signals. ^{1}H-NMR spectra also contain a large proton signal from water, which must be eliminated.

^{13}C and ^{15}N-NMR are not used in drug metabolite studies, because of their low natural abundance, low NMR sensitivity and the low concentrations of the drugs in the body fluids. The use of ^{31}P-NMR spectroscopy in drug metabolism studies is limited as few phosphorous containing drugs are used therapeutically. However, ^{31}P-NMR is used for intracellular pH measurements and metabolism studies, since signals are easily detected (Malet-Martino and Martino, 1991).

Many drug metabolism studies use ^{19}F-NMR, because fluorinated substituents are increasingly being used in new drugs (Wilson et al., 1989). Fluorine provides a unique, sensitive nucleus, that will not be subject to interference from endogenous molecules or water signals. Due to the large chemical shift range of the ^{19}F nucleus, it is very sensitive to changes in structure. It is therefore useful for drug metabolite studies as changes due to metabolism may be detected on carbon atoms up to nine bonds away.

2.3.5 Toxicology studies

Often the effects of novel therapeutic agents on the body are unknown. These compounds interact with cellular components and result in disturbances in the ratios and concentrations, binding, or fluxes of endogenous biochemicals, either by direct reaction with metabolites or by binding to enzymes that control metabolism. If these disturbances are large enough, this will result in toxic lesions or organ damage and therefore toxicology studies need to be carried out. These lesions cause a disruption of homeostasis, resulting in changes in metabolite profiles of extracellular fluids. Biofluid NMR spectroscopy reflects these changes and can be used to follow the biochemical response of the cells, thereby indicating the toxic effects of the drug. Each toxic

compound or class of compound produces characteristic changes in the concentration and patterns of endogenous metabolites. These changes depend on the interaction of the toxin with the particular cells. Therefore the excretion patterns detected by ^{1}H-NMR can indicate the location and severity of toxic lesions, and give insights into molecular mechanisms of toxicity (Holmes et al., 1998). For example nephrotoxins acting at the proximal tubule can be distinguished from toxins acting at the renal papilla and from hepatotoxins using ^{1}H-NMR of urine coupled to pattern recognition techniques (Gartland et al., 1990; Holmes et al., 1998).

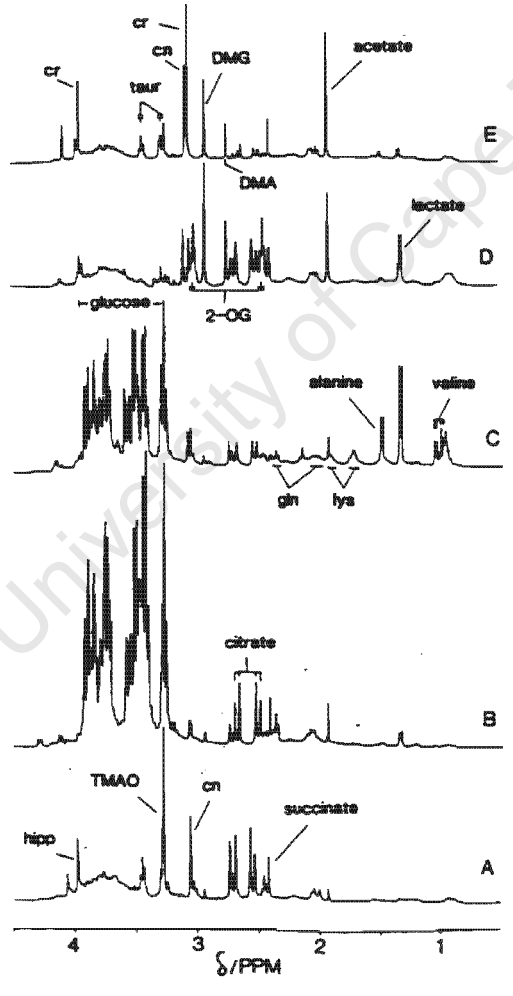


Figure 2.5 Shows 400 MHz ^{1}H-NMR MHz rat urine spectra from rats dosed with various nephrotoxins: (A) control rat urine; (B) sodium chromate; (C) *p*-aminophenol; (D) propylene imine; (E) bromoethanamine (reproduced from Nield et al., 1997).

^{1}H-NMR can also reveal novel markers of organ specific toxicity. This is useful in detecting side effects of novel drugs with unknown toxicology. Dose response and time course response to these novel therapeutic agents can also be followed (Beckwith-Hall et al., 1998). This has advantages over conventional histopathological techniques, as the onset of a particular toxic lesion can be monitored using metabolite profiles. Because NMR is non-invasive, a series of unique time related measurements can be taken and the biochemical events can thus be tracked. Figure 2.6 tracks the toxic effect and recovery of a rat dosed with nephrotoxin mercuric chloride.

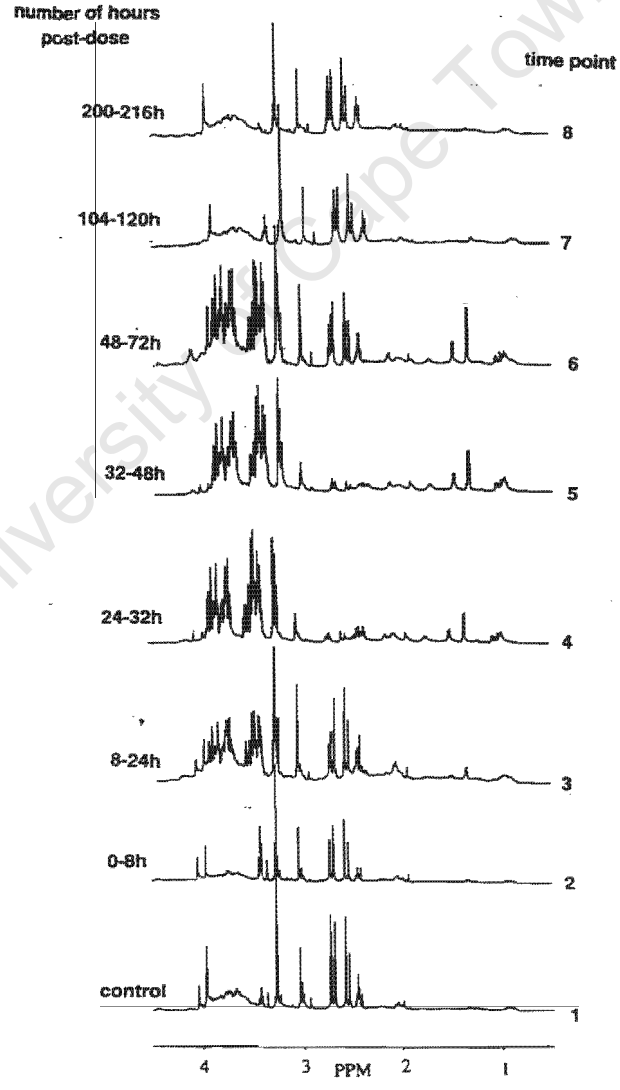


Figure 2.6. 400 MHz ^{1}H-NMR spectra of rat urine following a dose of renal cortical toxin mercuric chloride (reproduced from Neild et al., 1997).

2.4 ^{1}H-NMR SPECTROSCOPY OF BIOFLUIDS

2.4.1 Advantages and Limitations

A variety of techniques can be used to obtain metabolite profiles of body fluids. Most of these standard techniques require extensive sample preparation and careful selection of analytical conditions. This restricts the number of compounds that can be studied at a given time. Many body fluids contain a diverse range of molecules including amino acids, proteins and lipoproteins. The non-selective nature of NMR therefore makes it an ideal technique for obtaining metabolite profiles of body fluids. NMR can also give insights into molecular interactions in intact fluids.

^{1}H-NMR is insensitive by modern analytical standards. This limits the range of applications, but in many situations, absolute sensitivity is not the overriding consideration and NMR may be the best method. NMR has the advantage that it is non-invasive and non-destructive and allows multi-component analyses to be performed without separation or derivitization steps. It also provides structural information on observed metabolites, and changes in metabolic reactions as a function of time can be monitored. In order for ^{1}H-NMR to be useful, compounds must be present in 50-100μM concentrations (though they can be detected as low as 10μM) and contain NMR detectable proton containing groups. Detection is not usually limited by insensitivity but by the chemical noise of the sample, caused by the presence of hundreds of compounds near their limit of detection. These low intensity signals overlap and sum to give an irregular baseline that may mask the resonance's being studied.

In contrast to most biochemical analyses, NMR does not require precise pre-selection of analytical conditions to identify compounds. Chromatographic separations, for example, require a choice of column, solvent, flow rate and detector, which will limit the range of compounds studied. The spectrometer, however, acts as a non-selective detector permitting the detection of a diverse range of compounds in a single experiment, without pre-selection of conditions. The spectrum contains a considerable amount of structural information and can enable the detection and identification of unknowns.

NMR is rapid and requires a small sample (0.3-0.5ml). It does not disturb chemical equilibria in mixtures and therefore provides unexpected insights into the role of individual components in complex systems. Because NMR is non-destructive, it allows chemical equilibria and intermolecular and ionic interactions to be preserved and observed. Samples can then be reused for subsequent biochemical analyses. This is useful when there is limited sample and when compounds may decompose during sample purification or isolation.

The disadvantages of NMR spectroscopy for the analysis of these complex mixtures are four fold:

1. NMR's relative insensitivity makes it difficult to quantify substances present in low concentrations.
2. Dynamic range problems arise because of the use of a digital technique to detect signals from compounds present in low concentrations (mM or less) in the presence of the intense proton signal from water, which is about 10^5 times that of the metabolites. This can result in incomplete digitization of weak solute signals.

3. Chemical noise results from the overlap of signals from compounds that are individually low in concentration and often close to or below the limit of detection of the spectrometer, but collectively give rise to apparently broad and weakly featured signals. This limits spectral information content and accurate metabolite quantitation.
4. Macromolecule binding and chemical exchange phenomena cause resonance broadening. Small molecules binding to proteins have constrained molecular motions and therefore short T_2 values, causing broad resonances. Species with intermediate chemical exchange rates also have protons with short T_2 values. This is particularly prevalent in plasma, which contains large quantities of soluble plasma proteins such as immunoglobin and albumin. These give rise to a broad envelope of proton signals, which overlap and mask signals from highly mobile small molecules.

2.4.2 Practical considerations

Problems of insensitivity and overlapping peaks can be minimized by using the highest frequency spectrometers possible (Lindon and Nicholson., 1997). This will increase the sensitivity and signal dispersion, and decrease the amount of chemical noise. Spectral information can also be optimized by the use of 2-D correlation techniques or solid phase extraction with NMR detection. 2-D techniques reduce spectral overlap to gain more information on coupling constants and spin-spin connectivities, in order to facilitate assignment. Application of J-resolved (JRES) and homonuclear correlation spectroscopy (e.g. COSY) experiments increase the number of compounds that can be assigned (Foxall et al., 1993). Pulse field gradient double quantum filtered COSY (PFG-DQF-COSY) experiments are particularly useful as they

are quick and give good water suppression. This aids in rapid identification of metabolites of interest (Meshitsuka et al., 1999)

Recent development of simple solid phase extraction chromatography methods allow for simple rapid purification of biofluids prior to spectroscopy, and extends the number and type of compounds that can be identified and studied. Solid phase extraction chromatography with NMR detection (SPEC-NMR) can be used to reduce chemical noise (Nicholson and Wilson, 1989). It is used as a sample clean up procedure using a small disposable solid phase extraction column. It will allow analytes to pass through the column or be retained and concentrated prior to elution. Increasing concentration or decreasing the amount of macromolecules will reduce chemical noise and improve spectral resolution.

The dynamic range problems can be overcome by freeze drying samples and reconstituting them in D_2O (Wilson et al., 1989). This greatly reduces the water signal and concentrates the sample, increasing sensitivity. However, freeze-drying may result in the loss of volatile or unstable compounds (e.g. acetone and acetoacetone), the disruption of chemical equilibria and the structure of some macromolecules may be altered in the process. Selective deuteration of acidic protons on reconstitution with D_2O may also cause loss of metabolite signals and complicate spectral interpretation. Reconstitution with aprotic solvents will allow detection of exchangeable protons, but may result in changes in molecular mobility of components (Nicholson and Wilson, 1989). The time consuming task of sample preparation is also a disadvantage of this method.

Alternately, water suppression is used. This is the preferred method. It requires little or no sample preparation. The main methods of solvent suppression used are: the application of a secondary irradiation field at the water resonance frequency, to produce selective presaturation of the water signal; selective excitation methods based on T_1 relaxation time; augmentation of water T_2 relaxation by chemical means and subsequent attenuation of the water signal using a spin echo pulse sequence (These are summarised in Nicholson and Wilson, 1989 and Wright et al., 1995).

Presaturation by applying a gated or continuous secondary irradiation field at the water resonance frequency is effective on biofluids that contains small amounts of proteins, such as urine. This method requires no chemical pre-treatment of the sample. Its efficiency is limited by the large water line width. The centre of the water signal (at the irradiation frequency) is more strongly suppressed; leaving distorted 'wings' on either side. Chemical exchange and cross relaxation phenomena between water molecules in the sample allow some water suppression over the entire water line width, but local constraints such as interactions with protein solvation spheres, excludes some water molecules from cross relaxation phenomena, causing residual signal. Therefore this method is not effective for water suppression in protein rich biofluids like blood plasma.

WATR (water attenuation by T2 relaxation) is the method used for water suppression in biofluids rich is proteins. It is based on the reduction of the water T2 relaxation by adding ammonium chloride, guanidinium chloride, or urea, which exchange protons with the water, resulting in a broad water signal. The Carr Purcell Meiboom Gill (CPMG) spin echo method is then used to attenuate the water signal.

This also results in the elimination of broad lines from the macromolecules, thereby simplifying the spectra. This is particularly effective on protein rich biofluids.

Another alternative is to use pulse field gradient (PFG) water suppression techniques i.e. PFG-DFQ-COSY is particularly effective (Meshitsuka et al., 1999).

Many of these suppression techniques cause distortions or loss of peak intensity of signals near the water resonance, or signal molecules with short T2 relaxation times.

2.5 ^{1}H-NMR SPECTRA OF URINE

Of all biofluids studied using NMR, urine proton spectra are the least complicated by spectral overlap because of the low concentration of lipids and proteins, and high concentration of low molecular weight metabolites (Nicholson et al., 1995). However, the physical chemistry of urine is complex and the composition is highly variable and the composition depends on the functioning of the kidney and the biochemical state of the whole animal (due to homeostatic functioning of the kidney). The composition also varies considerably from species to species, with age, dietary composition and lifestyle (Lindon et al., 2000). This makes the spectral changes that occur, difficult to interpret. It is clearly important to differentiate between variation caused by diet and age, from those caused by disease or loss of homeostatic control. There is also enormous variation in the concentration range of NMR detectable metabolites in urine samples.

Urine contains a wide range of organic acids and bases, simple sugars, polysaccharides, heterocycles, polyols, low molecular weight proteins and polypeptides,

together with a variety of inorganic ions (Bales et al., 1984). Many of these contain protons that are NMR detectable. A typical 400 MHz ^{1}H-NMR spectrum of urine is shown in Figure 2.7. The proton resonances fall in the chemical shift range $0.8 < \delta < 9$ ppm. Table 2.2 contains a list of typical compounds that have been detected in urine by ^{1}H-NMR.

Table 2.2 Endogenous metabolites detectable in urine by proton NMR (adapted from Nicholson and Wilson, 1989).

Acetate, acetamide, acetone, acetoacetone, acetylcarnitine, *N*-acetylsugars, α-alanine, β-alanine, allantoin, anserine, ascorbate, betaine, butanone, carnitine, choline, citrate, creatinine, dihydroxyacetone, dimethylamine, dimethylglycine, ethanol, formate, glucose, glutamate, glutamine, glutarate, glycine, guanine, hippurate, histidine, hydroxybenzoate, 3-D-hydroxybutyrate, 2-hydroxyisocaproate, 2-hydroxyisovalerate, 2-hydroxy-3-methylvalerate, hydroxypropionate, indoxyl sulphate, inositol, isoleucine, isovalerylglycine, 2-oxoglutarate, lactate, leucine, lysine, methanol, methionine, methylamine, methylmalonate, *N*-acetyl glycoproteins, 2-oxoisocaproate, 2-oxoisovalerate, 2-oxo-3-methylvalerate, phenylalanine, propionate, propionylcarnitine, propionylglycine, succinate, taurine, trimethylamine, trimethylamine-*N*-oxide, threonine, triglycine, tiglate, tyrosine, urea, valine.

