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**THE DEVELOPMENT OF A METHOD TO
DETERMINE FELININE IN BODY FLUIDS
BY CAPILLARY ELECTROPHORESIS**

This thesis was presented in partial fulfilment of the requirements
for the degree of
Master of Philosophy in Chemistry,
Massey University,
New Zealand

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1999

ABSTRACT

Ion-exchange, paper-chromatography and high performance liquid chromatography were used in earlier studies for the determination of felinine in biological fluids. These methods were either inadequate and/or need laborious sample pre-treatments.

A new method for the determination of felinine by capillary zone electrophoresis has been developed. Preliminary investigations were carried out to address the conditions required for the separation of felinine. The separation of felinine can be performed on a fused-silica capillary with a 20 mM phosphate buffer (pH 2.0) and detection wavelength 200 nm. The separation principle was based on the different migration times due to the different molecular weights, molecular sizes and charges under an applied potential field.

The quantitative determination of felinine levels in cat urine has been achieved. The cat urine analysis was performed directly on the capillary electrophoresis without making any felinine derivative(s). The levels of felinine in different cat genders are reported. The results were compared with the results of an HPLC felinine derivatization method. Felinine levels in entire male cat urine were much higher than those in female and castrated male cat urine. A synthetic felinine was employed as standard felinine. Linear relationships between peak area and concentration of synthetic felinine calibrations are reported. Mean felinine recovery in cat urine was 95.9%. Taurine, urea, creatine and creatinine, which exist in large amounts in cat urine, showed no interference with the analysis of felinine by this method.

The new capillary zone electrophoresis method was then applied to the study of felinine stability. Conditions reported to influence the stability of felinine were investigated. These conditions included oxidation, storage temperatures and times, heating, acidic and alkaline solutions. Both synthetic felinine and felinine in cat urine were investigated. Storage temperature (-20°C to 20°C) had no significant influence on the stability of felinine while higher temperatures increased the decomposition of felinine. Felinine degraded at strong

acid and base conditions but was relatively stable under mild acid and base conditions. A similar stability of felinine in human urine is also reported.

The capillary zone electrophoresis method was also employed to study felinine in plasma and serum. Plasma and serum as well as urine can be analysed directly on the capillary electrophoresis after sufficient dilution. Conditions (eg. protein clean up, changing of injection time, 37°C heating) that might influence of felinine behaviour in plasma and serum are discussed. This study indicated that no traces felinine be found in cat plasma, within the detection limits of this new capillary electrophoresis method.

ACKNOWLEDEMENTS

First, I would like to express my appreciation to my supervisors Associate Professor David R. K. Harding and Dr. John McIntosh for all their guidance, inspiration and encouragement during the research and completion of my Masterate.

Second, my thanks to Dr. Wouter H. Hendriks for his suggestions and his contribution of feline and cat urine to my research.

Third, I would like to acknowledge Mr. Dick Poll for his constant instruction and assistance in helping me to become familiar with the use of CE and HPLC systems, especially for sharing his knowledge of the CE buffers and feline derivatization.

I would also like to thank members of Separation Science Group who have helped me in my research, both directly and indirectly.

Furthermore, thanks to all the academic staff and other members of Chemistry Section, especially Professor Andrew M. Brodie for his support and suggestions through my study at Massey.

Finally, I would like to thank my husband for his constant encouragement and support.

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LIST OF ABBREVIATIONS

AU	absorbance unit
AUFS	absorbance unit full scale
β -ME	β -mercapto-ethanol
BOC	tert-butyloxycarbonyl
BSA	bovine serum albumin
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focusing
CITP	capillary isotachopheresis
CMC	critical micelle concentration
CV	coefficient of variation
CZE	capillary zone electrophoresis
D.C.	direct current
DTBD	di-tert-butyloxy-dicarbonate
EDTA	ethylenediaminetetraacetic acid disodium salt dihydrate
EOF	electroosmotic flow
ESTD	external standard
fel	felinine
FSCE	free solution capillary electrophoresis
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
ISTD	internal standard
I. D.(or i.d.)	internal diameter
MEKC	micellar electrokinetic capillary
MS	mass spectrometry
N-(NO ₂)arg	N ω -nitro-L-arginine
OPA	O-phthalaldehyde
Pos	positive
R.S.D.	relative standard deviation
S.D.	standard deviation

SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
UV/Vis	ultraviolet-visible
WA	washing

CHAPTER ONE

INTRODUCTION

PART I FELININE: A URINARY AMINO ACID OF FELIDAE

1.1 Discovery, isolation and characterization of felinine

Felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid) is a urinary amino acid excreted in large quantities by domestic cats and several other Felidae. It was first discovered by Datta and Harris in 1951. At that time they were examining amino acids in the urine and plasma of various animals by two dimensional paper chromatography. Datta and Harris (1951) found a previously unknown amino acid that was frequently present in considerable quantity in the urine of domestic cats (*Felis catus*) and ocelots (*Felis pardinus*). They found that this material moved to a position similar to leucine in the two-dimensional paper chromatograms, when phenol and collidine-lutidine were used as solvents. They tentatively named this amino acid "cat spot". Soon after R.G. Westall (1951) became interested in this "cat spot" and isolated it as an amorphous solid from cat urine using large ion-exchange columns and renamed the amino acid, felinine. The simplest empirical formula that could be derived from degradation experiments was $C_8H_{17}O_3NS$, and the structure of this sulphur amino acid was provisionally determined as 2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid (**Figure 1.1**).

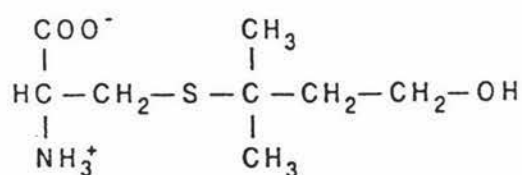


Figure 1.1. Molecular structure of felinine, 2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid (Westall, 1953)

Following these events, experiments by Datta and Harris (1953) showed felinine to be present in the urine of the leopard (*Felis pardus*) and the Indian leopard cat (*Felis bengalensis*). It was not found in the urine of any of the other animals investigated: lions, tigers, pumas, genets, dog, rabbit, rat, mouse, guinea pig, horse, cow or human. Roberts

(1963) found felinine in cat and bobcat urine but not in that of the leopard. **Table 1.1** shows their investigation for the presence of felinine in many mammals:

Table 1.1 Reported occurrence of felinine in the urine of different mammalian species (Hendriks, et al., 1995)

Species	Reference*		
	1	2	3
Cat (<i>Felis catus</i>)	Yes	Yes	Yes
Ocelot (<i>Felis pardalis</i>)	Yes	Yes	-
Leopard (<i>Panthera pardus</i>)	-	Yes	No
Indian leopard cat (<i>Felis bengalensis</i>)	-	Yes	-
Bobcat (<i>Felis rufus</i>)	-	-	Yes
Cheetah (<i>Acinonyx jubatus</i>)	-	-	No
Puma (<i>Felis concolor</i>)	No	No	No
Tiger (<i>Panthera tigris</i>)	No	No	No
Lion (<i>Panthera leo</i>)	No	No	No
Serval (<i>Felis serval</i>)	-	No	-
Fishing cat (<i>Felis viverrina</i>)	-	No	-
Dog (<i>Canis familiaris</i>)	No	No	-
Binturong (<i>Arctictis binturong</i>)	No	No	-
Genet (<i>Genetta tigrina erlangeri</i>)	No	No	-
Pole cat (<i>Putorius putorius</i>)	-	No	-
Bear (<i>Ursus</i> ?)	-	-	No
Rat (<i>Rattus norvegicus</i>)	No	No	-
Mouse (<i>Mus musculus</i>)	No	No	-
Guinea-pig (<i>Cavia porcellus</i>)	No	No	-
Golden hamster (<i>Mesocricetus auratus</i>)	-	No	-
Rabbit (<i>Oryctolagus cuniculus</i>)	No	No	-
Rhesus monkey (<i>Macacus rhesus</i>)	-	No	-
Horse (<i>Equus caballus</i>)	No	No	-
Cow (<i>Bos taurus</i>)	No	No	-
Goat (<i>Capra hircus</i>)	-	No	-

*Reference: 1, Datta and Harris (1951); 2, Datta and Harris (1953); 3, Roberts (1963).

-, not determined

The investigation of felinine in cat blood plasma was carried on as well. Datta and Harris (1953), Westall (1953) failed to show the presence of felinine in cat blood by using paper chromatography, Tallan et al., (1954) found traces of felinine in the plasma. Felinine was not found in tom cat plasma using modern HPLC analysis (Hendriks, et al., 1995).

The different quantitative data on levels of felinine in fluid and tissues of the cat have been published. Dietary factors influence the excretion of felinine, and the amount of felinine excreted in the urine of the cat is sex dependent. Westall (1953) estimated that felinine occurs in cat urine at a level of 1.0-1.2 mg/ml. Tallan et al. (1954) found levels of felinine in cat urine as high as 1.85 mg/ml, but with minimal quantities observable in

extracts of various tissues (**Table 1.2**). Acid-hydrolyzed extracts of tissues and fluids contained no felinine whatsoever. Excretion of more than 8mg felinine/milliliter (8 mg/ml) have been reported by Avizonis and Wriston (1959) in a cat fed a cystine enriched diet for 3 days. The lowest urinary concentration reported by them was 1.5 mg/ml by using ion exchange chromatography and Westall's (1953) "standard". Shapiro (1962) reported urinary excretion rates of 1.7 and 1.0 mg/ml for a male and female cat, respectively. More recently data is reported by Hendriks et al (1995b), who assayed 24-hr urinary felinine excretions in a total of 28 male (entire and castrated) and female (entire and spayed) cats. By using HPLC analysis and a synthetic felinine standard of known purity, the average felinine concentration in the urine of entire male cats was found to be 2.0 mg/ml (range 0.4-3.6 mg/ml) with entire females excreting on average 0.3 mg/ml. The higher concentration of felinine in the urine of entire male cats as compared with entire female cats was found to result in higher amounts of felinine excreted per day for the male cat. The average amount of felinine excreted (mg/24 hr) was 95 for entire male cats, 29 for castrated males, 19 for entire females and 13 for spayed females (Hendriks, et al., 1995).

Table 1.2 **Reported extracts of felinine in various tissues** (Tallan et al, 1954)

Tissues	felinine (per 100 g)
Bladder	3.4 mg (per 100mg)
Liver	<1.2 mg
Kidney	<1.0 mg
Brain	<0.6 mg
Plasma	0.4 mg
Gastrocnemius muscle	<0.3 mg

Felinine could not be detected in the urine of kittens of either sex by using paper chromatography (Roberts, 1963). The age of attaining sexual maturity is variable in female kittens but can be expected to occur around nine months (Beaver, 1992).

It was suggested by Tallan et al (1954), that felinine may be synthesised in the kidney and excreted directly into the urine or that it may be present in a bound form in the plasma.

Roberts (1963) detected felinine in tissue extracts of the liver, kidney and skin of the cat and suggested that felinine is carried in the blood, at low concentrations, and that it appears in many tissues and that the low blood levels too low to be detected would be maintained by a rapid excretion through the kidney.

1.2 Synthesis of felinine

Several chemical synthesis procedures of felinine were reported since 1957. It was first achieved by Trippett (1957) by the reductive S-debenzylation of 3-methyl-3-thiobenzylutan-1-ol in the presence of sodium in liquid ammonia and β -chloroalanine. The synthetic material behaved exactly as a sample of "natural" felinine obtained from Westall (1953). In 1962, Eggerer (1962) synthesised felinine by using a phosphate derivative of isopentenol in a reaction with cysteine. He also found two other routes, using isopentenol and cysteine in the presence of pyridine, yielded felinine. Schoberl et al. (1966,1968) reported a synthesis procedure yielding felinine up to 80%. More recently a new procedure for the synthesis of felinine leading to high yields of this amino acid was published by Hendriks et al. (1995b). To obtain a standard for high-performance liquid chromatograph (HPLC), Hendriks et al. also followed the synthesis procedures of Trippett (1957), Eggerer (1962), Schoberl et al. (1966, 1968), using nuclear magnetic resonance, mass spectrometry and an HPLC procedure.

1.3 Biosynthesis of felinine

Avizonis and Wriston (1959) studied the biosynthesis of felinine in the cat and found that cystine, leucine and mevalonic acid can contribute to the formation of felinine in the cat. They injected C^{14} -labeled compounds into a cat and isolated radioactive felinine from the urine. Felinine concentration increased when cystine and leucine were fed to the cats. Shapiro (1962) and Wang (1964) found similar rates of incorporation of radioactivity into felinine and cholesterol when 2- $[^{14}C]$ acetate was used. Shapiro (1962) suggested that mevalonic acid is diverted to the metabolic pathways for cholesterol and felinine and that these pathways use the same isoprenoid pool. Radioactivity from injected D,L- $[^{14}C]$ leucine was also incorporated into felinine (Shapiro, 1962). This supports the observations made by Avizonis and Wriston (1959) that leucine is a precursor to felinine (Hendriks, et al., 1995). Roberts (1963) indicated that cystine and methionine are not

possible solution to the question concerning the transportation of felinine in the blood was provided by Kuwaki et al. (1963). Using guinea pig liver homogenates, they showed that isovalthine could be formed *in vitro* as part of a glutathione-isovaleric acid conjugate (GSIV).

In vitro synthesis of felinine in liver homogenates of the cat has largely been unsuccessful. Roberts (1963) and Wang (1964) failed to find any felinine produced in their experiments. Wang (1964) also attempted to demonstrate the presence of a glutathione conjugates, but none could be detected and no glutathionase activity could be demonstrated in the kidney. If felinine is transported in the blood as a glutathione conjugate, it would be expected that hydrolysis of the blood would yield felinine. However, it has been observed that synthetic felinine is an acid-labile amino acid that is destroyed under the conditions of 2 and 6 M HCl hydrolysis at 110°C for 24 hr (Hendriks, et al., 1995).

1.4 Biological significance of felinine

The biological significance of felinine to the animal is still unknown. Although felinine and cholesterol seem to be synthesized from the same isoprenoid pool, no direct evidence for a regulatory effect of felinine on cholesterol or steroid metabolism has been found (Hendriks, et al., 1995). A more likely role for felinine, as a urinary component for territorial marking, was suggested by MacDonald et al., (1984). The observed differences in urinary felinine excretion rates between male and female cats (Roberts, 1963; Hendriks et al., 1995a) are in accordance with this role. Further evidence to support this hypothesis was published by Joulain and Laurent (1989). They observed that “fresh” cat (*F. catus*) urine was mostly odourless, but upon “aging” an odour developed reaching a peak at ~12-24hr. The extent of the odour depended on the diet and the period within the sexual cycle of the cats. Two volatile components, 3-mercapto-3-methyl-1-butanol (I) and 3-methyl-3-methylthio-1-butanol (II) (**Figure1.3**) (Hendriks, et al., 1995), which may be formed on degradation of felinine, were found in the urine of the tom cat (Joulain and Laurent, 1989) and bobcat (Mattina et al., 1991). Other components (disulfides and trisulfides) in the urine of these animals that are derivatives of compounds I and II were also found by these authors. Hendriks et al., (1995b) noted that odourless synthetic

felinine stored as a lyophilisate at -20°C and room temperature developed an odour that was similar to the odour of tom cat urine. They suggested that felinine degrades to another compound or other compounds to give rise to the distinct tom cat urine smell.

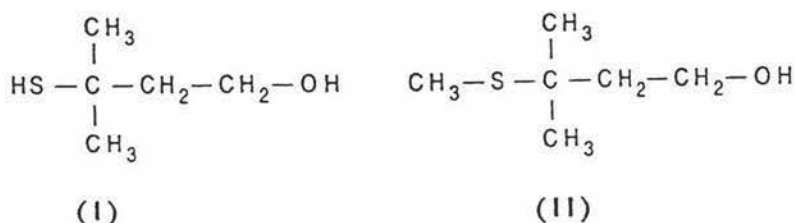


Figure 1.3 Molecular structure of 3-mercapto-3-methyl-1-butanol (I) and 3-methyl-3-methylthio-1-butanol (II) (Hendriks et al., 1995)

Although bobcat urine has been shown to have a repellent effect on the snowshoe hare (Sullivan et al., 1985a), the black-tailed deer (Sullivan et al., 1985b) and the white-tailed deer (Swihart, 1991), there is no direct evidence that felinine degrades to volatile compounds I and/or II whether any of these compounds or derivatives of these compounds actually have a repellent effect. Related to the possible repellent effect and territorial marking roles of felinine, is its possible role as a pheromone to attract the opposite sex (Hendriks, et al., 1995). Although no direct evidence is known to substantiate this hypothesis, Joulain and Laurent (1989) noted that the sexual cycle has some effect on the occurrence of the odour of tom cat urine. Further research into the function of felinine as a pheromone is warranted.

PART II CAPILLARY ELECTROPHORESIS

1.5 Background and advantages

Capillary electrophoresis is usually said to date from 1981, when Jorgenson and Lukacs (1981) described spectacular separations of peptides using free zone electrophoresis in glass capillaries of 75 μm i.d.; however, due to the limitations of light transmission through glass, fluorescence detection was needed. This capillary format enabled the Joule heating, which occurs in all electrophoretic separations and degrades the resolution by thermal mixing, to be efficiently dissipated. It was thereby possible to use much higher voltages (up to 30 kV) for electrophoretic separations and so shorten the analysis times. Later, glass capillaries were superseded by polyimide-coated silica capillaries which are much more robust in use and, more importantly, transmit ultraviolet light (Perrett, 1999).

Today, capillary electrophoresis has become a modern analytical technique for the separation and analysis of chemical compounds. Separations are based on the differences in electrophoretic mobilities of ions in electrophoretic media inside small capillaries (Jorgenson and Lukacs, 1981, 1983 and Karger *et al.*, 1989). Many methods of analysis that currently use high-performance liquid chromatography or slab gel electrophoresis will probably be converted to capillary electrophoresis because it offers highly efficient and fast separations, relatively inexpensive and long lasting capillary columns, small sample size requirements, and low reagent consumption (Wiktorowicz and Colburn, 1990; Novotny *et al.*, 1990 and Jorgenson, 1983). CE is characterized by its ability to resolve, using a high applied D.C. voltage (field strengths up to 500 V/cm), the components of complex aqueous samples with very high resolution ($N > 200000$ plates/m), analysing with analytical precision of less than 10 nL of sample (Perrett, 1999). The separated components are identified by on-line detection during the analysis, and the results are obtained in a matter of minutes, sometimes seconds. High-resolution separations of a wide variety of sample types can be done by capillary electrophoresis. It can be used for analysis of polar ionic, polar nonionic, and nonpolar nonionic compounds, as well as high molecular weight biomolecules (Lauer and McManigill, 1986; McCormick, 1988; Messana *et al.*, 1997 and Dolnik, 1997), and chiral compounds (Nishi, 1997; Hage, 1997 and Fanali, 1997).