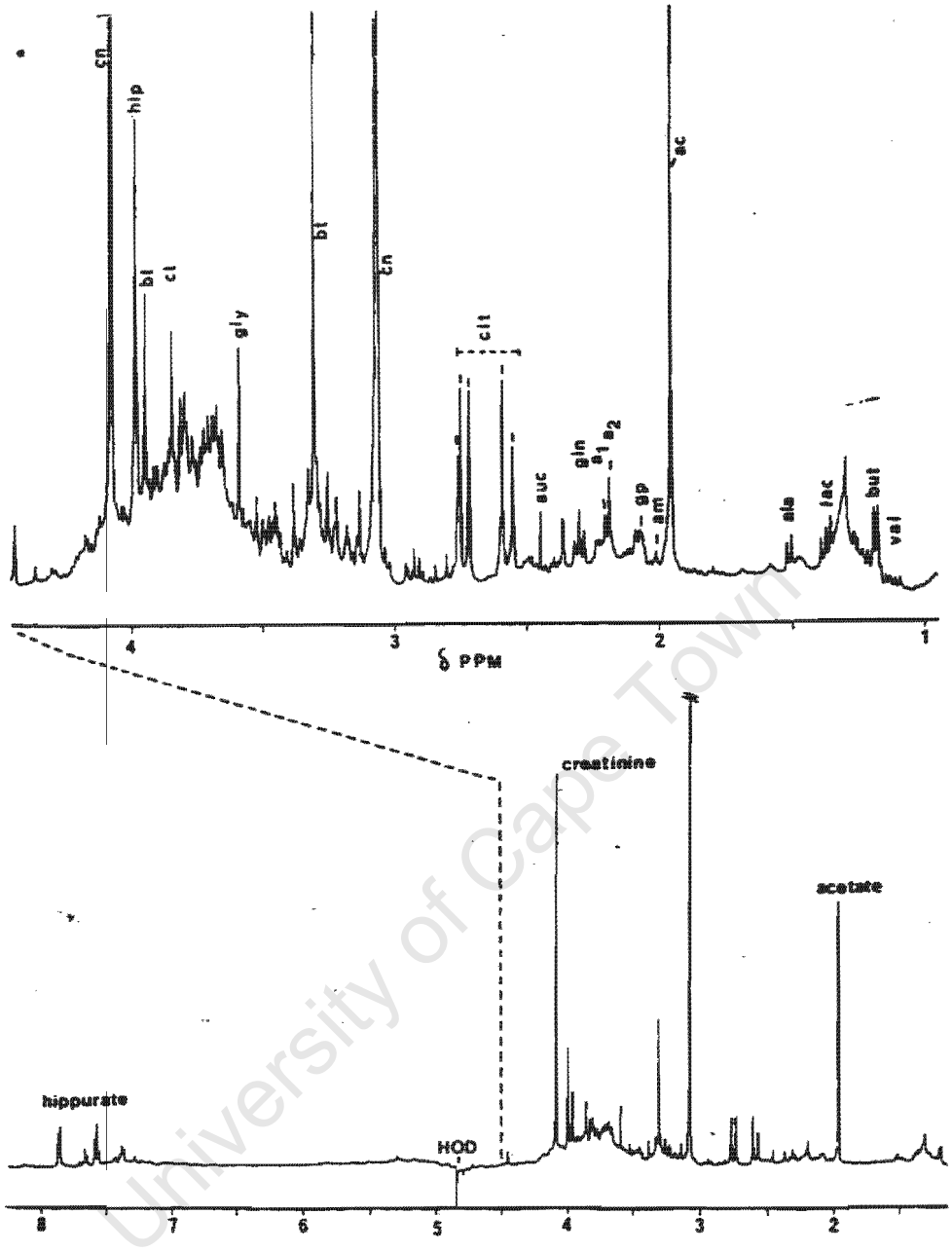


Figure 2.7 400 MHz ^{1}H-NMR spectrum of human urine showing assignments of major signals (reproduced from Nicholson and Wilson, 1989). Val, valine; But, β-hydroxybutyrate; lac, lactate; ala, alanine; ac, acetate; am, acetamide; a_1 and a_2, *N*-acetyl glycoproteins; gln, glutamine; suc, succinate; cit, citrate; cn, creatinine; bt, betaine; gly, glycine; ct, creatine; hip, hippurate.

Creatinine is usually present in millimolar concentrations, and gives rise to two large singlets. Many amino acids are present in low concentrations, but can be quantified using integration of peaks, for example, alanine, glycine and histidine. Glucose is present, and resonances are found in the crowded spectral region between 3.2 and 4.2 ppm. Selected organic acid metabolites are also visible e.g. acetoacetone, 3-D-hydroxybutyrate and formate.

Normal urine contains small amounts of macromolecules, but resonances from these are broad, because of their low mobility in solution. They are therefore seen as weak featureless signals that cause chemical noise and an uneven baseline (Bales et al., 1984). Resonances from small molecules that bind to macromolecules or complex to paramagnetic ions also become broad or unobservable. Resonances from chemical species with highly coupled spin systems may also be difficult to detect.

Some compounds present such as inorganic phosphates and metals are undetectable because they have no protons. Others are undetectable because they only contain exchangeable protons such as NH, OH, SH or COOH protons. These protons exchange with water to give broad or undetectable signals. Examples of such compounds are urea (which may be present in up to 0.7 molar concentration) and oxalate which is undetectable.

pH values of urine samples may vary considerably according to prevailing physiological conditions in the body. pH usually lies between 6.5 and 7.5 in normal human urine, but has been known to vary between pH 5 and 8 (Bales et al., 1984).

Resonances for compounds with pKa values in this pH range can shift significantly depending on the pH of the sample.

Urine osmolarities vary over a range of 150 to 1300 mOsmoles/ 1000grams in normal human urine (Lindon et al., 2000). Ionic strength of samples is also variable, but not enough to adversely affect spectrometer tuning except at particularly high field strengths (Bales et al., 1984).

^{1}H-NMR measurements on urine are relatively simple, once the water has been suppressed (see Section 2.4.2). Many of the low molecular weight metabolites can therefore be monitored this way. Assignments of signals are done on the basis of chemical shift and spin-spin coupling. 2-dimensional correlation techniques are sometimes used to aid in the spectral assignment.

2.6 OBJECTIVES

The difference in chemical composition of the urine between kidney stone patients and healthy subjects is important in the diagnosis and management of stone disease. Kidney stone formation is a very complex chemical process that depends on and is controlled by several urinary factors which act either individually or synergistically. Hyper- or hypo-values of any one component does not necessarily result in stone formation. Researchers are thus faced with the daunting challenge of identifying some chemical, biochemical or physiological factor or property in urine that is able to discriminate between stone formers and normals. It is perfectly normal to hypothesise

that some such discriminator must exist, because calcium oxalate crystallization and stone formation occurs in one type of urine, but not the other.

^{1}H-NMR studies of urine have been used in metabolic monitoring (see Section 2.3.1) effectively indicating kidney functioning and overall metabolic functioning. It is suited to the analysis of whole urine samples without pre-treatment leaving all physiological interactions and chemical equilibria intact. It is therefore proposed as a novel means of studying the urine of kidney stone patients. Although many of the traditional urinary risk factors related to stone disease (see Section 1.5.1) cannot be directly measured by NMR, for example calcium cannot be detected and oxalate only has exchangeable protons, ^{1}H-NMR can effectively measure multiple metabolites simultaneously. Hence it is not envisaged that a major change in the urine of stone forming patient will be seen. Rather it is anticipated that subtle changes in the relative concentration of the different components will occur. In order to detect these subtle changes it will be necessary to employ pattern recognition methods. The aim of the present study, then, is to determine whether ^{1}H-NMR data coupled with pattern recognition methods can discriminate between the urine of kidney stone patients and healthy subjects.

CHAPTER 3

PATTERN RECOGNITION

3.1 INTRODUCTION

Chemometrics is the application of modern statistical methods to the interpretation of chemical data (Adams, 1995; Sharaf et al., 1986). Modern analytical laboratory instruments allow for the rapid analysis of samples and therefore generate large amounts of numerical and graphical data. Data interpretation therefore becomes a limiting factor of sample analysis. Chemometrics uses mathematical and statistical data manipulation, along with high-speed computers, to aid in the classification and interpretation of chemical data, making it faster and less labour intensive.

The ease with which we can obtain multiple analyses on a sample leads to multivariate sample data matrices. This means that each sample has more than one measurement. The use of chemometrics greatly helps to reduce the amount of data and to produce more manageable and interpretable data. Most spectral data including NMR spectra are inherently multivariate i.e. each spectrum gives us a quantitative measurement of the compounds present in a sample and therefore each sample can be described by many variables.

An example of these multivariate statistical techniques is pattern recognition (PR). This is an exploratory data analysis technique used to identify patterns in numerical data. Pattern recognition is defined as the ability to identify and interpret meaningful regularities in complex environments (El-Deredy, 1997). Given a collection of samples

characterized by a set of measurements made on each, the aim of PR is to find or predict a property of the samples that is not directly measurable, but is described by a pattern in the measurements (i.e. a combination of the measurements).

3.2 THE PATTERN RECOGNITION PROCESS

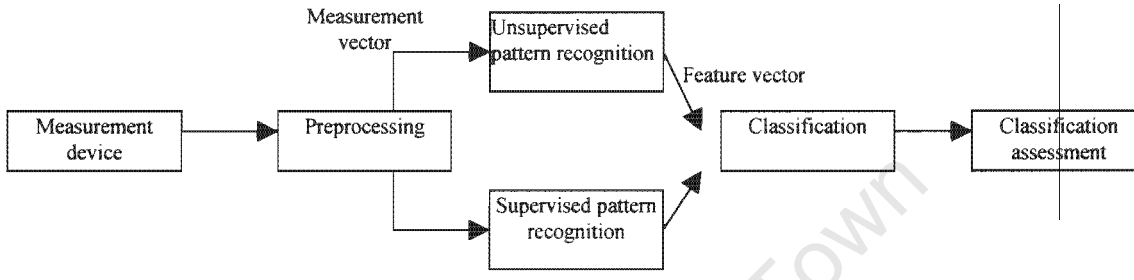


Figure 3.1 A flow diagram of the pattern recognition process.

PR processes are summarized in El-Deredy, 1997 and illustrated by Figure 3.1. First the data is preprocessed to produce the measurement vector. Feature extraction then produces a feature vector, which can be used to develop a classification model.

The PR process starts with the conversion of a physical or biological phenomenon into a set of numbers, which are preprocessed into a pattern. For example the urine sample is converted into a fid which is Fourier transformed into a spectrum, which is then integrated or digitized and expressed as numerical data. This is preprocessed to enhance the information representation of the original data by numerically transforming it (usually by scaling data) to bring variables into comparable ranges. Preprocessing includes phasing, baseline correction, integration and standardization of resonances to an internal reference. Other preprocessing methods are normalization of the data,

scaling of the data, autoscaling and feature weighting (Kowalski and Bender, 1972). The result of this is called the measurement vector.

The PR process uses the measurement vector to predict some unknown property or characteristic about the observed phenomenon. This is done by transforming the measurement vector into a feature vector. The feature vector is a mathematical representation of the measurement vector, usually with reduced dimensionality. This is achieved by either selecting a subset of the variables from the measurement vector, which are representative of the process, while discarding redundant, less relevant measurements (known as feature selection), or by combining the original measurements to form a new variable set with fewer features (known as feature extraction). The aim is to reduce the dimensionality of the data so that it is easier to interpret, while still preserving the information content. These techniques assume that the nearness of points in multi-dimensional space reflect the similarity of the properties of the samples. The distance (d_{ij}) between two points in multi-dimensional space is usually calculated according to the Euclidean distance formula (Kowalski and Bender, 1972):

$$d_{ij} = [\sum_{K=1}^{NV} (x_{ik} - x_{jk})^2]^{\frac{1}{2}}$$

where i and j are two samples containing k variables.

The learning and classification section of PR methods encompasses both unsupervised and supervised learning techniques. Unsupervised PR techniques look for patterns in the data without prior assumptions about group membership of the samples. The aim is to assist in the uncovering of intrinsic clusters or patterns in the data.

Supervised PR techniques involve the classification of samples based on the advanced knowledge of their group membership. The aim is to maximise the group separation (of a training data set), by manipulation of the descriptors and to develop a classification rule or algorithm to classify unknown samples into these groups. This classification rule is then used to allocate unknown observations (test data set) into the pre-defined groups.

Performance of the classification rule is an important part of the PR process. Performance is assessed practically by counting the number of errors a classification model makes when predicting the group membership of known samples. This is done by using independent samples or the leave-one-out error estimate method. In this method, one sample is removed at a time from the data and the remaining samples are used to determine the classification rule. The sample that is left out is then classified using this rule. This is repeated until all the samples are independently tested.

PR processes can be parametric or non-parametric. Non-parametric techniques make no assumptions about the underlying statistical distribution of the data, whereas parametric methods depend on knowing the probability functions of the data. Parametric technique classification results are therefore also accompanied by confidence estimates.

3.3 APPLICATION OF PATTERN RECOGNITION TECHNIQUES TO THE NMR SPECTRA OF BIOFLUIDS

3.3.1 The use of PR to interpret NMR spectra

A major advantage of biofluid NMR is that it allows observation of a large number of metabolites simultaneously. This, however, poses difficulties when trying to interpret the measurements or associated changes in measurements in more than a few metabolites. For example, the combination of all the biochemical components in a sample of urine represents a unique "metabolic fingerprint" of the sample. Knowledge of the excretion of three substances in the urine would give you a 3-dimentional view of the urine. Similarly, measurement of all the NMR detectable components simultaneously, gives a unique 'n' dimensional representation of the biochemical composition (where n is the number of metabolites measured or signals in the spectrum). When metabolic changes occur in the body, the levels of many of the endogenous compounds in the urine change. This is reflected in changes in the relative intensities of many NMR signals. The subtle biochemical alterations taking place, the complexity of the spectral patterns and the chemical noise due to signal overlap makes these changes extremely difficult to interpret. Therefore computer based pattern recognition techniques are used to explore the chemical or biological significance of the information presented in the spectra.

This is done by converting the NMR signal intensities into a multidimensional array of metabolite concentrations. These complex data sets are then reduced by PR

methods to produce more easily visualised and interpretable data. Often samples can be separated into different classes or groups according to their spectral patterns i.e. diseased patients urine may contain an unusual metabolite or high levels of an endogenous metabolite that may distinguish patient spectra from normal subject's urine spectra. PR techniques can also be used to develop a classification model and to predict the groups to which unknown samples belong i.e. unknown samples can be classified as patients or controls. A training set of NMR data is used to construct a mathematical model that predicts correctly the class of each sample. This training set is then tested with independent data to determine the robustness of the model.

3.3.2 Preprocessing of data

As mentioned, preprocessing is used to enhance the information representation of the original data by numerically transforming it. This includes manipulation of the FID by phasing, baseline correction and integration of spectra. It also involves the standardization of resonances to an internal reference, normalization of the data, mean centering, autoscaling and feature weighting

Mean-centering and autoscaling are the most popular preprocessing methods. Mean centering data involves subtracting the calculated average of a variable from the data so that the mean is zero. Mean centering is the most common procedure, when all data are the result of one spectroscopic technique (Hagberg, 1998).

Autoscaling refers to the division of each variable by the standard deviation for that variable (Kowalski and Bender, 1972). It removes any inadvertent weighting of the

variables and can therefore help extract important changes in metabolites present in low levels i.e. the variables with the largest values do not dominate the PR analysis.

3.3.3 Pattern recognition techniques

The most frequently used PR methods for the analysis of NMR data (as reviewed by El-Deredy, 1997, Hagberg, 1998 and Adams, 1995) are principle component analysis (PCA) (Nicholson and Wilson, 1989; Holmes et al., 1998; Holmes et al., 1994; Holmes et al., 1998; Beckwith-Hall et al., 1998; Nicholson et al., 1995; Holmes et al., 2000), factor analysis, non-linear mapping (NLM) (Nicholson and Wilson, 1989; Gartland et al., 1990; Holmes et al., 1994), cluster analysis, artificial neural networks (ANN) (Corne, 1996; Lisboa et al., 1988), soft independent modelling of class analogy (SIMCA) (Holmes et al., 1998; Beckwith-Hall et al., 1998), discriminant analysis, and canonical variate analysis. Most of these studies involve the classification of toxicological data and disease screening, in particular the diagnosis of inborn errors of metabolism.

NMR/PR also has many other uses, especially in the food industry where it has been applied to studies on substances such as orange juice (Le Gall et al., 2001). In this study NMR combined with PCA allows orange juice to be distinguished from pulp wash (a lower quality product) and points to the chemical compounds responsible for the discrimination between the two. It can therefore be used to identify pure orange juice and adulterated products.

Other studies have been published that use a variety of analytical measurement techniques and statistical PR methods (Defernez and Kemsley, 1997). These include studies on wine authenticity (Arvanitoyannis et al., 2000), characterisation of apple cider cultivars (Blanco-Gomis et al., 1998) and the classification of coffee cultivars (Murota, 1993). Most of these studies use supervised learning PR techniques such as discriminant analysis, canonical analysis and canonical discriminant analysis.

3.3.4 Pattern recognition techniques used in this study

The interpretation of many multivariate problems can be simplified by considering not only original variables, but also linear combinations of them. A new set of variables can be calculated each of which contains a sum of the original variables each suitably weighted. This can lead to a representation of the data using fewer variables (a reduction in the dimensionality of the data) and a better representation of the original data for subsequent analysis and interpretation. These linear combinations are derived by established mathematical techniques such as principle component analysis or canonical analysis.

3.3.4.1 Principal component analysis

Chemical data contains many sources of variation, such as different chemical composition of samples, systematic variation associated with the instrumentation used to measure the data and random noise. Principal component analysis is a multivariate data reduction technique which can separate these sources of variation by combining pooled correlation information contained in the data matrix into a new set of variables,

principal components. This is done by rotating and transforming the original n axes each representing an original variable, into new axes so that they lie along the direction of maximum variance of the data.

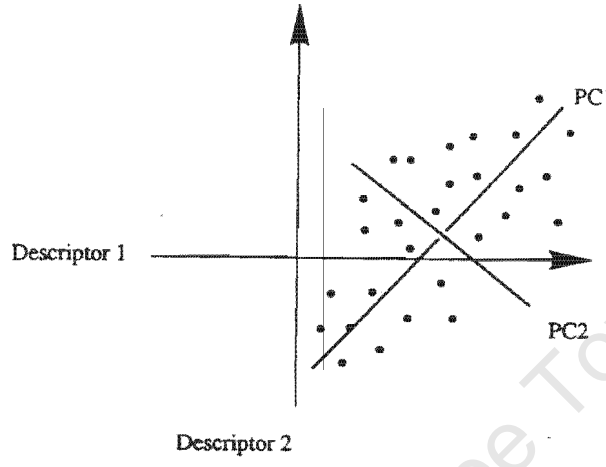


Figure 3.2 Geometrical representation of principal components extraction.

Principal component analysis can be simply illustrated geometrically in 2D as in Figure 3.2. The co-ordinates of each point with respect to the two axes are the values of the two descriptors for each sample. Principal component analysis attempts to fit these points with a straight line so that the residual distance of the points from the line is minimised. This line is equivalent to the first principal component (PC), as minimising the residual is the same as maximising the variance. The residuals can be described by a second line orthogonal to the first. This corresponds to the second PC. In the case of n-dimensional data, where each sample is described by n measurements the procedure is continued until the scatter of points in n-dimensional space is completely described.

PC's are therefore new variables created from linear combinations of the original data. Each principle component in turn contains less and less of the variation and is orthogonal to each new principle component i.e. new variables are uncorrelated.

The majority of the variance is described by the first few PC's and therefore the dimensions of the data are reduced. Principal component analysis can also reveal those variables or combinations of variables that determine some inherent structure in the data, which may be interpreted in chemical or physico-chemical terms.

For a more in depth mathematical description of principal component analysis, refer to (El-Deredy, 1997; Wold et al., 1984; Wold et al., 1987; D'Agostino, 1998; Adams, 1995).

3.3.4.2 Canonical discriminant analysis

Canonical discriminant analysis is a supervised learning PR technique, which can be used for prediction and classification of data (Cary, 1985).

In general, the aim of discriminant analysis is to find a classification rule to assign observations to one of several predefined groups. It uses a set of training data with known classification and extracts a discriminant function (combinations of the original variables) such that group separation is optimised. It then uses this function to classify unknown test data. A training set is projected along the discriminant functions and the co-ordinates of the centroid i.e. average point in multidimensional space, is calculated for each group. To classify unknowns, test data is then projected along the discriminant

functions and the distance between samples and the centroid of each group is calculated. The sample is then assigned to the group to which it is closest.

Canonical discriminant analysis is a form of discriminant analysis that finds functions of the variables that optimally predict the group membership. It is a dimension reduction technique that summarises between class variation by calculating linear combinations of the original variables that represent sources of successively maximised $\frac{\text{between group variance}}{\text{within group variance}}$ in the data i.e. canonical variables simultaneously minimise the spread of and maximise the distance between the pre-defined groups (Defernez and Kemsley, 1997).

Given samples of known group classification and several quantitative variables, canonical variable analysis derives canonical variables that have the highest multiple correlation within groups. The maximal multiple correlation is called the first canonical correlation. The variable defined by the linear combination is the first canonical variable. The second canonical variable is obtained by finding the linear combination, uncorrelated with the first canonical variable, that has the highest possible multiple correlation with the groups. The number of canonical variables equals the number of original variables or the number of classes minus one, whichever is smaller.

The coefficients of the linear combination are called canonical coefficients. When the canonical coefficients are applied to the data, a score is generated for each sample. A scores plot of the canonical variables will depict the relationship between samples in n dimensional space, into two or three dimensions, thereby making interpretation easier.

CHAPTER 4

EXPERIMENTAL PROCEDURES

4.1 SAMPLING

4.1.1 Subjects

Urine samples were collected from calcium oxalate kidney stone patients and healthy control subjects. The training data set consisted of 98 subjects of varying ages and of both gender. The control group contained 38 subjects: 14 females, ages ranging from 22-54 years, and 24 males, ages ranging from 17-55 years. The kidney stone patient group contained 60 patients: 34 females, ages ranging from 21-71 years, and 26 males, ages ranging from 26-67 years. Both groups had diverse racial backgrounds. All patient samples were obtained from the Urology Department Groote Schuur Hospital. All control samples were obtained from staff and students in the Chemistry Department. A test data set, consisting of four patients and four controls were also collected. All subject details are tabulated in Appendix 1

4.1.2 Sample volume

Most urine analyses to determine the risk of kidney stone formation are performed on 24h urine samples. This is necessary because urine composition varies throughout the day. A 24h sample collection enables a standardised urine composition to be reported. Urinary volume is also a factor in stone formation, and therefore a standardised collection period is useful.

However, 24h human urine samples cannot be effectively used for NMR urine analysis as they are very dilute and the large water signal cannot be adequately suppressed. To avoid dynamic range problems and to allow the observation of solute signal in the presence of the proton signals from water, spot samples were collected for NMR analysis. These spot samples were collected at the early morning voiding because this is when the urine is most concentrated. Early morning specimens have been widely used in NMR studies on human urine samples (Holmes et al., 1994). It is recognised that a spot sample is not fully representative of all components that may be excreted throughout the day, and that additional components may be excreted due to dietary variables and other external factors. However, practical considerations dictate that 24h urines are not feasible in studies such as those described in this thesis.

4.1.3 Preservatives

When 24h samples are collected, a preservative needs to be added to prevent bacterial degradation of the urine during the collection period. A common preservative used when collecting human samples is benzoic acid. However, when ^{1}H-NMR is to be carried out on the samples, it is advantageous to use a non-protonated preservative so that its proton signals do not interfere with sample signals in the proton spectrum. Benzoic acid is thus unsuitable. Sodium azide is often used for the preservation of urine samples for ^{1}H-NMR urinalysis. However, this substance is toxic, and is therefore not used in human sample collection. An alternative approach when using spot samples is to freeze them at -40 °C immediately after collection. The problem with this method is that upon defrosting the samples for analysis, precipitation of protein occurs in the

samples. It can be argued that other substances in the urine may co-precipitate, thereby altering the composition of the urine. A variation of this method is to analyse fresh samples, immediately after collection, before bacterial degradation can occur. This allows accurate measurement of urine composition as found in vivo. For these reasons NMR was run within a few hours of sample collection, in this study, using early morning spot samples.

4.1.4 Urine selection

Samples were tested for blood, glucose and nitrite, using urine test strips (Combur[10] Test). Samples testing positive for any of the above were discarded. Blood in the urine is indicative of kidney injury or infection. Such samples cannot be used. If there are large amounts of glucose in the urine, the glucose signals will obscure all other compound signals in the proton spectrum, so that they cannot be measured. These samples therefore cannot be compared with other patients' samples, as overriding differences in spectrum will be due to glucose. Nitrite is indicative of nitrite producing bacteria in the urine. Bacteria will alter the urine metabolite profile by their metabolic processes. Therefore nitrite positive samples were also discarded.

4.2 SAMPLE PREPARATION

4.2.1 pH adjustments

As mentioned earlier, many of the compounds present in urine have pK_a values within the normal pH range of urine. This gives rise to a pH dependent shift in NMR

chemical shifts of these compounds. Because the spectra are being compared by integration of spectral regions, and observation of the increase or decrease in these signals is desired, elimination of pH induced shifting of NMR signals is necessary. The pH of the samples were therefore maintained between 6.7 and 7 by buffering with phosphate buffer. Exact pH measurement is not necessary, but the pH must be maintained above the pK_a of the substances of interest.

NMR samples were made up in 5 mm outer diameter sample tubes containing 600µl fresh urine and 200µl of a 200 mM phosphate buffer. The buffer contains D_2O and 5 mM sodium 3-trimethylsilylpropanoate (TSP). The D_2O provided the deuterium field frequency lock, and the TSP acted as an internal concentration standard and a NMR chemical shift reference.

4.3 NMR ANALYSIS

4.3.1 Water suppression

As discussed in Section 2.4.2, the main difficulty with acquiring ^{1}H-NMR of urine is the presence of a large signal from the protons of water. The water concentration can be reduced by freeze drying the sample and reconstituting in D_2O. This would entail more sample preparation and would result in a loss of exchangeable proton signals and possibly the loss of volatile compounds in the urine. For this reason, NMR solvent suppression techniques were investigated as a means of eliminating the water proton signal.

The main water suppression techniques that have been reported for the acquisition of ^{1}H-NMR on urine samples are (Wright et al., 1995):

- Presaturation. This is gated secondary irradiation at the water resonance, thus saturating the resonance. The main advantage of this is simplicity and speed, but the disadvantage is that a residual water signal may still remain and analyte signals too close to or under the water signal will also be distorted or lost.
- WATR (water attenuation by T2 relaxation). This method selectively attenuates the water signal by reducing the water T2 relaxation time. The water T2 relaxation time is reduced by the addition of urea, guanidinium salts or ammonium chloride, and making appropriate pH adjustments to obtain maximal water line widths. The resulting broad water signal is then selectively attenuated using the CPMG spin-echo sequence (Nicholson and Wilson, 1989). Signals from exchangeable protons and other broad resonance's such as macromolecules with short T2 relaxation times are also attenuated. Signals close to the water signal or exchangeable protons may be distorted.
- 1-D NOESY presaturation. This is a standard homogated 1-D NOESY pulse sequence, with both the transmitter and decoupler frequencies set to the water resonance with presaturation during the delay between pulses. The NOESY sequence is essentially removing the transverse components of the residual water signal after presaturation via the phase cycling routine. This method has been shown to give good suppression of the water signal and a 2 fold increase in the signal to noise ratio over the WATR method. It also does not distort resonance close to the water signal.

Another commonly used pulse sequence used for solvent suppression, although not reported for acquisition of 1D spectra of biofluids, is WATERGATE (water suppression by gradient tailored excitation) (Piotto, M et al., 1992). This is a gradient

echo sequence that uses a non-selective RF pulse to excite all resonances uniformly, and a subsequent symmetrical echo segment with two short field gradients to eliminate the water. The echo segment of the sequence is formed by two short field-gradient pulses of the same amplitude and sign with a centrally placed 180° selective RF pulse. All coherences dephased by the first field gradient are rephased by the second one. The echo is designed so that the water resonance approaches zero while the rest of the resonances are flipped by 180°. Therefore no water signal is left when acquisition starts. This principal can be incorporated into many 2D pulse sequences for solvent suppression.

A Varian Unity Plus 400 MHz spectrometer was used in this study,. The pulse sequences available on this spectrometer for solvent suppression were: presaturation (presat), CPMG, TOCSY WATERGATE, NOESY1D and a special gradient presat sequence.

Firstly, it was decided not to use the WATR method because of the time consuming task of sample preparation and accurate pH adjustments. Therefore the CMPG sequence was not attempted. Secondly, the NOESY1D and gradient presat sequences require shaped pulses, which were not available on our instrument. Therefore these pulse sequences could not be attempted. The presat and TOCSY WATERGATE pulse sequences were investigated to find the most appropriate method for solvent suppression to be used when acquiring the spectra. The TOCSY WATERGATE was run as a 1D, by only using one increment, but was found to be difficult to optimise, and did not give improved water suppression when compared to presat.

Presaturation was therefore chosen as the method of water suppression because of its simplicity, ease of use and comparable results obtained with the more complicated WATERGATE pulse sequence. Water suppression was found to be adequate, and because the region surrounding the water peak in urine does not contain any solute signals of interest, none of the metabolite signals were affected by irradiation at the water frequency.

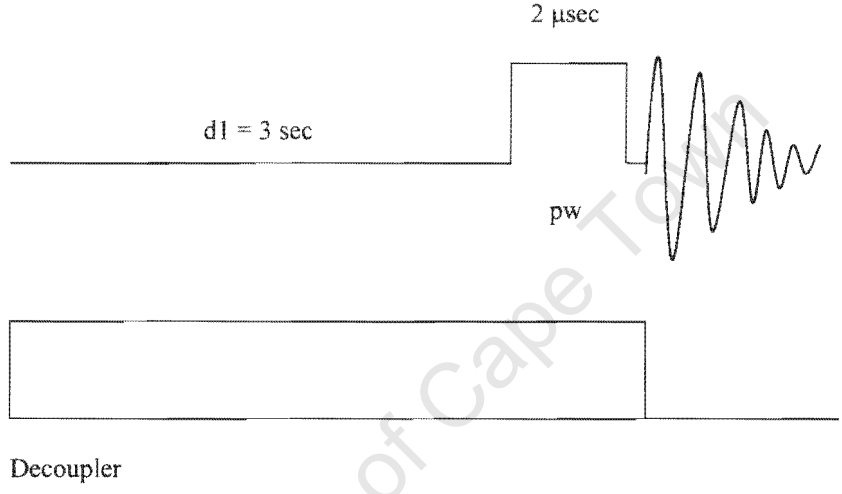


Fig 4.1 Drawing of pulse sequence used.

4.3.2 NMR data acquisition

^{1}H-NMR spectra of each urine sample were obtained on a Varian Unity Fourier transform spectrometer, operating at 400 MHz (7.4 Tesla). The temperature was kept constant at 303K. Spectra were run spinning at 20Hz, accumulating 30 transients. The following acquisition parameters were used:

A 3 second water presaturation pulse (decoupler frequency set to water at 4.64 ppm) was followed by a 2 microsecond (26 degree) observation pulse. A spectral

window of 6000 Hz was accumulated in an acquisition time of 3.7 seconds. The FID's were collected with 45K data points (7.5 points per Hz).

4.4 DATA PROCESSING

The urine spectra were processed using VNMR 6.1c Varian software. The FID's were Fourier transformed using a line broadening function of 0.5 Hz. The methyl peaks of the TSP were referenced to 0 ppm. Each spectrum was phase corrected, automatically drift corrected and baseline corrected. Because spectra are being compared quantitatively, processing methods are important and spectra need to be treated identically. An automated baseline correction method was devised by identifying baseline regions in the spectra and specifying these as zero for baseline correction in all spectra.

Peak assignments were made on the basis of literature chemical shifts and couplings. To maximise the use of all metabolic information in the spectrum and to provide a reproducible procedure, each spectrum was integrated over 37 consecutive regions between δ 9.0 and δ -0.03. Integral regions were identified by considering the regions of all the spectra that contained signals, and defining these as the integral regions for all the spectra. The region around the residual water signal between δ 6.7 and δ 4.2 (including the broad urea NH resonance) was excluded, because of the variable levels of suppression of the water signal. There were no significant signal peaks found in this region, only urea, which cannot be quantified because of variability due to cross relaxation effects.