The same capillary can be used for separations and analyses of polar compounds, nonpolar compounds, chiral compounds, and large biomolecules at ambient temperature and low pressure without the need for a liquid pump. Separation can be performed on the basis of the size of the compounds in a mixture through the use of a gel-filled capillary. Compared to high-performance liquid chromatography columns, capillaries are relatively inexpensive, easy to use, and last a long time. Now, capillary electrophoresis is being used by more and more analysts in the fields of analytical chemistry and biochemistry.

1.6 Capillary electrophoresis system

The main components of a capillary electrophoresis system, shown in **Figure 1.4**, are a sample vial, source and destination vials, capillary, detector, high-voltage power supply, and a data output and handling device, such as an integrator or computer (Baker, 1995).

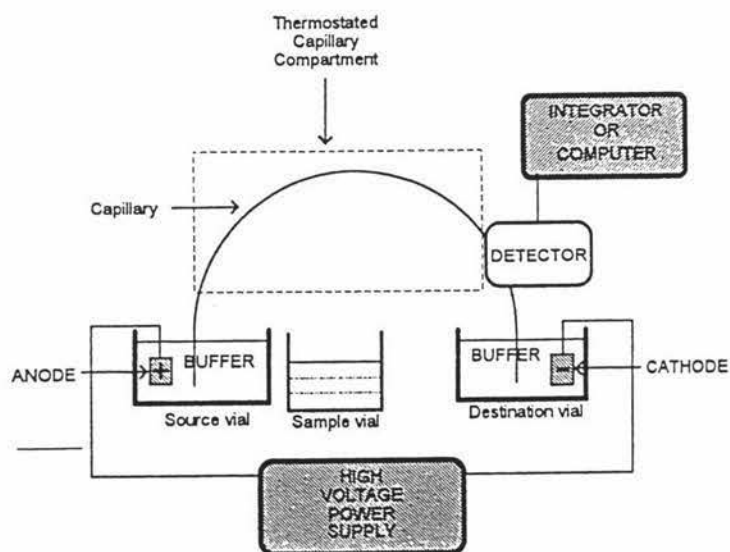


Figure 1.4 Schematic representation of a capillary electrophoresis system (Baker, 1995)

Electrophoresis is performed by filling the source vial, capillary, and destination vial with an electrolyte, usually an aqueous buffer solution. The capillary inlet is placed into a sample vial, the sample is introduced, then the capillary inlet is placed back into the source vial, and an electric field is applied between the source and destination vials. As the solutes

migrate through the capillary, they are detected by the detector and its output is sent to an integrator or computer. Since different solutes pass through the detector at different times, the separated compounds appear as peaks with different *migration times* in the electropherogram.

Sample injection

The most commonly employed injection methods for CE are direct on-column methods such as hydrodynamic (Huang *et al.*, 1989; Rose and Jorgenson, 1988 and Honda *et al.*, 1987) and electrokinetic (Jorgenson and Lukacs, 1981 and Rose and Jorgenson, 1988) injection. Hydrodynamic injection can be performed by pressure or siphoning. In electrokinetic injection, an electric field is applied to the sample vial, causing the sample components to migrate into the capillary.

Capillaries

Fused silica capillaries, which are externally coated with a polymer such as polyimide to improve their mechanical strength, that are 30-100 cm long with inner diameters of 50-75 μm and outer diameters of 375 μm are typically used. The cell window of a capillary can be made by simply burning or scraping off a small section of the polyimide outer coating of the capillary. This section of the capillary is then placed in the light path of the detector.

Detectors

A variety of detectors have been used in capillary electrophoresis, including: UV/Vis absorbance, fluorescence, laser-induced fluorescence, mass spectrometric, conductivity, amperometric, radiometric, and refractive index. The most widely used are UV/vis absorbance detectors.

Power supply

The purpose of the power supply is to provide an electric field across the capillary. It can be operated in either the constant voltage, constant current, or constant power mode and

have the ability to reverse the polarity. Voltages up to 30 kV, currents up to 300 μA , and power up to 6 W are used.

Data Handling

An electropherogram is a plot of detector response versus time, as shown in **Figure 1.5**. This is the same way data are displayed in a chromatogram obtained from a high-performance liquid chromatograph or a gas chromatograph, so the same type of data handling that is used in these techniques can be used in capillary electrophoresis.

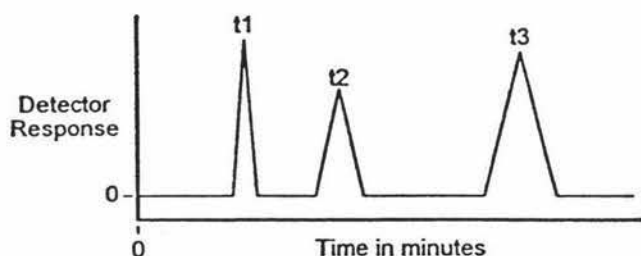


Figure 1.5 Drawing of a capillary electropherogram. t_1 , t_2 , and t_3 represent the migration times of sample components, measured at the apices of the peaks (Baker, 1995).

Qualitative analysis could be done by measuring the migration time of a compound of unknown identity and matching its migration time to that of a compound whose identity is known. By comparing migration times of unknowns and standards, we can determine which peak corresponds to which compound. Quantitative analysis is done by injecting a standard containing known concentrations of each of the components of interest, and measuring either the heights or areas of each of the resultant peaks. Then, a sample containing unknown concentrations of the sample components is injected and its peak heights or areas are measured. By comparing areas or heights obtained from the standard to those obtained from the sample, the concentrations of components in the sample can be determined.

1.7 Modes of capillary electrophoresis

Different modes of capillary electrophoresis separations can be performed using a standard CE instrument. The most often used modes of CE are capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic capillary chromatography (MEKC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), and capillary isotachopheresis (CITP). The mechanisms by which solutes separate in the six techniques are illustrated in **Figure 1.6** and **Figure 1.7** (Li, 1992). The migration of each type of charged species under the influence of the applied voltage is represented by an arrow in **Figure 1.6**, and the distribution of electrolytes and a two-component sample are shown at three different times in **Figure 1.7**.

1.7.1 Capillary zone electrophoresis (CZE)

Capillary zone electrophoresis (CZE) is also referred to as free solution capillary electrophoresis (FSCE). Currently CZE is the most commonly used separation mode available within CE. Many compounds can be separated rapidly and easily. It can be used to separate almost any ionized compounds that are soluble in a buffer. Samples as diverse as small inorganic anions (Harrold, et al., 1993 and Jandik and Jones, 1991) and large biomolecules (Knox, 1988) have been separated by CZE. The separation in CZE is based on the differences in the electrophoretic mobilities of the ions. The mobility differences result in different velocities of migration of the ionic species in the electrophoretic buffer contained in the capillary.

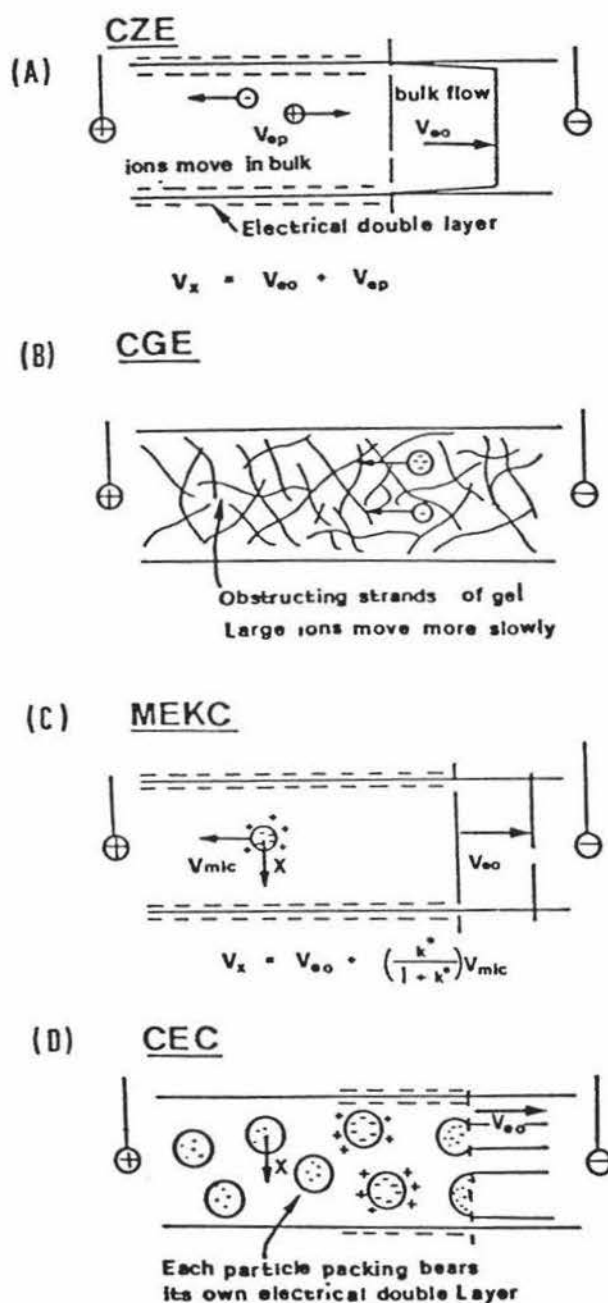


Figure 1.6 Diagrammatic representation of (A) capillary zone electrophoresis (CZE), (B) capillary gel electrophoresis (CGE), (C) micellar electrokinetic chromatography (MEKC), and (D) capillary electrokinetic chromatography. v_x is the linear migration velocity of the analyte X. v_{eo} is the electroosmotic velocity, v_{ep} is the electrophoretic velocity and k' is the phase capacity ratio (Li, 1992).