This resulted in 37 integral area descriptors (variables) for each sample. The data was tabulated as a 98x37 data matrix. The integrals were then normalised to the area of the methyl groups of the TSP to eliminate instrumental variation and each data set was normalised to the total sum of all the integrals. The integral definitions for each variable and the data matrix are shown in Appendix 1.

The data set was preprocessed by mean-centering and data autoscaling. Mean centering data involves subtracting the calculated average of a variable from these data so that the mean is zero, whereas autoscaling refers to the division of each variable by the standard deviation for that variable. This is done to eliminate the dominance of large signals during data analysis.

4.5 MULTIVARIATE DATA ANALYSIS

Data were analysed by commercial statistical software packages, Statistica and SAS, that carry out a variety of statistical data analyses. First principal component analysis (PCA), using Statistica, was conducted on both normalised and autoscaled data to look for any structure in the data. The entire data set was imported into the Statistica factor analysis module and all the variables were included in the analysis. Statistica calculates the principal components (PC's) that describe the data. A scores plot of the first few principal components were examined to see if any of the first PC's (describing the largest variation in the data), described the variation between patients and control subjects.

Canonical discriminant analysis was carried out using the SAS software package. Three analyses were carried out. The first containing all the data with the samples coded into four groups: male patients (PM), female patients (FP), male controls (MC) and female controls (FC). This was then used as a dependant variable. The second and third analyses contained males and females separately, coded as controls (C) and patients (P). Each analysis used the CANDISC procedure (Cary, 1985) to determine the canonical variables, canonical scores and the eigenvalues of each canonical variable. The scores for each sample were plotted to give a graphical representation of the canonical variables describing each data set.

Finally test samples were used to validate the canonical discriminant model developed. This entailed collecting fresh samples as independent test data. Test samples comprised two control females, two control males, two female patients and two male patients. Data were obtained by identical procedure to that used for the original data. Determination of classification using the canonical discriminant model entailed applying the canonical coefficients to each variable of the test sample to obtain scores. This was carried out using the SAS program shown in Appendix 2. These scores were plotted on the scores plot with the original data. The test samples were then assigned to the group to which they were closest. This was done by calculating the 95% confidence interval for each group and assigning each test sample within these.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 NMR ANALYSIS

400 MHz ^{1}H-NMR spectra of urine from healthy control subjects and calcium oxalate stone patients were obtained. A wide range of urinary metabolites were assigned by comparison with chemical shift values reported in the literature (Bales et al., 1984; Bell and Sadler, 1995) but many minor resonances from metabolites present in low concentrations could not be assigned. In the chemical shift range 1 to 4.2 ppm, there are many signals that cannot be identified because they either originate from proteins or compounds present in low concentrations. This, together with the overlapping of signals, causes an irregular baseline and makes assignment of signals difficult. Fewer resonances are found in the region between 7 and 9 ppm. Table 5.1 lists signals that have been identified.

A typical ^{1}H-NMR spectrum of urine from a healthy control subject is shown in Figure 5.1 and a typical ^{1}H-NMR spectrum of urine from a calcium oxalate stone patient is shown in Figure 5.2. Signals that have been identified are labelled on each spectrum.

Table 5.1 ^{1}H-NMR assignments of control human urine.

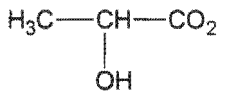
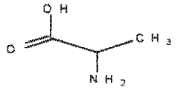
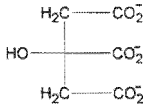
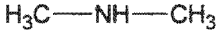
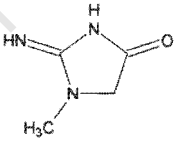
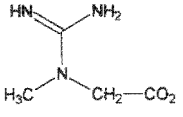
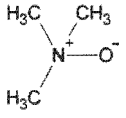
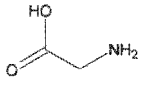
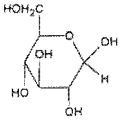
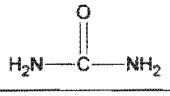
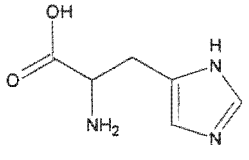
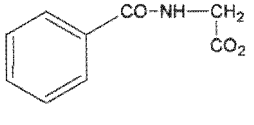
Chemical Shift (ppm)	**Multiplicity**	**Assignment**	**Compound**	**Structure**
1.33 4.11	d q	CH_3 CH	Lactate	
1.47 3.76	d q	β-CH_3 α-CH	Alanine	
2.67 2.80	d d	CH_A CH_B	Citrate	
2.72	s	CH_3	Dimethylamine	
3.03 4.05	s s	CH_3 CH_2	Creatinine	
3.04 4.00	s s	CH_3 CH_2	Creatine	
3.27	s	CH_3	TMAO	
3.56	s	α-CH_2	Glycine	
3.2-4	m	All	Glucose	
5.75	Broad	NH_2	Urea	
4.04 7.36 6.56	t s s	α-CH C4H C2H	Histidine	
3.97 7.55 7.64 7.84	d t t d	CH_3 m-CH p-CH o-CH	Hippurate	
8.46	s	CH	Formate	HCO_2^-

Figure 5.1 400MHz ^{1}H-NMR of typical control subject urine

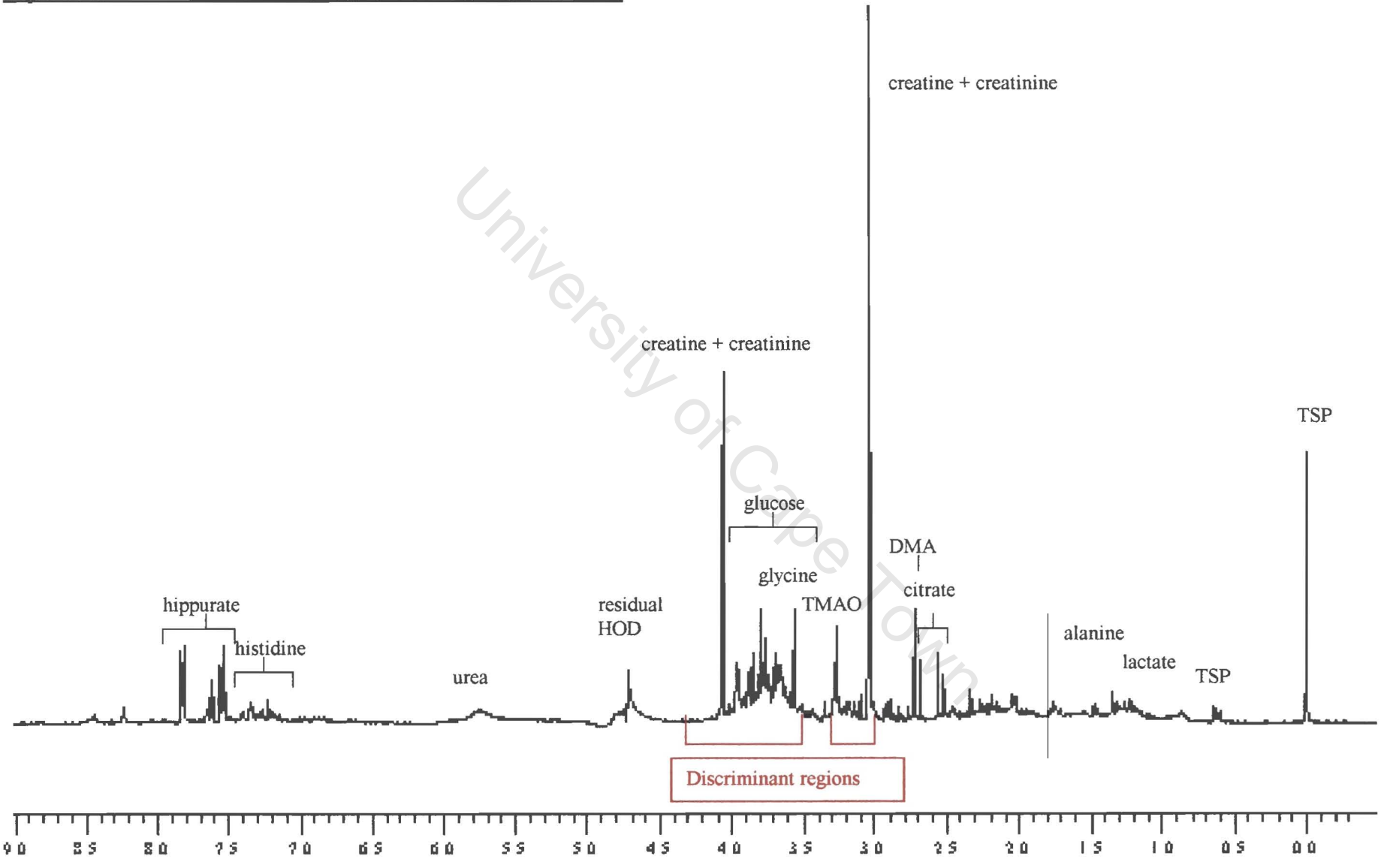


Figure 5.1 (B) An expansion of the aromatic region of the control spectrum.

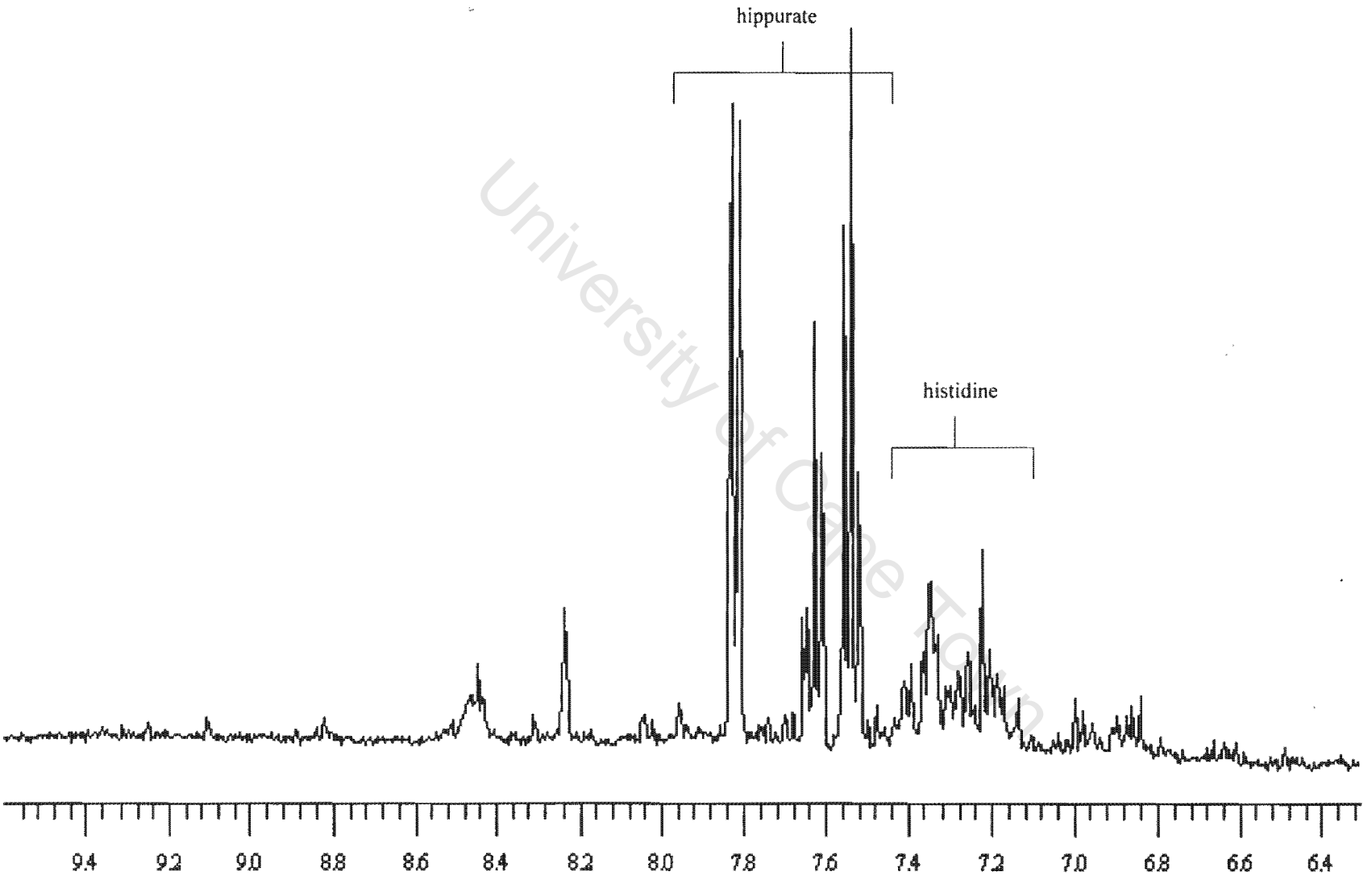


Figure 5.1 (C) An expansion of the aliphatic region of the control spectrum.

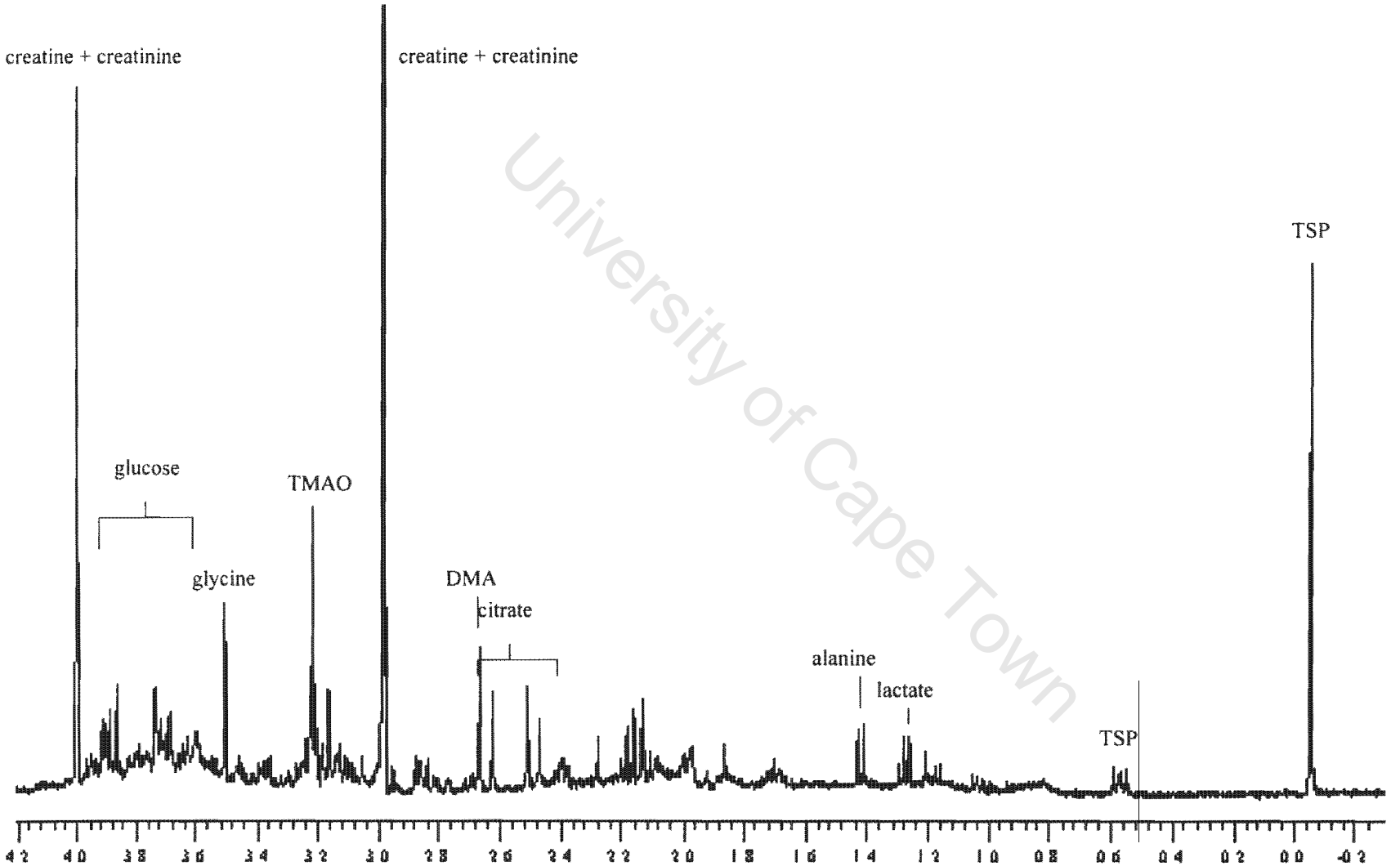


Figure 5.2 (A) 400 MHz ^{1}H-NMR spectrum of calcium oxalate kidney stone subject urine