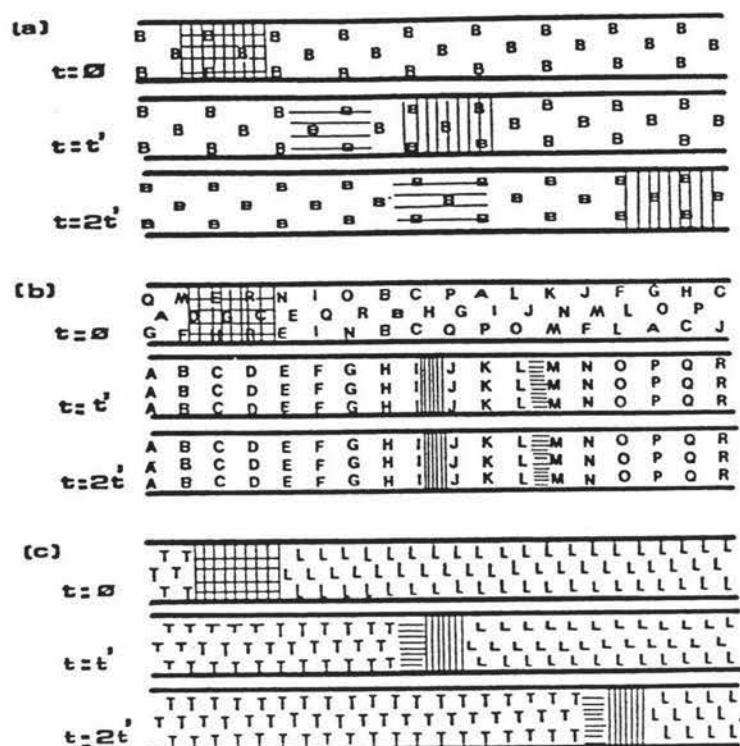


Figure 1.7 Schemes of electrophoretic techniques: (a) zone electrophoresis, (b) isoelectric focusing, and (c) isotachopheresis. The distribution of electrolytes and a two-component sample are shown at three different times: the start of the analysis ($t = 0$), the time interval t' after the start ($t = t'$), and the double time interval after the start ($t = 2t'$) (Li, 1992).

The separation mechanism is mainly based on differences in solute size and charge at a given pH. **Figure 1.8** represents a CZE separation. A sample is injected into the capillary, which is filled with a buffer, and when a voltage is applied, the solutes migrate through the capillary in zones. Solutes are separated as they move through the capillary due to differences in their rates of migration, which are dependent on their electrophoretic mobilities. Electroosmotic flow moves the solutes through the capillary from the anode to the cathode. The order of elution in CZE is cations, neutrals, then anions. Neutral compounds are not separated in CZE, whereas ions are separated on the basis of their charge-to-size ratios. Cations are attracted towards the cathode and their speed is augmented by the electroosmotic flow, with small, highly charged cations eluting first, followed by cations with reduced charge/size ratio. Neutral molecules, which move through the capillary under the influence of only the electroosmotic flow and are not separated

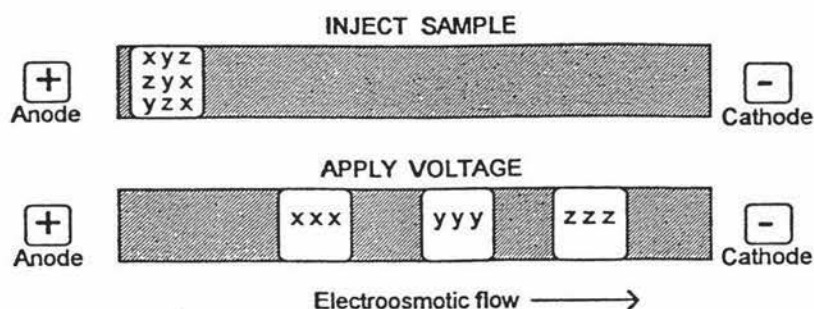


Figure 1.8 Separation by capillary zone electrophoresis with “normal” electroosmotic flow and polarity. The x’s, y’s, and z’s represent ionic solutes with different charge-to-size ratios, with z having the highest ratio and x the lowest (Baker, 1995).

from each other, elute after the cations. Anions, which are attracted to the positive electrode and consequently tend to migrate in the opposite direction to the electroosmotic flow, elute last. Anions with lower charge/size ratio migrate earlier than those with greater charge/size ratio. The anions with the greatest electrophoretic mobilities migrate last. **Figure 1.9** is a representation of the elution order in CZE.

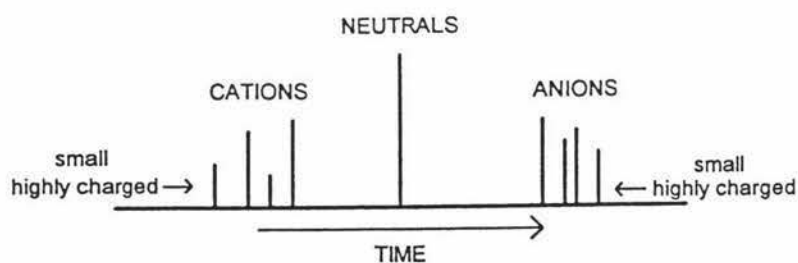


Figure 1.9 Drawing of a capillary zone electropherogram indicating elution order. Small, highly charged cations elute first (Baker, 1995).

1.7.2 Capillary gel electrophoresis (CGE)

Capillary gel electrophoresis (CGE), which combines the principles of slab gel electrophoresis with the instrumentation and small diameter capillaries of CZE, was introduced by Cohen and Karger (1987). The separation efficiency which they achieved was extremely high using gel-filled capillary columns. The capillaries were filled with

polyacrylamide gels which contained sodium dodecyl sulfate. This technique is referred to as a capillary SDS-PAGE separation and has been used for the separation of proteins, polynucleotides and DNA fragments (Hjerten, et al., 1987).

In CGE, the capillary is filled with a gel, which is usually either a polyacrylamide/bisacrylamide crosslinked polymer or a linear, noncrosslinked polyacrylamide polymer. There are pores within these gels, and as charged solutes migrate through a gel-filled capillary, they are separated by a molecular sieving mechanism on the basis of their sizes. Small molecules are able to pass through the pores and elute first, whereas larger molecules are retarded by the gel and elute later. In CGE, the solutes are separated on the basis of size, so it is well suited for the analysis of charged molecules that vary in size, but not in their charge-to-size ratios, regardless of their chain lengths, such as oligonucleotides, DNA restriction fragment, or proteins.

1.7.3 Micellar electrokinetic capillary chromatography (MEKC)

Micellar electrokinetic capillary chromatography was introduced by Terabe and co-workers (1984, 1989). It is the most popular of the electrokinetic chromatographic techniques (Terabe, et al., 1991). Separation in MEKC is based on a combination of electrophoretic mobility and chromatographic partitioning between the slower moving micellar “pseudo stationary phase” and the solution phase. The separation mechanics have been illustrated by Dulfier (1990). The technique provides a way to separate both ionic and neutral analytes. Also, large peptides have been separated by MEKC (Yashima, et al., 1992).

MEKC is most commonly performed with anionic surfactants, especially sodium dodecyl sulfate (SDS). Micelles form in solution when a surfactant is added to water in concentrations above its critical micelle concentration (CMC). In the case of anionic surfactants, the hydrophobic hydrocarbon molecules are in the centre of the micelle and the charged head groups around the surface of the spheres. The micelles can be considered as small droplets of oil with a highly polar surface which is negatively charged.

They are used with ionic substances as well as organic modifiers which can lead to significant changes in resolution. The micelles of MEKC can also be replaced with any

material that reacts differentially with the analytes of separation and affects their velocity through the capillary.

1.7.4 Capillary isoelectric focusing (CIEF)

Capillary isoelectric focusing (CIEF) is a relatively new mode of capillary electrophoresis introduced by Hjerten and Zhu (1985). CIEF is a focusing type of capillary electrophoresis in which the solutes, usually proteins, are separated on the basis of their isoelectric points or pI values (Hjerten and Zhu, 1985). In CIEF, the protein samples and a solution that forms a pH gradient are placed inside a capillary. The anodic end of the column is placed into an acidic solution (anolyte), and the cathodic end in a basic solution (catholyte). When an electric field is applied, charged proteins migrate through the capillary until the pI is reached (where they become electrically neutral) and therefore stop migrating. At this time, a steady state is attained. Sharp peaks with good resolution are obtained, and a large peak capacity is observed.

1.7.5 Capillary isotachorphoresis (CITP)

Capillary isotachorphoresis is a moving boundary technique in which the sample is sandwiched between two buffers. The main feature of CITP is that it is performed in a discontinuous buffer system with “leading” and “terminating” electrolytes. The leading electrolyte (or buffer) has the ion with the highest mobility whilst the terminating electrolyte (or buffer) has the ion with the lowest mobility (Schwer and Kenndler, 1990; Jougenon, 1986). These leading and terminating buffers, act to separate the anions of the sample from each other. Although CITP separates analytes on the basis of their electrophoretic mobilities, the means of separation and the output derived from the separation are significantly different from other electrophoretic techniques. In CITP, the isotachopherogram obtained contains a series of steps, with each step representing an analyte zone. The quantitation in CITP is mainly based on the measured zone length which is proportional to the amount of sample present. CITP is useful only for the separation of ionic materials, and it is not possible to separate anions and cations in the same run.

1.8 Principles of separation in capillary zone electrophoresis (CZE)

1.8.1 Electroosmosis

An important phenomenon in capillary electrophoresis is electroosmosis, which refers to the flow of solvent in an applied potential field. At pH values above 3, the fused silica capillary has negative surface charges due to ionization of silanol groups. Positive ions in the buffer will be attracted to these fixed anionic sites, forming an electrical double layer. When high voltage is applied, migration of hydrated cations in this double layer causes movement of bulk fluid in the direction of the cathode. This phenomenon is called electroosmosis, as represented in **Figure 1.10**. The magnitude of electroosmotic flow (EOF) increases as a function of pH at a rate much greater than the migration speed of sample ions. This can be an advantage when analyzing mixtures of anionic and cationic species, since all analytes will eventually swept past the detection point by EOF.