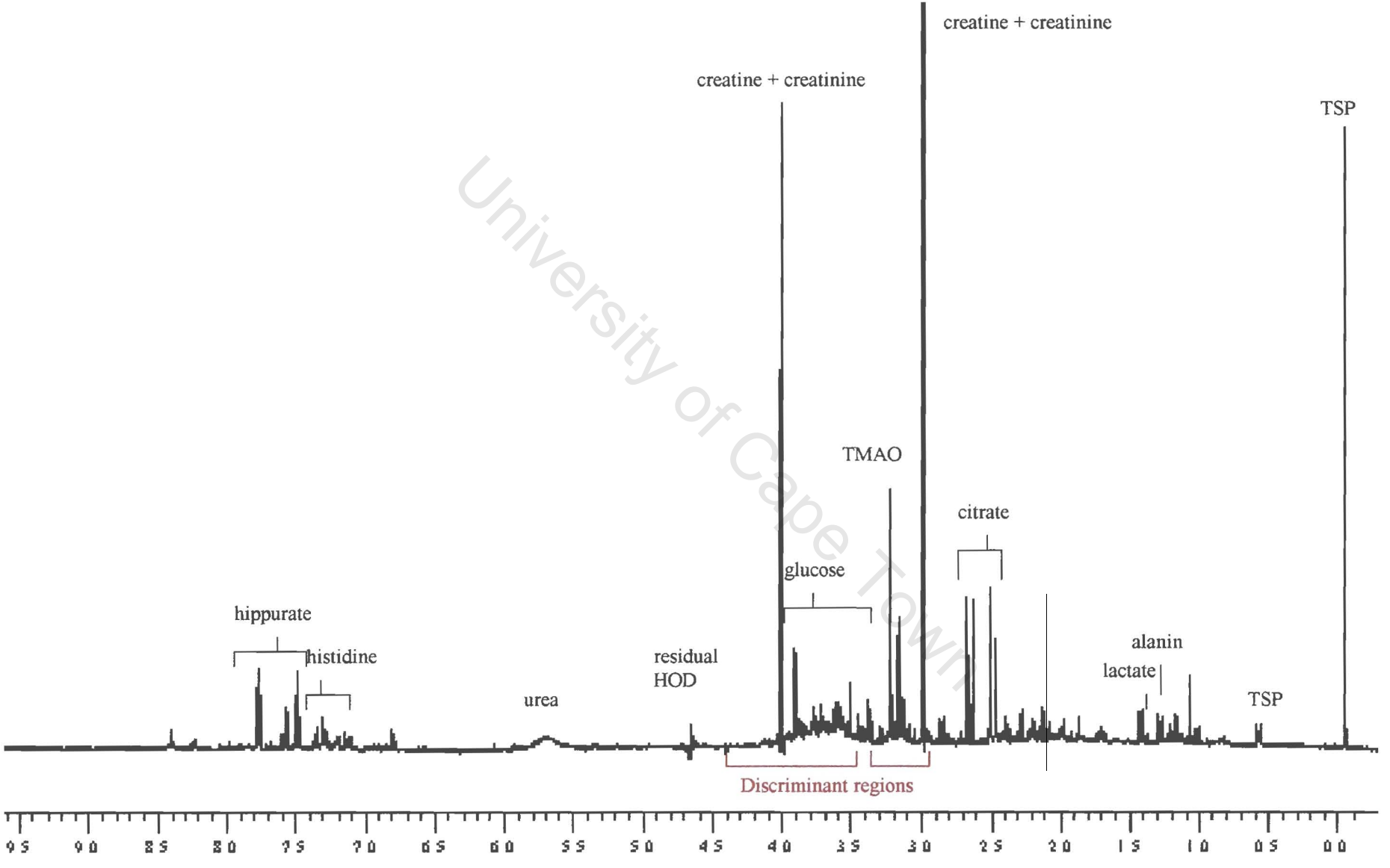


Figure 5.2 (B) An expansion of the aromatic region of the patient spectrum.

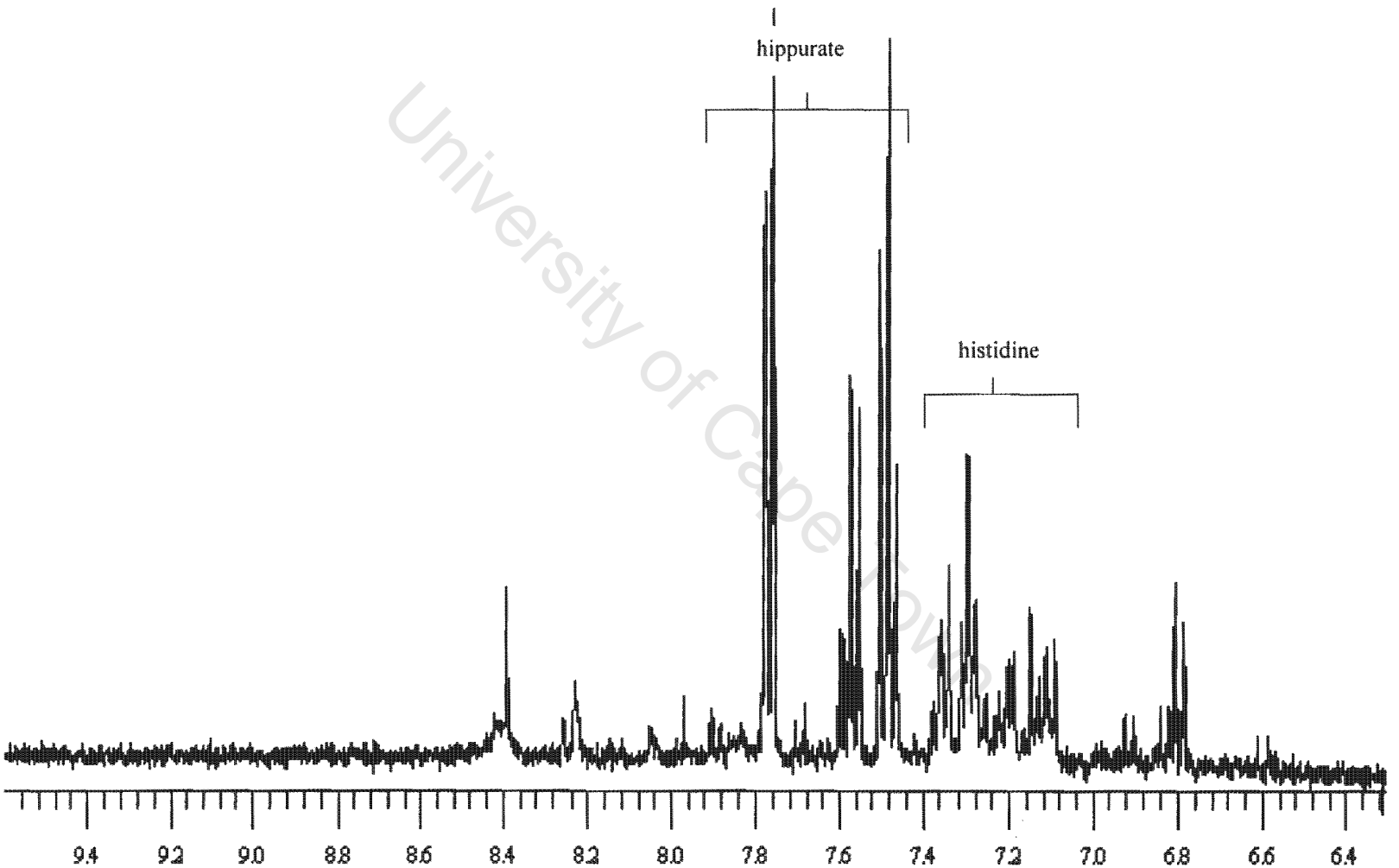
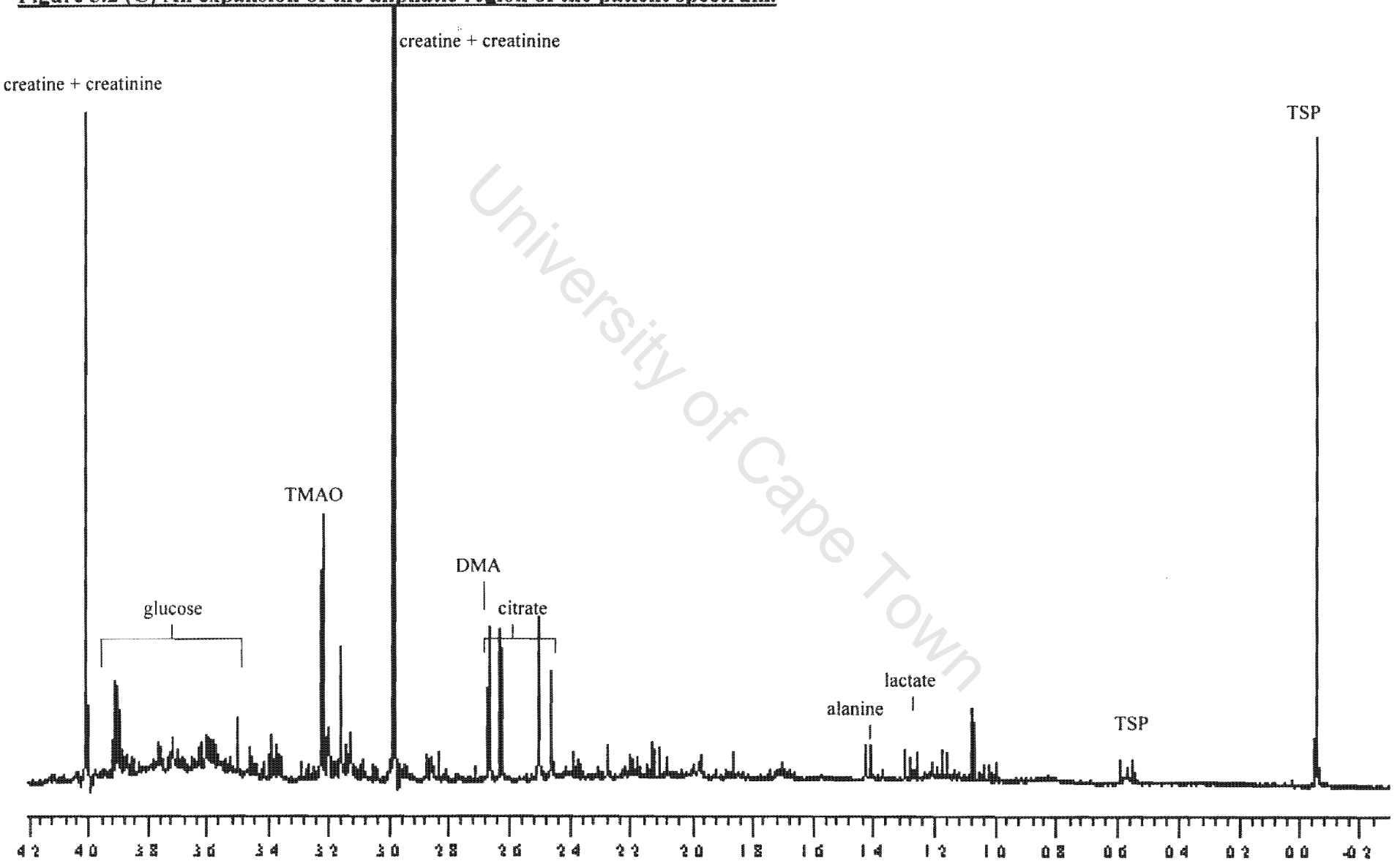


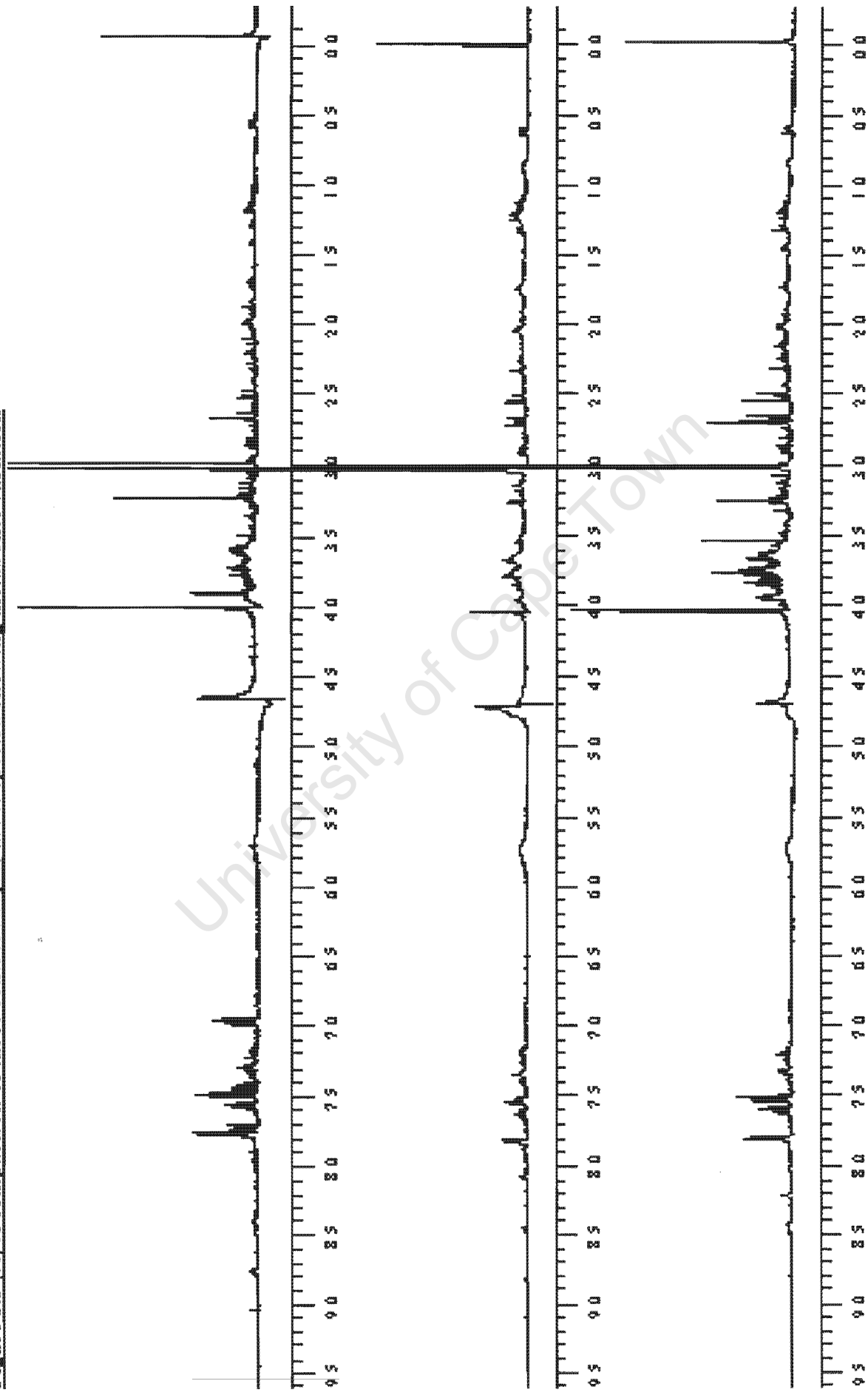
Figure 5.2 (C) An expansion of the aliphatic region of the patient spectrum.



The strongest signals at 3.03 and 4.05 ppm were assigned to the creatinine and creatine CH_3 and CH_2 protons, respectively. The region between these two peaks contains signals for glycine, trimethylamine-N-oxide and multiple signals for glucose. Citrate protons show a doublet of doublets at about 2.6 ppm, close to the singlet for dimethylamine at 2.72 ppm. Doublets at 1.33 and 1.47 were assigned to lactate and alanine respectively. A residual water signal remains at 4.64 ppm. The broad signal at 5.75 ppm was assigned to the urea protons. It is broad because these protons are exchanging with the water. Signals from formate, hippurate and histidine were also assigned. Full assignment was not necessary, as spectra were converted into numerical descriptors for comparison.