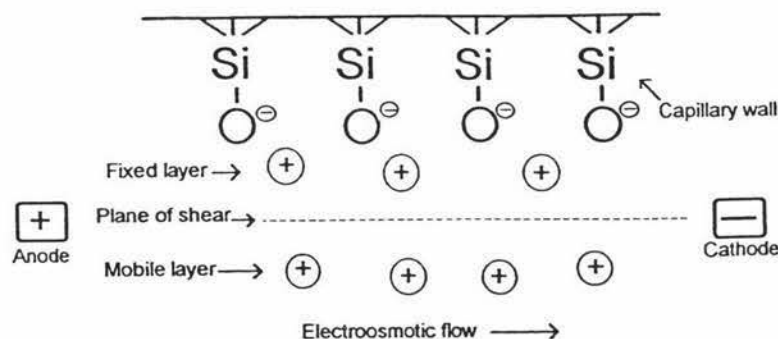


Figure 1.10 Representation of electroosmotic flow in a capillary. Electroosmotic flow is caused by the negatively charged Si-O⁻ groups on the inner wall of the capillary attracting the positively charged cations, represented by the circled +’s, forming the fixed layer. The mobile layer of cations is pulled toward the cathode, dragging the bulk buffer solution with it. The anions and the solvation of the cation are not shown (Baker, 1995)

The potential across the layers is called the zeta potential, denoted by ζ , which is given by the Helmholtz equation:

$$\zeta = 4 \pi \eta \mu_{eo} / \varepsilon \quad (1.1)$$

where η is the viscosity, ε is the dielectric constant of the solution, and μ_{eo} is the coefficient for electroosmotic flow (Li, 1992). It is important that electroosmotic flow be

consistent from run to run, and control of EOF often requires extensive capillary conditioning before and between analyses. If the flow varies, the migration times of the solutes will change, which may cause peaks to be identified incorrectly or errors in quantitation.

The velocity of the electroosmotic flow, v_{EOF} is given by

$$v_{\text{EOF}} = \varepsilon \zeta E / 4 \pi \eta \quad (1.2)$$

where E is the applied electric field in volts/cm.

The electroosmotic mobility, μ_{EOF} , of the buffer is given by

$$\mu_{\text{EOF}} = \varepsilon \zeta / 4 \pi \eta \quad (1.3)$$

Note that electroosmotic mobility is dependent solely on buffer characteristics, that is, dielectric constant, viscosity, pH, and concentration (which influence the zeta potential) and is independent of the applied electric field.

There are several approaches to alter electroosmotic flow. The methods used involve changing the zeta potential across the solution-solid interfaces; increasing the viscosity at the interface; adjusting the pH and the ionic composition of the buffer and introducing additives to the buffer to alter the zeta potential developed across the capillary solution interface. Other approaches of varying or elimination electroosmotic flow include covalently bonding γ -methacryloxypropyltrimethylsilane to the glass surface or coating the capillary wall with a polymer such as methylcellulose (Li, 1992).

1.8.2 Electrophoretic mobility

An electrically charged solute will migrate through a buffer under the influence of an electric field with an electrophoretic velocity, v_{EP} , given by

$$v_{\text{EP}} = \mu_{\text{EP}} E \quad (1.4)$$

where μ_{EP} is the electrophoretic mobility and E the applied electric field. Separation is achieved because solutes migrate through the capillary at different velocities. Electrophoretic mobility is related to its charge-to-size ratio as given by

$$\mu_{EP} = q / 6 \pi \eta r \quad (1.5)$$

where q is the charge of the ionized solute, η the buffer viscosity, and r the solute radius. Anything that affects the charge or size of a solute or the viscosity of the buffer will cause changes in the electrophoretic mobility. For a given applied electric field and buffer, the greater the charge-to-size ratio (q/r), the higher the electrophoretic mobility and velocity. Small, highly charged molecules move through the capillary the fastest, and large molecules with a lower charge move slower. Neutral molecules have an electrophoretic mobility of zero.

1.8.3 Factors affecting performance in capillary electrophoresis

Joule heating is an important factor to consider when performing any electrophoretic separation. A major limitation on the speed, resolution, and scale of electrophoretic separations is the ability to dissipate the Joule heat that is generated as a result of the electric current passing through the electrophoresis buffer. This Joule heating and the resulting temperature gradient can negatively affect the quality of the separation.

Migration time, efficiency, selectivity and resolution are the parameters of an electrophoretic separation. They are influenced by one or more of the electrophoretic parameters, including voltage, electrophoretic mobilities, electroosmotic flow, and capillary length.

The migration time of a solute, t_m , is the time it takes it to migrate the effective capillary length, l , from the inlet to the detector. Higher voltages, shorter capillaries, and high electroosmotic flows give shorter migration times and, therefore, faster analysis times. Efficiency, N , is expressed as the number of theoretical plates, and can be calculated by measuring the migration time and the peak width. If the peak width is measured at half the peak height, $w_{1/2}$, N can be calculated by

$$N = 5.54 (t_m / w_{1/2})^2 \quad (1.6)$$

It can be seen that the narrower a peak and the longer its migration time, the higher its efficiency.

Selectivity, α , has to do with how far apart adjacent solutes are when they pass through the detector, that is, the distance between the apices of adjacent peaks in an electropherogram. Selectivity is dependent on differences in electrophoretic mobilities. The most effective way to affect a change in selectivity is to change the pH of the buffer.

Resolution, R , is the most important separation parameter. It shows how well the components in a mixture are separated. Resolution is influenced by the applied voltage, buffer pH and composition, capillary length and electroosmotic flow. It is increased by increasing the applied voltage, by optimizing the buffer pH and composition, increasing capillary length, and by optimizing the electroosmotic flow.

1.9 Applications of capillary electrophoresis in biological fluids

The application of CE methodology to the analysis of biological fluids has provided great promise for its use in clinical chemistry. Recently, a few studies employing CE for the rapid analysis of biological fluids have been reported. Analytical methods based on CE for diagnosis and studies of several types of human diseases have been developed (Jellum, et al., 1991). Separation of urinary porphyrins by MEKC has been performed (Weinberger, et al., 1990) and amino acids in human urine were separated by CE (Yu and Dovichi, 1989). Schoots et al. (1990) used CE to identify hippuric acid, 2-hydroxyhippuric acid, and uric acid in the blood serum of chronic renal-failure patients. Miyake et al. (1991) used MEKC to quantify the levels of creatinine and uric acid in human plasma and urine. Guzman et al. (1990), Lee et al. (1992) and Guo (1998) used CZE to quantify creatinine and creatine in serum and urine. Barbas, et al. (1998) developed a CE method to separate, identify, and measure the short-chain organic acids in urine. Petucci et al. (1995) reported a CZE method for the analysis of biological fluids for low-molecular-mass species. CE is suitable

for detecting important changes in the metabolic profiles of body fluids and provides a rapid and simple alternative to other techniques in routine analysis.

Since capillary electrophoresis has been widely and successfully applied for the analysis of various biological fluids, it should be possible to analyse the amino acid, felinine, by this modern method. The aim of my study is to develop a capillary electrophoresis method for simple, rapid and accurate determination of felinine in biological fluids.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Reagents

L-Methionine, L-methionine sulfone, L-methionine sulfoxide, L-cysteic acid, L-cysteine, N_ω-nitro-L-arginine, creatinine, creatine and urea were from Sigma Chemical Co., St Louis, MO, USA. Taurine was from New Zealand Pharmaceuticals Ltd., Linton, N.Z; 1,4-dioxane was from Ajax Chemicals, a division of Clyde Industries Limited, Australia; acetonitrile, propan-2-ol, ethanol 96% v/v and hydrochloric acid (all HPLC grade), acetic acid (AR), β-mercaptoethanol and 2,4,6-collidine were from BDH Laboratory Supplies, Poole, England; methanol was from Scharlau, Spain; n-butanol was from Univar (conforms to ACS); sodium dihydrogen phosphate (AR) and triethylamine were from Riedel-de Haen, Selze, AG, Germany; di-tert-butyl dicarbonate (99%) was from Lancaster, Eastgate, White Lund, Morecambe, England; diethylamine was from MERCK-Schuchardt, Hohenbrunn bei Munchen; ninhydrin (AR) was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England; formic acid (AR) was from May & Baker Ltd., Dagenham England; bovine albumin fraction V and sodium chloride (ACS grade) was from Life Technologies Inc., Grand Island, N.Y., USA.

TLC aluminum and cellulose sheets were from MERCK Darmstadt, Germany; 0.22 μm pore size filters were from Millipore Corporation, Bedford, MA 01730.

All other reagents were analytical grade wherever possible. Water was Milli-Q grade.

Synthetic D, L-felinine, cat urine and plasma were obtained from the Best Friend Feline Nutrition Research Unit at Massey University; human plasma and serum were from Nutrition Laboratory, Institute of Food Nutrition & Human Health, Massey University; amino acids standard mixture were from Pierce, Rockford, Illinois 61105, U.S.A

2.2 Apparatus

2.2.1 HPLC system

The reversed phase HPLC system used was from Waters Associates, U.S.A. and comprised a model 680 automated gradient controller, 2 model 510 pumps, a model 440 absorbency detector and a Reodyne injection system. A Pye Unicam PU 8610 spectrophotometer was used for absorbency measurements. A Phenomenex Jupiter C5, 150×4.6 mm; 5µm column was used throughout the study. Detection at 214nm with 2.0 AUFS was used. Buffer A was water + 0.1% TFA; buffer B was 90% acetonitrile + 0.1% TFA.