The composition of urine is known to be highly variable and many of the metabolite concentrations are found to vary widely from subject to subject within both the control group and patient group. Dietary, diurnal and hormonal variations may influence the composition of the urine. Variation may also be due to age related changes in renal function (Hesse et al., 1986), or natural biorhythms of the subject, which may cause variation according to collection time. Concentrations of metabolites found in the urine also vary due to water loading and state of physical exertion of patient. Given these types of variation, it is important to try to closely match control and patient samples by age and gender. Figure 5.3 shows a stacked plot of control subject spectra illustrating this variation. Largest variation can be seen in formate, histidine, TMAO, glycine and citrate signals.

Figure 5.3 (A) A comparison of three control subject urine spectra showing metabolite variation



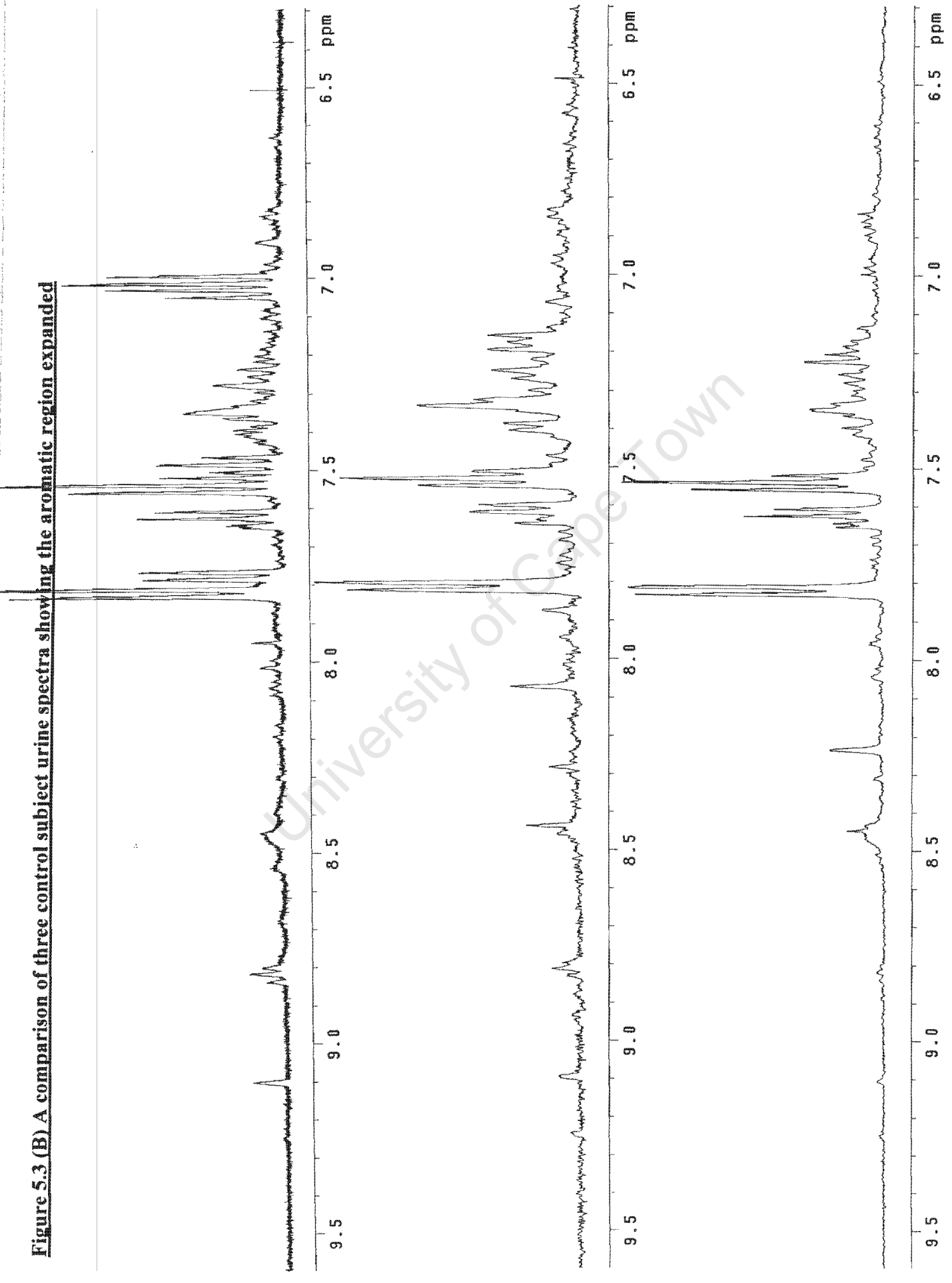


Figure 5.3 (B) A comparison of three control subject urine spectra showing the aromatic region expanded

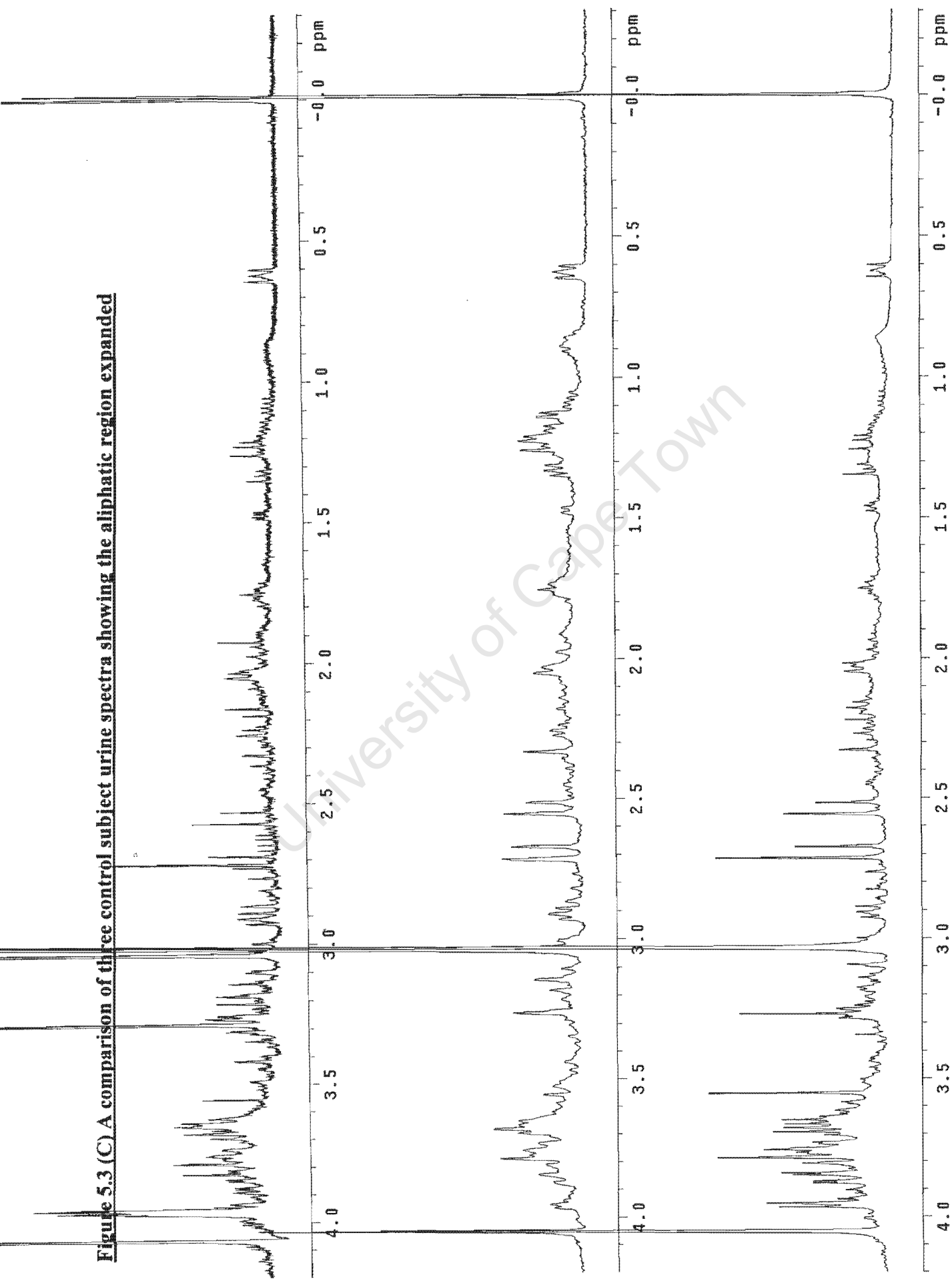


Figure 5.3 (C) A comparison of three control subject urine spectra showing the aliphatic region expanded

Other sources of variation are instrumental and are introduced by spectral processing. The importance of processing of spectra was mentioned in Section 4.4. When FID's are phased, baseline corrected and integrated it is important that no operator bias is introduced. Therefore as much automated processing as possible should be carried out. Baseline correction for example can be very subjective, and may cause apparent differences in concentrations (peak integrals). This is a drawback of using integration as the means of spectral comparison. To reduce the variation due to subjective baseline correction, this was automated by predefining baseline regions, and applying these to all spectra.

From a visual comparison of patient and control subject urine spectra, no obvious differences in 'metabolic fingerprints' are evident. Within group variation is equivalent to between group variation. This is illustrated in Figure 5.4, which shows a patient and control spectrum. They look very similar with regards to composition. Any differences which may appear are therefore either masked by chemical noise, or may be due to subtle changes in a number of metabolites simultaneously, or due to a macromolecule, whose signals are not well defined. As discussed in Section 3.3 the complexity of the spectra make it difficult to visually determine inter group differences. Therefore pattern recognition techniques need to be used to aid in the spectral interpretation.

Figure 5.4 (A) Comparison of a patient (a) control subject (b) spectrum

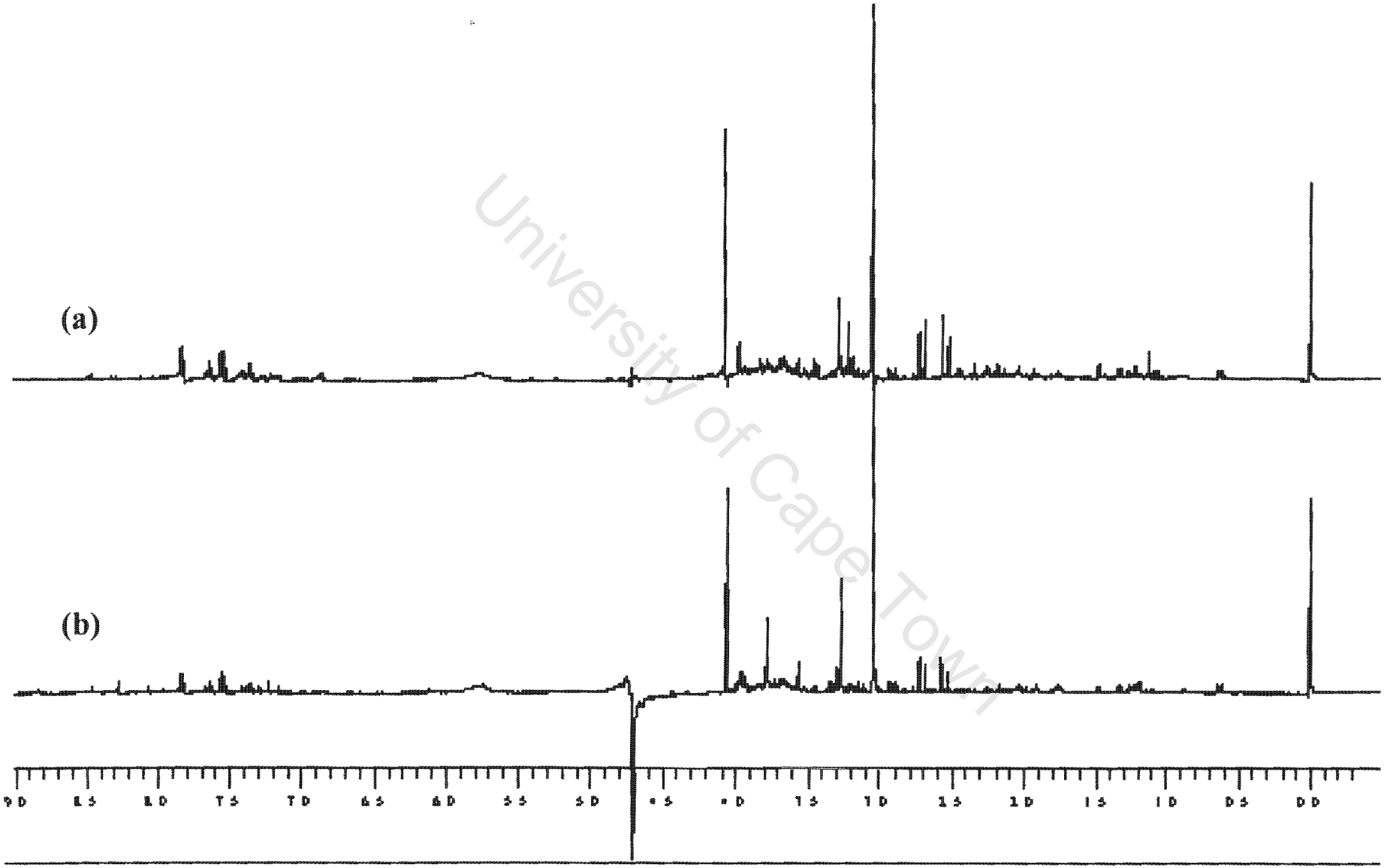


Figure 5.4 (B) Comparison of a patient (a) control subject (b) spectrum showing the aromatic region expanded

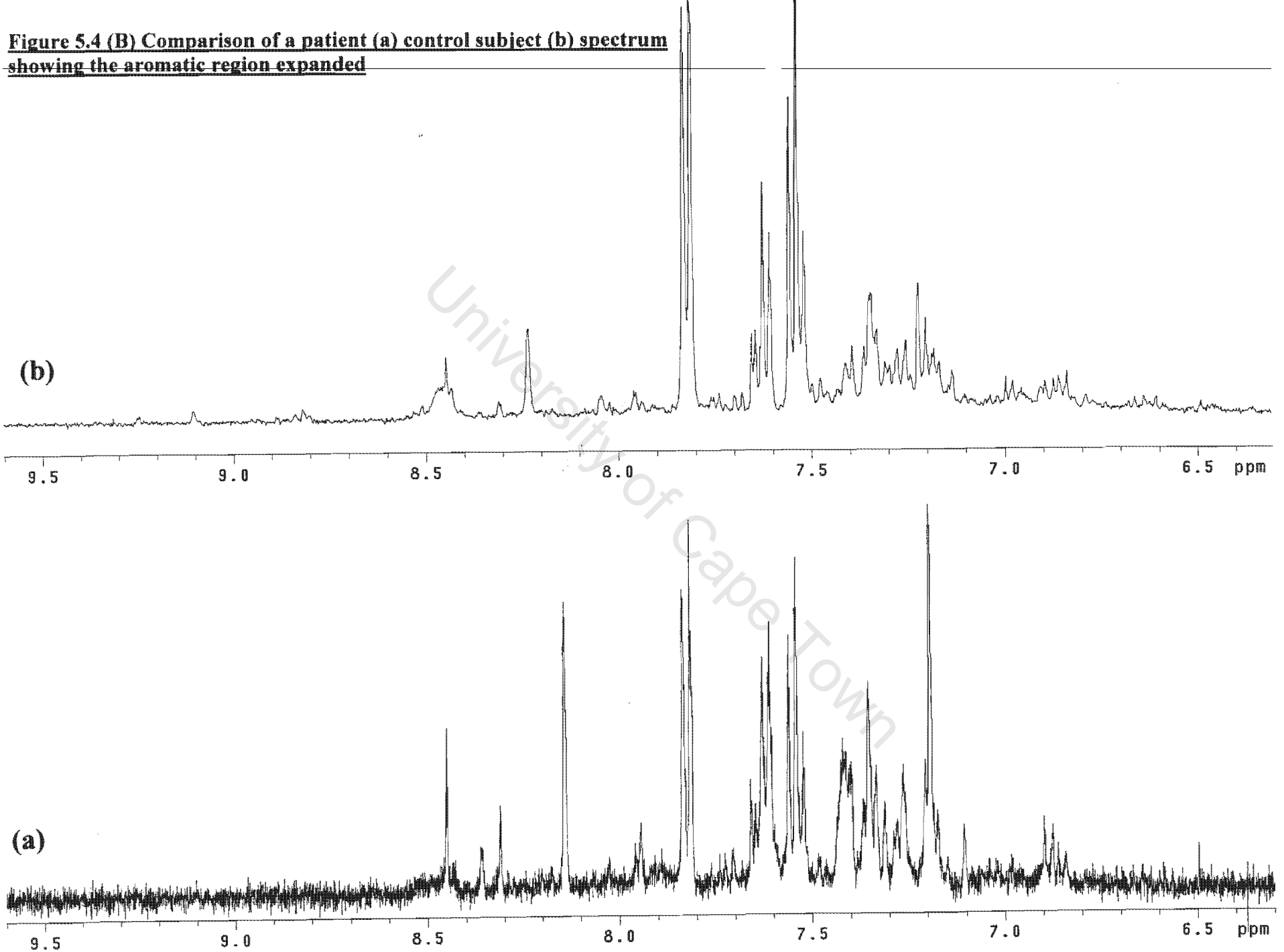
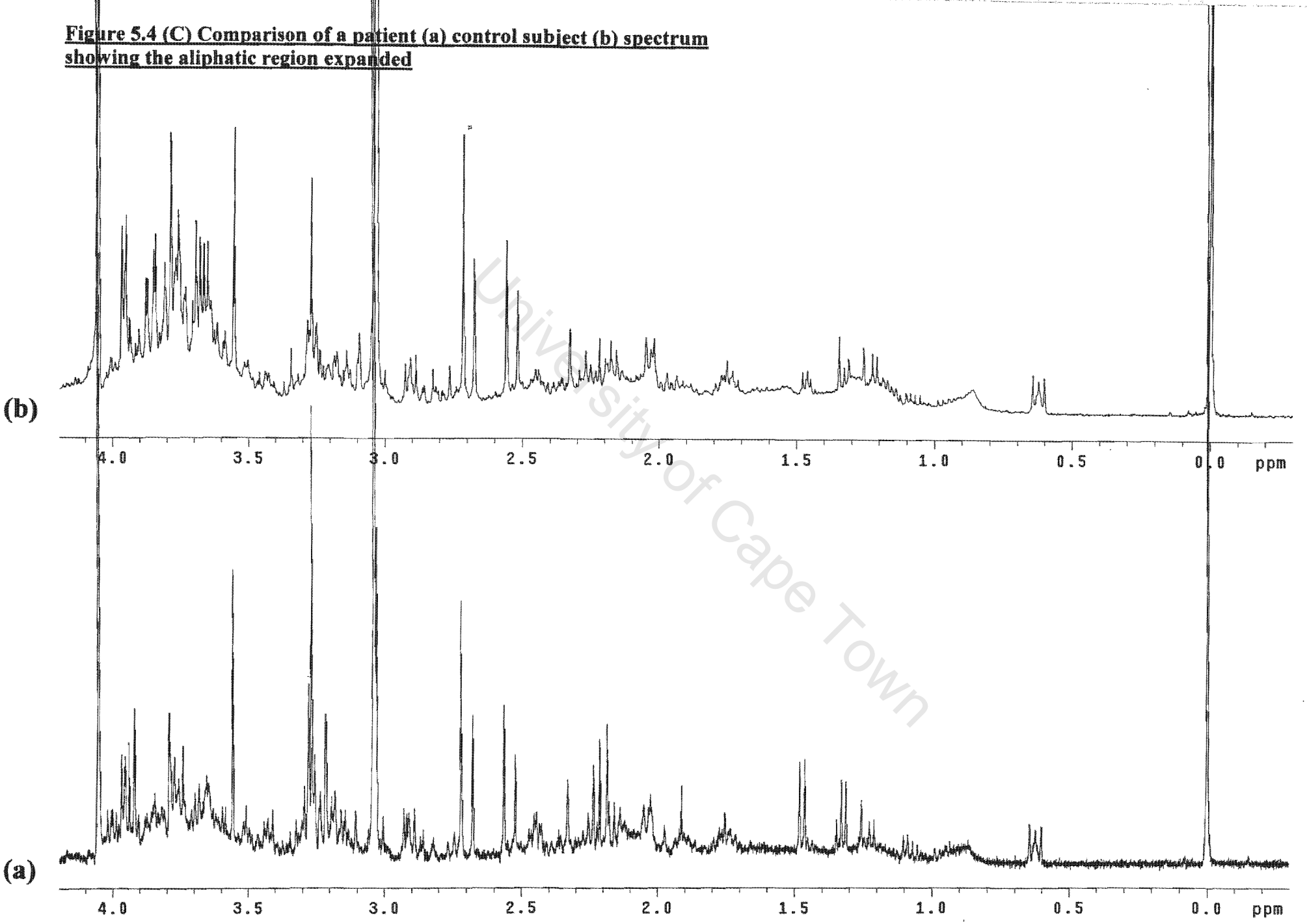


Figure 5.4 (C) Comparison of a patient (a) control subject (b) spectrum showing the aliphatic region expanded



5.2 PATTERN RECOGNITION

Data were normalised to TSP, and the integral of all the signals normalised to a constant to eliminate instrumental variation. Before PR analysis was carried out, the data were also mean centred and autoscaled to eliminate the dominance of large signals. These standardised variables are calculated by subtracting their mean and dividing by their standard deviations. This puts all the variables on a comparable footing as regards variability within groups. Appendix 1 shows the normalised data for patient and control samples.

5.2.1 Principal component analysis (PCA)

There are many PR methods available for the analysis of multivariate data. As discussed in Section 3.3, PCA is an exploratory analysis tool. It was therefore used as the first means of looking for structure in the data. The objective of PCA is to find a lower dimensional representation of the data that accounts for most of the variance of the variables; it does not use a discriminant function. Because it looks for principal components (PC's) that maximise the variation in the data, with PC1 showing the largest variation, and PC2 the next largest variation, it is useful to carry out PCA and look at a scores plot of the samples using the first few PC's. This gives a summary of the variation in the data with reduced dimensionality in an easily interpretable graphical fashion.

Patients and control groups will separate in a scores plot of PC1 if the largest variation in the data is found to be due to the difference between patient and control

spectra, or PC2 if the second largest cause of variation in the data is due to this difference, and so on. There is no guarantee that large variation features in the data will necessarily qualify as being important factors for discrimination between patients and controls e.g. if the metabolite with the largest variance does not play a role in the cause or effect of the disease, it will dominate PC1, resulting in difficult classification. In such a case PCA will not distinguish diseased patients in PC1. PCA generally displays effective discriminant ability in cases where the largest variation is due to a metabolite that is altered directly as an effect of the disease state, such as an inherent metabolic disorder.

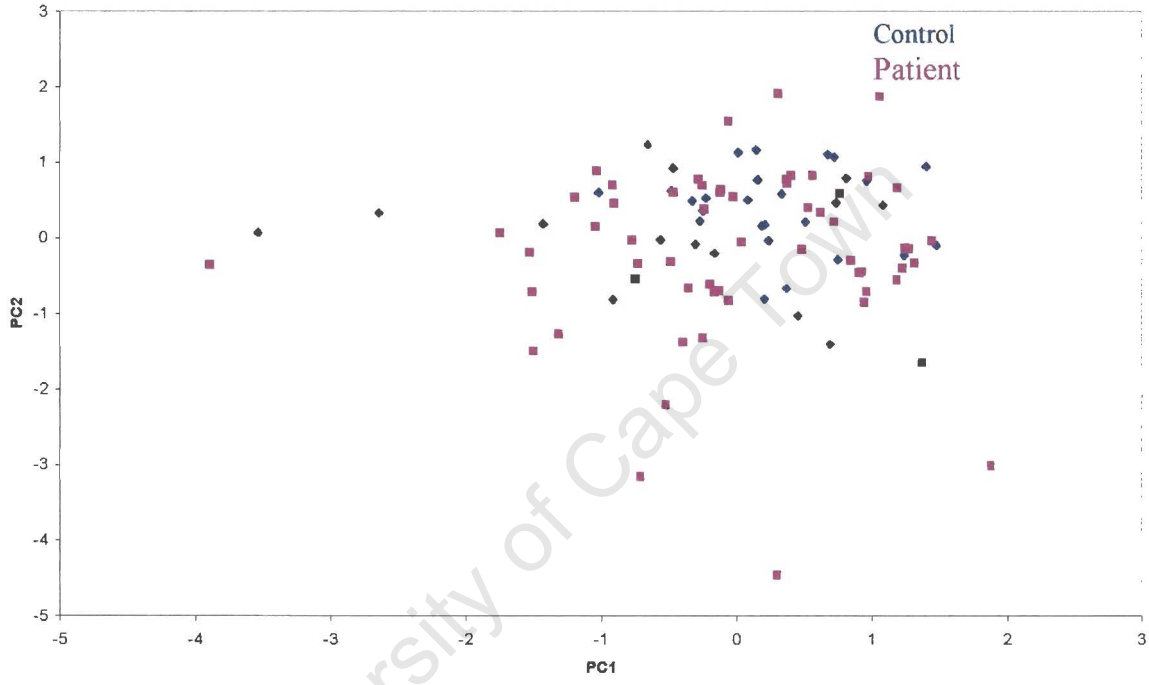
When both normalised and autoscaled data (the kidney stone patient and control data) generated in this study were analysed by PCA, it was found that the sample variation was spread over quite a few principal components. The eigenvalues are shown in Table 5.2. This shows that PC1 accounts for 25.6% of the variation, PC2 13.9% and PC3 only 8.7% and so on.

Table 5.2 Eigenvalues for principal component analysis of urine data.

STAT. FACTOR ANALYSIS	Eigenvalues Extraction: Principal components			
Value	Eigenvalue	% total variance	Cumul. Eigenvalue	Cumul. %
1	9.23	25.64	9.23	25.64
2	5.00	13.90	14.23	39.54
3	3.13	8.68	17.36	48.22
4	2.77	7.74	20.17	55.96
5	2.49	6.92	22.64	62.88
6	1.66	4.60	24.29	67.48
7	1.54	4.29	25.84	71.77
8	1.37	3.82	27.21	75.59
9	1.23	3.43	28.45	79.02
10	1.16	3.23	29.61	82.24

The scores plots of the first five PC's were examined, but none of these PC's displayed a patient discriminant ability. The resultant PC1 vs PC2 scores plot is shown in Figure 5.5. All other PC scores plots and PCA results are given in Appendix 3.

Figure5.5 PC1 vs PC2 scores plot



The above results indicate that the differences between patient and control subject spectra are smaller than other sources of variation in the data. This was expected after visual inspection of the spectra. As mentioned earlier, normal variation within each group is quite large. This being the case, it would be of great interest to identify which parts of the spectra , if any, are different in the two groups i.e. to establish whether the spectra can be used to discriminate between patients and controls. Therefore canonical discriminant analysis was performed on the data.

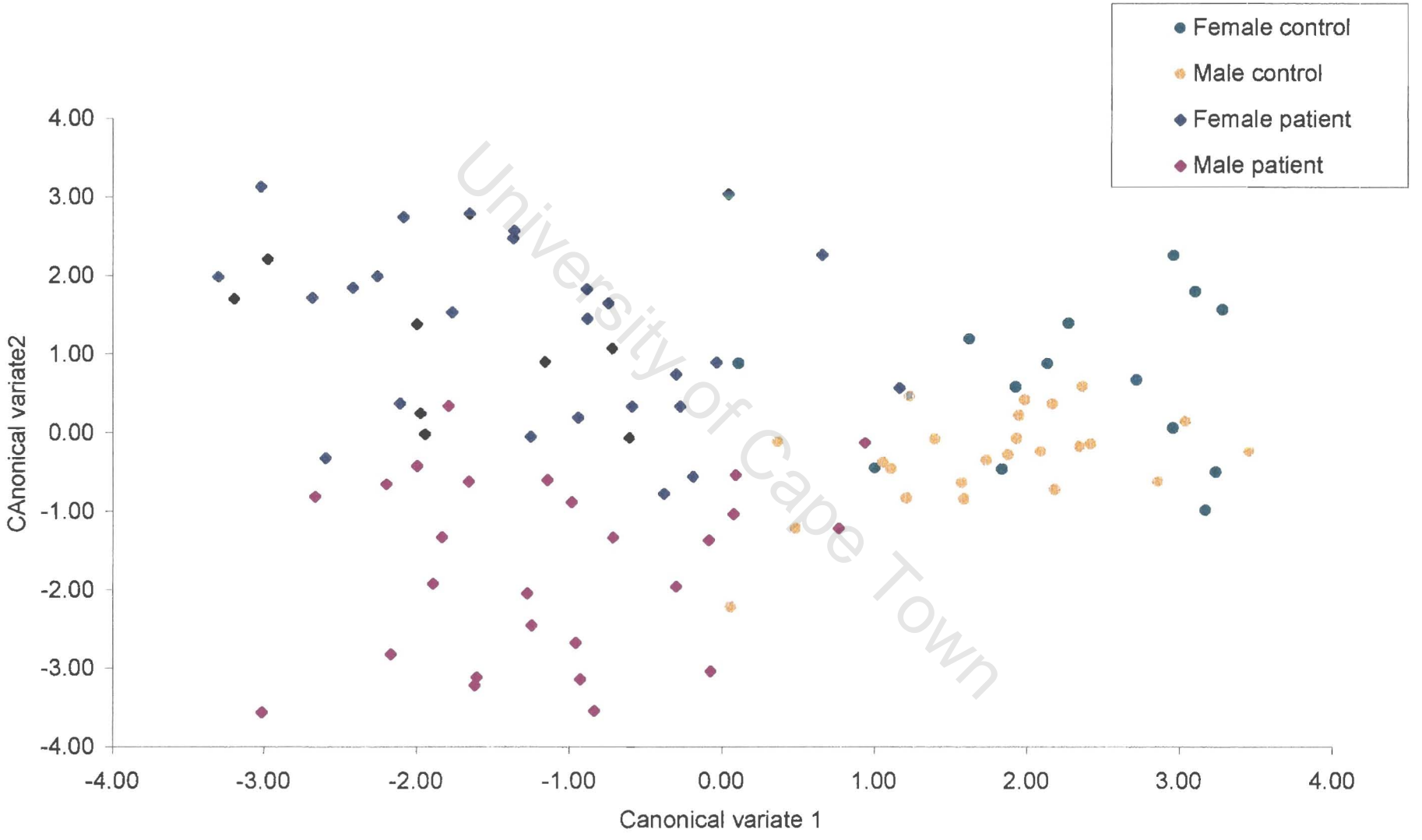
5.2.2 Canonical discriminant analysis

Canonical discriminant analysis calculates linear combinations of the variables (canonical variables) which maximise the difference between group means (see Section 3.3.4.2). In this case four classes were defined: male patients (MP), female patients (FP), male controls (MC) and female controls (FC). Three canonical variables were therefore calculated, the first accounts for the largest proportion of the between groups variation, the second the next largest proportion and so on. This was done using the SAS program as discussed in Section 4.5. The output for the canonical discriminant analysis is summarised in Appendix 5.

The canonical scores are the values of the canonical variables that are obtained when the standardised canonical coefficients are applied to the original variables. These canonical scores are tabulated in Appendix 4. **A canonical scores plot of canonical variables 1 and 2 is shown in Figure 5.6. This shows that samples separate into groups: female control, male control, female patient and male patient. The plot further shows that canonical variable 1 (CV1), which accounts for the largest of the between groups variation (56.4%) discriminates between patients and control subjects. This shows that NMR/PR analysis can indeed discriminate between calcium oxalate kidney stone patients and healthy control subjects.** It is noted that there is some overlap between the patient and control groups.

Males and females are discriminated by canonical variable 2 (CV2), which accounts for 31.2% of the between groups variation. There is good separation between

Figure 5.6 Scores plot of CV1 and CV2



males and females in the stone patient group, whereas the control male and female groups are largely overlapped.

To assess the contribution of each variable to the canonical variable scores, the standardised canonical coefficients need to be considered. The larger the coefficient, the larger the contribution from that variable towards the canonical variable score, and hence its importance in distinguishing between groups. The values for the three canonical coefficients for the data are shown in Table 5.3.