2.2.2 Capillary electrophoresis system

An Applied Biosystems Model 270A Analytical Capillary Electrophoresis System was used throughout this study. The buffer volumes of the vials at the injection end and at the detector end were 4ml and 12.5 ml respectively. Eppendorf sample vials (0.5 ml) were used in all experiments. The polyimide-coated fused-silica capillary (I.D. 75 µm) was purchased from Phenomenex, U.S.A.. The total length of the capillary was 75 cm and the effective length was 50 cm. The window for the on-column detector cell was created by burning off a small section (~ 0.4 cm) of the polyimide-coating, and the excess residue was then wiped off with methanol. The electropherogram was recorded and evaluated on a Kipp and Zonen DB 40 recorder. Detection at 200nm with 0.02 AUFS was used.

2.2.3 Others

A Buchi 461 Water Bath (made in Switzerland) with Rotavapor and cooling system was used in preparation of the felinine derivative. A Reacti-Therm™ Heating module (made by the Pierce Chemical Company) was used in all heating experiments.

2.3 Preparation of reagents

Standard felinine

Standard felinine solution was prepared by dissolving 10mg of felinine in 10ml of Milli-Q water in a volumetric flask to make a 1 mg/ml felinine solution and diluted to other desired concentrations as necessary. The standard solutions were filtered through 0.22 μm filter before running on the CE. This standard solution was stored at refrigerator temperature.

Buffer solutions

Phosphate buffer was prepared at the required concentration by dissolving NaH_2PO_4 in water. By dissolving 600mg, 480mg, 360mg and 240mg NaH_2PO_4 in 100ml H_2O respectively, 50mM, 40mM, 30mM and 20mM phosphate buffer solutions were made. Adjustment of pH to the required value was achieved by the addition of 1 M and 0.1 M H_3PO_4 . The buffer solutions were filtered and stored in the refrigerator.

Internal standard solution

$\text{N}-(\text{NO}_2)\text{arg}$ was prepared by dissolving 20.00mg $\text{N}-(\text{NO}_2)\text{arg}$ in water in a 100ml volumetric flask. 0.02% NaN_3 was added to the solution to kill bacteria. The solution then was filtered and stored in the refrigerator. This internal standard solution was used throughout the experiments.

Urine collection

Cat urine samples were collected in the Best Friend Feline Nutrition Research Unit at Massey University. The cats were fed of standard, commercially available canned cat food (g component / 100g cat food): protein, 80; fat, 8.0; fibre, 0.8; salt, 0.6; calories, 85). Entire and castrated male cats and female cat were transferred to a stainless steel urine collection metabolic cage specially designed for accurate collection of urine. Food and water were withheld during the collection period (24hr). The cat was returned to its colony cage after the collection. Urine samples then were filtered through a 0.22 μm filter and analysed for felinine on the same day or the day following collection. Samples then were kept in the freezer (-20°C), refrigerator (5°C), room temperature (20°C) and exposed in air respectively.

Urine samples were filtered again just prior to loading on the HPLC or CE systems when kept for some time. Some urine samples were diluted up to 10-20 fold with Milli-Q water before assay.

Fresh human urine was collected into a plastic cup. Pure felinine was added to the urine after the collection and the mixture filtered through a 0.22 μm filter, then run on the CE as required.

Plasma and serum collection

Venous blood from entire male and female cat was collected into a vacutainer tube containing EDTA, and plasma was obtained without delay by centrifuging the blood samples at 2000g for 10min at room temperature within 30min after collection. Human plasma was obtained in the same way as above. Plasma and serum samples were stored at -20°C until analysed, usually within 14 days. Human serum was collected into a vacutainer tube and then the blood was allowed to clot. The clotted blood was centrifuged and the serum removed.

Preparation of the BOC derivative of felinine

Felinine (8mg) was dissolved in 1 ml water and 8ml methanol in a round bottom flask. 1ml triethylamine was added followed by 1 ml of BOC-reagent (di-tert-butyl-oxy-dicarbonate, DTBD, in dioxan 10mg/ml). The solution was evaporated to dryness on a Rotavapor, the bath temperature was no higher than 40°C . The derivative then was dissolved in 8 ml of 20% ethanol (giving the equivalent of 1mg felinine/ml), filtered and used for HPLC.

Cat urine and serum were treated similarly starting with 100 μl and adding water and ethanol as above. Samples were dissolved in 1 ml 20% ethanol, filtered and used for HPLC.

Initial experiments showed that 1ml of reagent resulted in only 95% of the felinine being derivatized. Complete derivatization was obtained when 2 or 3 ml of reagent were added to the reaction mixture (**Figure 2.1**). No negative effects were noted at all with either

excess reagent. Condition used throughout the remainder of the study used 3ml (30 mg) of DTBD per 100 μ l of cat urine.

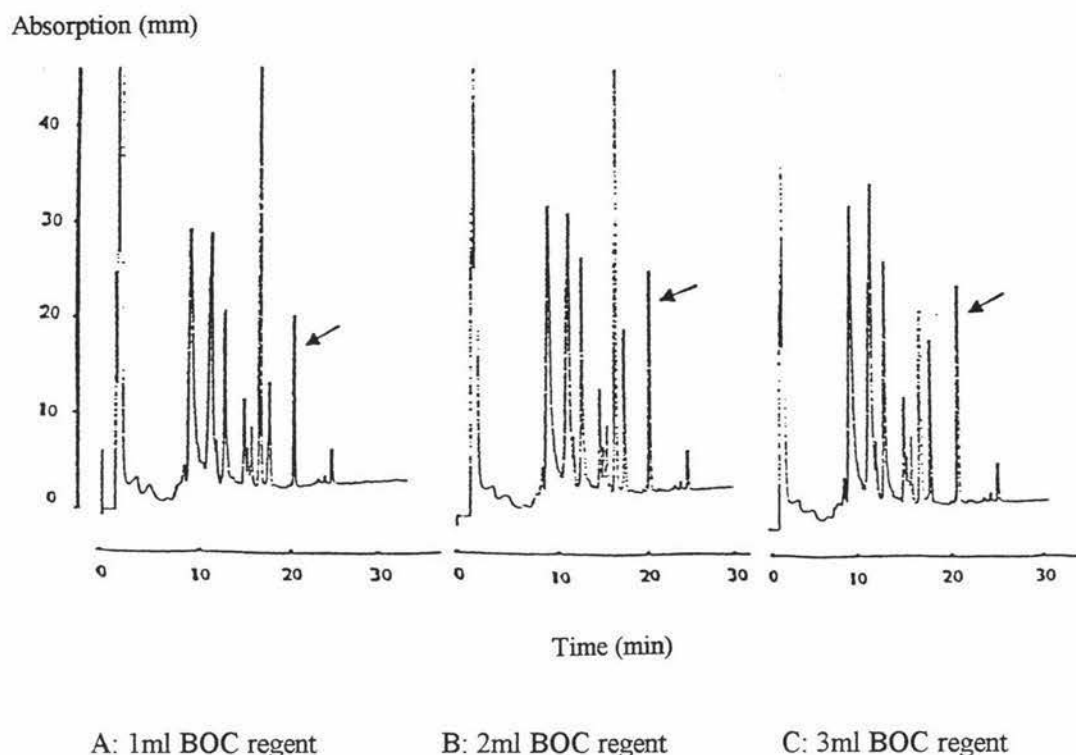


Figure 2.1 HPLC chromatograms of BOC-felinine derivative
* The peak of felinine is indicated by an arrow.

OPA derivative of felinine analyses

The OPA derivative of felinine was analysed by the Nutrition Laboratory, Institute of Food Nutrition & Human Health, Massey University. Felinine analysis was performed on a Waters (Millipore, Milford, MA) ion exchange HPLC system employing post-column derivatization with O-phthalaldehyde and detection by fluorescence spectrometry. A Waters ion-exchange amino acid analysis column was used with HPLC conditions as described by manufacturer.

2.4 General procedures

2.4.1 Capillary electrophoresis

The capillary was loaded as described by manufacturer. The following was the routine operation of the capillary electrophoresis:

Cycle: WASH (0.1 M NaOH)

Time: 1 min	Vial: WA	Pause: No
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Cycle: BUFFER

Time: 2 mins	Vial: B1	Pause: No
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Cycle: MARKER

Time: secs	Vial: MA	Mode: Vacuum
Polarity: Pos	Level: 5	Pause: No

Cycle: SAMPLE

Time: secs	Vial: S	Mode: Vacuum
Polarity: Pos	Level: 5	Pause: No

Cycle: DETECTOR

Risetime: 2.00	Range: 0.02	Autozero: YES
Change: YES		

Cycle: TIME

Time: mins	Buffer: B1	Wavelength: 200 nm
Voltage: kV	Temperature: 35°C	Polarity: Pos

The rate of the chart recorder was 0.2 cm/min.

The adsorption (y-axis) values were obtained as measurements in millimetres (mm) from the original traces. Full scale deflection of 20 cm is equivalent to 0.02 absorbance unit (A.U.). The peak areas (mm²) were calculated from the height and half-height width measurements before use.

The new capillaries were washed with 1M NaOH for 10 minutes, then water for 5 minutes.

2.4.2 Measurement of pH

Measurement of pH was made using a glass combination pH electrode and a digital pH meter (Orion Research digital ionalyzer). The same electrode was used throughout the experiments. The electrode was calibrated using pH 7.0 ± 0.02 standard solution (BDH Chemicals Ltd., Poole, England). The pH meter was equipped with a built-in temperature compensation system with a thermometer probe.