Table 5.3 Standardised canonical coefficients

Variable	Standardised canonical coefficients		
	CAN1	CAN2	CAN3
1	1.46	-0.93	2.13
2	3.16	-0.67	1.01
3	4.18	-1.50	5.62
4	2.43	-1.42	2.96
5	2.74	-1.49	2.61
6	3.97	0.20	4.72
7	15.52	-3.23	16.28
8	5.58	-0.72	4.34
9	4.94	-4.03	7.47
10	18.08	-5.30	19.20
11	16.31	-6.87	19.02
12	8.62	-1.07	7.68
13	6.52	-1.98	7.10
14	5.71	-2.37	6.72
15	6.44	-3.41	9.76
16	0.69	-0.41	1.48
17	2.81	-0.54	1.89
18	28.85	-9.77	31.76
19	56.84	-18.21	63.05
20	16.90	-5.55	18.30
21	57.99	-18.69	63.89
22	36.01	-12.55	40.06
23	15.26	-5.25	17.72
24	12.38	-3.97	12.48
25	17.32	-5.36	19.70
26	5.80	-1.97	5.96
27	18.77	-5.55	20.10
28	4.91	-0.80	5.43

Table 5.3 continued....

Variable	Standardised canonical coefficients		
	CAN1	CAN2	CAN3
29	8.62	-2.66	10.01
30	13.81	-4.31	13.89
31	7.50	-2.03	7.71
32	5.66	-3.75	8.18
33	18.99	-6.64	20.45
34	9.54	-3.32	11.19
35	9.19	-2.87	11.78
36	0.00	0.00	0.00

From Table 5.3, the variables that are most important in distinguishing between patients and controls are variables 18,19,21 and 22. These have coefficients 28.85, 56.84, 57.99 and 36.01 respectively. These variables translate to chemical shift regions 2.95 to 3.32 ppm and 3.54 to 4.41 ppm on the spectra, indicated in Figures 5.1 and 5.2. These regions contain many signals, including signals for creatine and creatinine, TMAO, glycine, glucose, signals from macromolecules and unidentified signals from substances present in low concentrations. Many of these signals are overlapped making the identification of the exact resonance that discriminates between these two groups non-trivial. Identification of the discriminating substance is possible, but is beyond the scope of this study. The use of large integral regions as spectral descriptors makes the identification of the discriminating signals difficult. Dividing the spectrum into 0.5 ppm regions may be an alternative. This was not attempted, because slight shifts in signals are sometimes seen and this would cause some signals to be spread over two integral regions. Therefore these regions cannot be compared between spectra. Digitising spectra, to express each data point by frequency and intensity, is another alternative. This was not attempted as the data size becomes unmanageable.

As mentioned earlier the separation between male and female control subjects was not very good. As the gender of each sample is known, this variable can be eliminated by analysing male and female subjects separately. Data were therefore divided into male and female data sets, defining patients and controls for each. Canonical discriminant analysis was performed on both data sets separately. Each analysis therefore gives one canonical variable. The SAS output is given in Appendix 6 for female subjects and Appendix 7 for male subjects.

The canonical scores plots for each gender are shown in Figures 5.7 and 5.8. Plots also show the mean for each group (patients and controls) and a 95% confidence interval for each. The 95% confidence interval is calculated as the mean _ 2(standard deviation).

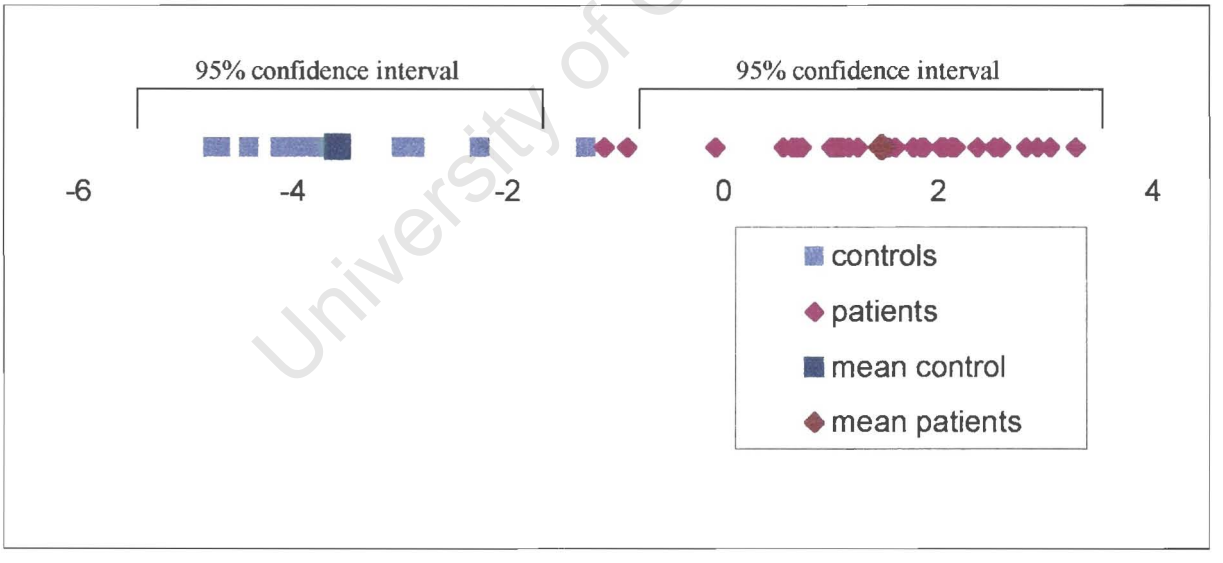


Figure 5.7 CV1 scores for the female data set showing the mean of patient and control groups and their 95% confidence intervals.

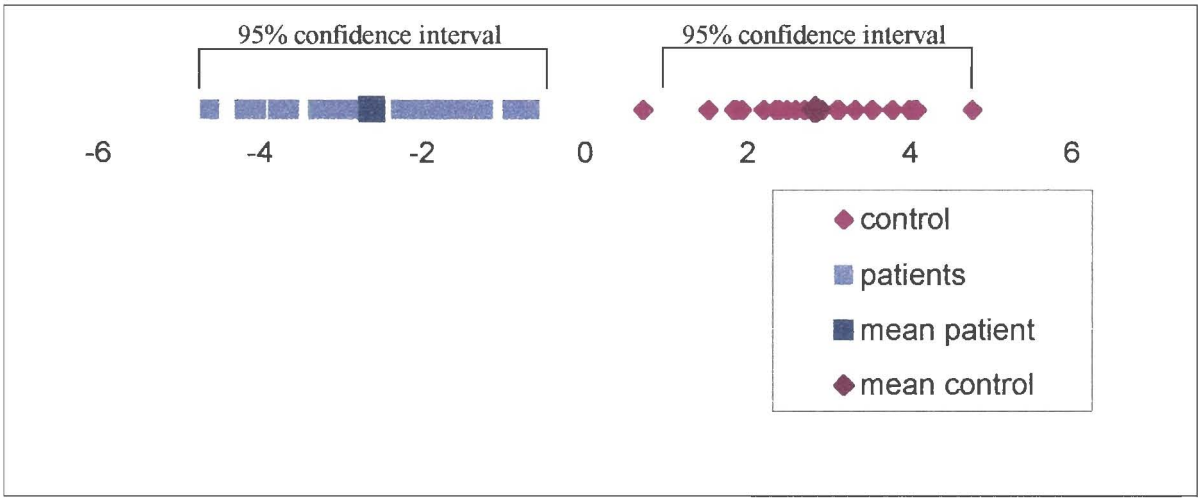


Figure5.8 CV1 scores for the male data set showing the mean of patient and control groups and their 95% confidence intervals.

This method shows excellent discrimination between patients and controls for both genders with much less overlapping of patients and controls than seen in the analysis where both genders were combined. Since the gender of the subjects are known in advance, this may be a better way of classifying the risk of kidney stone formation. Prediction of unknown samples using this model was not attempted, due to the smaller sample size generated by dividing the data into gender groups. Prediction should be attempted with a larger sample size for each gender.

5.3 VALIDATION

The canonical discriminant model containing both genders was validated by the classification of eight independent test samples into the predefined groups. The resulting scores for eight test samples are shown in Table 5.4 and plotted in Figure 5.9. Included in this plot are the 95% confidence regions for each group. These are indicated by

circles centered on the group means. Each 95% confidence has a radius of 2.45. This is because we have used standardised canonical coefficients to obtain the scores for each group. This means that the variance is 1 and that each group has a Chi-squared distribution (X^2) with two degrees of freedom.

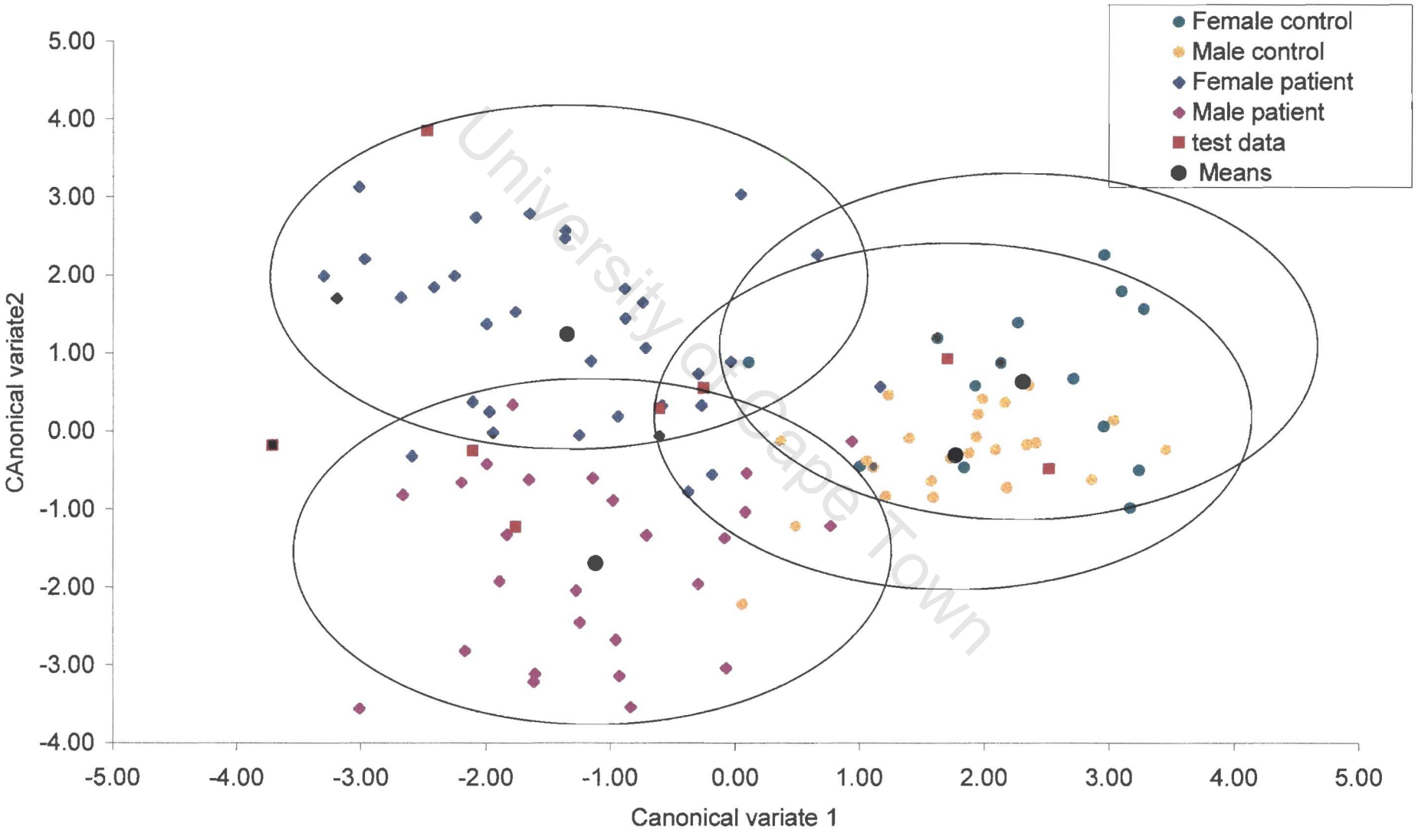
Each test sample should fall within the 95% confidence interval to be correctly classified. Table 5.4 shows the results of the test data.

Table 5.4 Table of test data scores showing classification of each sample.

Known classification	Can1	Can2	Model prediction
Female control	-0.25246	0.551363	MC or FP or MP
Female control	2.519712	-0.48107	FC or MC
Male control	1.705175	0.927627	MC or FC
Male control	-0.60636	0.294551	MC or FP or MP
Female patient	-3.71291	-0.17979	none
Female patient	-2.47176	3.851525	FP
Male patient	-1.75943	-1.22974	MP
Male patient	-2.10347	-0.25451	MP

Table 5.4 shows that the classification of three of the test samples could be accurately predicted (both of the male patient samples and one female patients samples). The other female patient did not fall into any of the 95% confidence regions, but from Figure 5.9 we can see that it is definitely in the patient region.

Figure 5.9 Scores plot of CV1 and CV2 including test sample scores and 95% confidence ellipses



The control samples are more difficult to assign into either male or female as the groups are largely overlapped. Some samples are found in the overlapping regions of two groups so that they are classified into two or more groups with 95% confidence. It is evident that a larger data sample or a better class separation is needed before unknown samples can confidently be classified.

5.4 CONCLUDING COMMENTS

This study demonstrates that ^{1}H-NMR urine analysis can be used to discriminate between the urine of calcium oxalate kidney stone patients and healthy subjects, by running a simple ^{1}H-NMR spectrum of each urine sample, converting the spectra into numerical data and carrying out pattern recognition analyses. The procedure gives a canonical discriminant model of the data which is able to distinguish calcium oxalate stone patients' urine from healthy control urines. This model can potentially be used as an indicator of the risk of stone formation, by indicating if an unknown sample falls into the patient population or the control population.

This study has described, for the first time, a physicochemical procedure for discriminating between male and female stone patients and control subjects. As such the objective described in Section 2.6 has been realised. NMR urine analysis provides a fast method of distinguishing between urine samples from calcium oxalate kidney stone patients and healthy control subjects. It requires a small sample and minimal sample preparation making it ideal for rapid screening of samples. As such it provides a powerful tool for the clinical evaluation of patients as well as persons who are at the risk of stone disease. While this technique is not routine, specialised laboratories might

provide an analytical service for the classification of a given urine sample as "control" or "patient". Conservative intervention such as diet management or medication could perhaps also be monitored in an ongoing process to see if the risk of stone formation decreases as the treatment continues.

5.5 FURTHER CHALLENGES

- Improving the separation of patient and control groups in the canonical discriminant analysis. This could be made possible by increasing the number of samples used in the training set and by reducing the number of variables used for canonical discriminant analysis. The number of variables can be reduced by eliminating redundant variables that do not have any influence on the discrimination between patients and controls.
- To identify the urinary factors that influence discrimination between groups. This can be done by identifying the NMR signals that cause the group discrimination. To achieve this either we need to use a higher field spectrometer, which would increase signal dispersion and spectral sensitivity, thereby allowing the identification of more of the ^{1}H-NMR signals and allowing for smaller integral regions to be used as spectral descriptors. Or another method of obtaining spectral descriptors for PR should be attempted. Either the use of 0.5 ppm integral regions, or the digitisation of the spectra should be employed to obtain more specific spectral descriptors, so that the PR can be used to identify smaller regions of the spectra or individual signals that are responsible for the class discrimination. Once these have been identified then 2D-NMR techniques

can be used to try to identify the substance or substances responsible for these signals.

- The study should be extended to 24 hour urine samples, using some method of concentrating the urine such as solid phase extraction (Nicholson and Wilson, 1989) or removal of some of the water by rotary evaporation (Hallson and Rose, 1978). These results can then be compared to conventional urinalysis techniques.
- Other variables such as age or population group, could be included in this data, instead of gender. It would be interesting to see if variation between these groups could be identified in the urine.
- To determine whether NMR urine analysis can be used to determine the efficacy of treatment of kidney stone disease. This could be a useful application. The ^{1}H-NMR urine spectra of subjects placed on conservative treatments, such as diet management or medication, could be followed throughout their treatment programme and compared to a PR model of patients and controls to examine whether their CV1 canonical score values shift in the direction of "controls".

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