2.4.3 Thin layer chromatography

TLC cellulose aluminum sheets were cut into 10×10cm size square pieces. Two lines were drawn on about 1.0 cm from the bottom and left hand side for two-dimensional TLC. Cat urine was spotted one cm in and one cm up from the lower right side corner and feline reference was spotted at the lower left side corner and upper right side corner. The plate was developed with two solvent systems in succession.

The two solvents systems below were selected in the order indicated:

System 1: BuOH-acetone-diethylamine-H₂O (20:20:4:10)

System 2: Isopropanol-acetic acid-H₂O-ninhydrin (40:2:10:0.1)

System 1 was chosen as the solvents for development in the first dimension. After the solvent had run almost to the upper line of the plate, the plate was removed from the tank and dried in air approximately for 24 hours. It was then developed in the second dimension with System 2. Again, after the solvent had ascended almost to the line, the plate was removed from the tank and air dried at least three hours at room temperature. The part of the plate that was not developed with System 2 was then sprayed with System 2 (which contained ninhydrin) in order to make feline reference visible. The plate was then air dried for another three hours and photographed next day.

2.4.4 Heating

Samples were placed to a glass tube with Teflon screw cap to prevent evaporation, then heated in the heating block at different temperature as required. Samples were taken after

heating at different time and filtered through a 0.2 μm filter. The internal standard was then added to the sample just before the sample running on the CE.

2.4.5 Hydrolysis

Hydrolysis at 110 °C

For hydrolysis of the urine and felinine, 100 μl urine or felinine solution (1mg/ml) was taken and placed into a side-arm glass tube followed by 1ml 6M HCl (with 1mg/ml phenol). The tube was put in liquid air to freeze the sample and then evacuated to remove air. The cap was then quickly screwed down. The tube was placed in a preheated oven at 110°C 24 hrs. The tube was removed from the system and cooled. Hydrolysed urine and felinine were filtered and run on the CE.

Hydrolysis at room temperature

Cat urine and felinine were hydrolysis in the same solvent as above but different HCl concentrations (6M, 4M, 3M, 1M and 0.5M) at room temperature. One drop of β -ME was added to protect the thiol (-SH) group. After reaction different time, the samples were filtered and run on the CE.

2.4.6 The separation

L-methionine, L-methionine sulfone, L-methionine sulfoxide, L-cysteic acid, L-cysteine and/or L-cystine were dissolved together in water. The concentration of these samples were varied but mostly were 1 mg/ml. The mixture was filtered through a 0.22 μm filter and run on the CE with felinine. The mixture then was stored in refrigerator. The solution normally was kept 2 or 3 days, then filtered again prior to use.

CHAPTER THREE

FELININE SEPARATION

3.1 Introduction

Felinine has been recognized since 1951 as a urinary amino acid excreted by domestic cats and several other felidae. The biological role of felinine in the animal is still uncertain but its function as a precursor to a pheromone seems likely but remains unproven (Hendriks, 1995).

In early work felinine was determined and separated by paper chromatography, thin layer chromatography and ion-exchange chromatography (Greaves and Scott, 1960; Avizonis and Wriston, 1959; Fukutome, 1961; Roberts, 1963). Although the amount of felinine was given by these studies, the sensitivity and accuracy of the methods are suspect. A better analytical method, high performance liquid chromatography (HPLC), was introduced by Hendriks (Hendriks, 1995) which gives better quantitative determination of felinine. Even though HPLC method is faster and more sensitive than the previous methods, it is still complicated and time consuming due to the need for felinine-OPA derivatisation.

Further study of felinine requires a more rapid, efficient, precise and easier analytical method. Capillary electrophoresis probably is the best option to date. Capillary electrophoresis complements the older separation techniques of high-performance liquid chromatography, gas chromatography, and slab gel electrophoresis. Capillary electrophoresis offers higher efficiencies, faster separation times, ease of automation, smaller sample size and reagent requirements than HPLC. It is expected that many of the analyses currently done by HPLC will be done by capillary electrophoresis in future.

Literature searches revealed that capillary electrophoresis (CE) had not previously been applied to felinine separation and determination. Since felinine is a sulphur containing amino acid, this study started from the separation of felinine with some sulphur containing compounds and the 20 amino acids found in proteins as a standard mixture. The aim of this chapter was to establish the conditions under which felinine can be separated by capillary electrophoresis.

3.2 Results and discussion

3.2.1 Selection of the mode of CE

Capillary zone electrophoresis (CZE) is the most popular mode of CE, because it is relatively simple. Most separations can be performed using an untreated fused silica capillary. Analysis can be carried out in free aqueous solution. Its application area is wide, ranging from simple to complex samples. The separation is on the basis of differences in charge-to-mass ratios of solute ions at a given pH. It is possible to change the charge-to-mass ratio of many ions by adjusting the pH of the buffer. CZE mode was used through out this study.

3.2.2 Capillary selection

Teflon, Pyrex and fused silica capillaries are three types of capillaries used in CE. Although both Pyrex and Teflon capillaries were used by early workers (Baker, 1995), in recent years, nearly all capillary electrophoresis separations have been performed in polyimide-coated fused silica capillaries. Fused silica capillaries have good thermal and optical properties in the UV range. They are transparent to a wide range of UV wavelengths and hence can be used with a UV detector. Absorbancy measurement is directly through the capillary. A small section of the polyimide coating is removed from the capillary to provide an optical window for detection. Fused silica capillaries are easy to work with as they are flexible and are easy to handle and cut. Most importantly, high-quality silica capillaries with internal diameters below 100 μm are readily available. They were chosen in this study.

The most commonly used capillaries are 50-100 μm I.D. A large inner diameter capillary gives better detector response when a UV/Vis detector is used. However, less Joule heat is produced in a capillary with a smaller inner diameter. If the Joule heat is not dissipated, a capillary with a large inner diameter may give poorer separation than a capillary with a small inner diameter.

Capillary length affects both peak spacing and separation time. The longer the capillary, the longer the separation time and the wider the peak and the spacing between peaks with all other conditions being equal. Migration times are proportional to capillary length and resolution is

proportional to $(l/L)^{1/2}$ where l is the capillary length to the detector and L is the total capillary length. In order to have the solutes separated in the fastest time, the shortest capillary that gives the required resolution should be used.

In this study, capillaries with 75 μm I.D and 25 cm and 50 cm effective length (from injection end to the detector window) with total length 47 cm and 75 cm respectively were used.

3.2.3 Injection mode selection

To begin a separation in CZE, the sample is usually injected (or introduced) into the end of the capillary at the high-voltage electrode. This sample injection process can affect the sample quantity injected, the run-to-run reproducibility of the quantity injected, and the electrophoretic separation efficiency (Rose and Jorgenson, 1988). A number of techniques are used for sample injection in CZE. The most commonly used modes are *hydrodynamic injection* and *electrokinetic injection*. These two methods are suitable for quantitative analysis as they give good linearity for peak area response (Schwartz, et al, 1989). For the most precise work, hydrodynamic injection is desirable whilst for the most sensitive work, electrokinetic injection is better (Harakuwe, 1995). Injection reproducibility with both methods depends on the relative ionic strength and pH of the running buffer and the sample, as well as the pI of the analyte.

Hydrodynamic Injection also called hydrostatic injection. It can be performed by gravity flow, pressure or vacuum suction. Picolitre to nanolitre sample volumes are possible. The mean hydrodynamic flow (v_{HD}) in the capillary during hydrodynamic sampling is given by Poiseuille equation.

$$v_{HD} = \rho g r \Delta h / 8 \eta L \quad (3.1)$$

where ρ = solution density, r = capillary radius, Δh = difference in height of the capillary ends, η = electrolyte viscosity and L = capillary length.

The sample zone length (l) introduced into the capillary using a sampling time of t_i is

defined by Eq.3.2.

$$l = v_{HD} t_i \quad (3.2)$$

As can be seen from the above equations, hydrodynamic injection sensitivity is affected by sample viscosity. This means temperature control is vital. It also means that internal standards may be more reliable than external standards which bear little resemblance to the sample matrix (Harakuwe, 1995).

Electrokinetic injection is also called electromigration injection. To perform electrokinetic injection, the electrode is removed from the buffer vial and placed into the sample vial. The buffer reservoir at the high-voltage electrode is replaced with the sample vial such that the capillary and electrode dip into the sample solution. An injection voltage is then applied for a brief period of time, causing sample to enter the end of the capillary by electromigration. Electromigration injection includes contributions from both electrophoretic migration of charged sample ions and electroosmotic flow of the sample solution (Li, 1992).

The instrumental configuration for electrokinetic injection is simpler than for hydrodynamic injection. Although simple, electrokinetic injection has the disadvantage of being discriminating and may not introduce the same amount of ions due to differences in their mobilities. Furthermore, quantification is difficult due to the dependency of injection quantity on sample composition.

Model 270A CE offers both hydrodynamic (vacuum) and electrokinetic injection methods. In hydrodynamic (vacuum) injection, a precisely controlled preset vacuum is applied to the detector end of the capillary for a selected time interval. The pressure differential aspirates sample fluid into the capillary (**Figure 3. 1**).

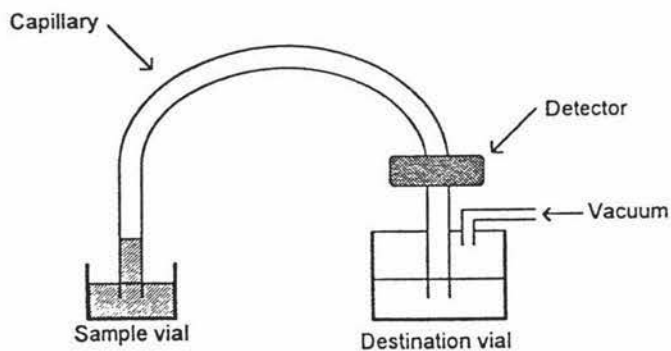


Figure 3.1 Hydrodynamic injection by vacuum. A vacuum is applied to the destination vial, pulling the sample solution into the capillary. The volume of sample injected depends on the magnitude and duration of vacuum applied, sample solution viscosity, and capillary dimensions. After injection, the capillary is placed back into the source vial and an electric field applied (Baker, 1995).

The hydrodynamic injection method was chosen to be the way of sample injection in this study.

3.2.4 Buffer selection

The buffer plays a central role in capillary zone electrophoresis. It is the semiconducting property of the buffer that makes the migration of analyte ions possible. A wide variety of electrolyte systems have been used in CE to effect the required separations. The majority of these are aqueous buffers. The choice of the electrolyte system in a CE separation involves consideration of many factors, such as the solubility and stability of the analytes in the electrolyte, the degree of ionization of the analytes, the influence of the anions and cations present in the electrolyte on the electromigration of the solutes, the effect of pH, and the dissipation of heat generated in the electrolyte during the passage of the current (Li, 1992).

The composition, pH and concentration of the buffer are important parameters in adjusting retention, selectivity, and peak shape in CZE. In order to attain symmetrical peaks in the electropherogram, the mobility of the buffer should match that of the solutes. Electrophoretic mobility is proportional to the charge-to-size ratio of an ion. So a buffer anion should be used that has a similar charge-to-size ratio as the solute anions (Baker, 1995).

Buffer composition

Buffer composition can alter retention time (migration time) and selectivity. Different ions in the run buffer will interact differently with the capillary wall and can change the electroosmotic flow. Also, the buffer ions may interact with solute ions, and changing the buffer composition may change the selectivity (Baker, 1995). Therefore, a change of buffer composition can dramatically influence the separation.

As felinine has not been previously analysis by CE, and its pKa was not available, separation using a variety of buffers having pH values from 2.0 to 7.5 were studied. The result was showed in **Table 3.1**.

Table 3.1 Felinine separation at different buffers

Sample	Buffer	pH	Peak	Migration time (R_t)	Apply voltage
Felinine 1mg/ml	30mM NaCl +0.1% HCOOH	3.0	One	21.6 mins	20KV
Felinine 1mg/ml	30mM NaCl +0.1% HCOOH	4.0	none		
Felinine 1mg/ml	50mM NaH ₂ PO ₄	4.5	none		
Felinine 1mg/ml	20mM Na ₂ B ₄ O ₇ + HCl	2.0	One	27.0 mins	15 KV
Felinine 1mg/ml	20mM Tris	7.5	none		
Felinine 1mg/ml	30mM NaH ₂ PO ₄	3.0	One	36.2 mins	12 KV
Felinine 1mg/ml	30mM NaH ₂ PO ₄	2.0	One	34.8 mins	12 KV
Felinine 1mg/ml	20mM NaH ₂ PO ₄	2.0	One	31.0 mins	12 KV

As we can see from **Table 3.1**, when the pH was higher than 4.0, no felinine peak appeared. Phosphate buffer with pH 2.0 showed advantages for the separation of felinine (**Figure 3.2**). Moreover, phosphate buffer is widely employed for amino acid, protein and peptide separations with pH at 1.14-3.14 and 6.20-8.20. Therefore, phosphate buffer was chosen as the run buffer

through out this study.

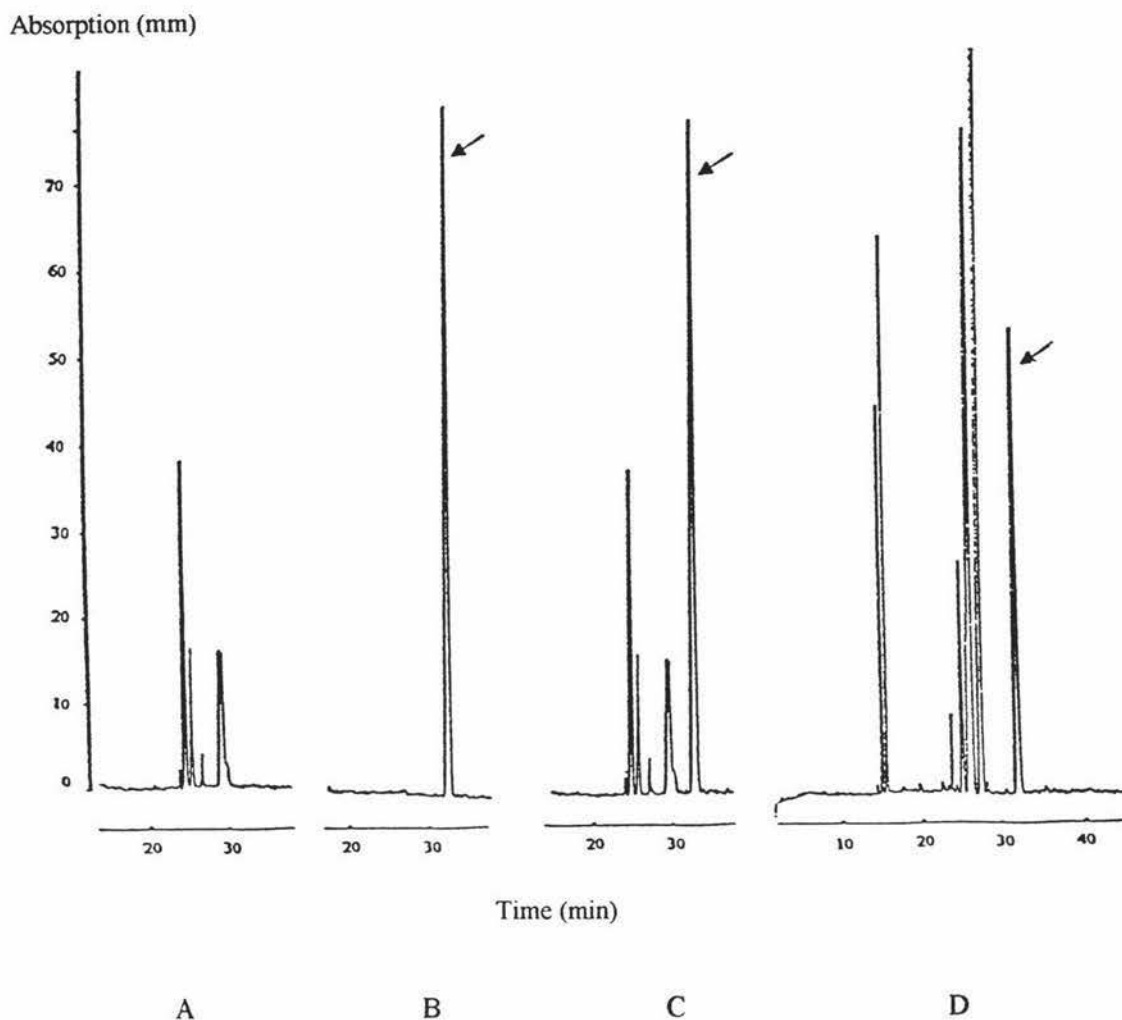


Figure 3.2 Felinine separation (* The felinine peak is indicated by an arrow.)
 Conditions: capillary, 75 $\mu\text{m} \times 75$ cm, 50 cm to the detector; apply voltage, 12 kV;
 buffer, 20 mM NaH_2PO_4 , pH 2.0.
 A: L-methionine, L-methionine sulfone, L-methionine sulfoxide and L-cysteine
 B: felinine
 C: felinine (0.6 mg/ml) with L-methionine, L-methionine sulfone, L-methionine
 sulfoxide and L-cysteine
 D: felinine (0.3 mg/ml) with standard amino acid mixture

Buffer pH

The pH of the 'run' buffer can have a significant influence on migration times and selectivity. As the 'run' buffer pH is changed, the electro-osmotic flow changes due to

changes in the degree of ionization of the silanoate (Si-O^-) groups on the capillary inner wall. Buffer pH also influences the electrophoretic mobilities of solutes, as the degree of ionization of many species present in the electrolyte system depends on the pH of the solution. Differences in the degree of ionization of the analytes result in differences in their mobilities which are important for achieving separation in the CE system. The most important consideration in the selection of the buffer is the choice of a pH where the analytes are ionized. If a pH is selected that is above the pK_a for bases or below the pK_a for acids, separation will not occur, since there is no charge developed on the analyte. In **Figure 3.3** felinine was separated from amino acid standard and mixed sulphur containing analytes at pH 2.0, 2.1 and 2.2. It shows that a change of only 0.2 units in pH can greatly change the migration times of the separation. Lower pH gives fast separation for felinine. When the buffer pH was changed from 2.0 to 2.2, the migration time of felinine changed from 14.7 minutes (A) to 23.0 minutes (C).

The effect of further changes of buffer pH on the separation performance were investigated. In addition to pH 2.0 and 2.2, separations using buffers having pH values of 1.8, 2.5 and 2.8 were studied. As can be seen in **Figure 3.4**, the elution time of felinine increases when pH changes from 2.0 to 2.8. For pH 2.8 and pH 2.5, the efficiency and resolution were poorer (loss of baseline resolution) than those at pH 2.0 and pH 2.2. At pH 2.8, the separation really deteriorated. This loss of resolution presumably arises because the analytes begin to adsorb onto the silica capillary, resulting in tailing (McCormick, 1988). The selectivity and efficiency observed at pH 1.8 and 2.0 were very similar, and the separation time had no significant difference. Buffer pH 2.0 has greater advantages for the separation than pH 1.8 as lower pH combined with the higher current generates and produces more Joule heat.

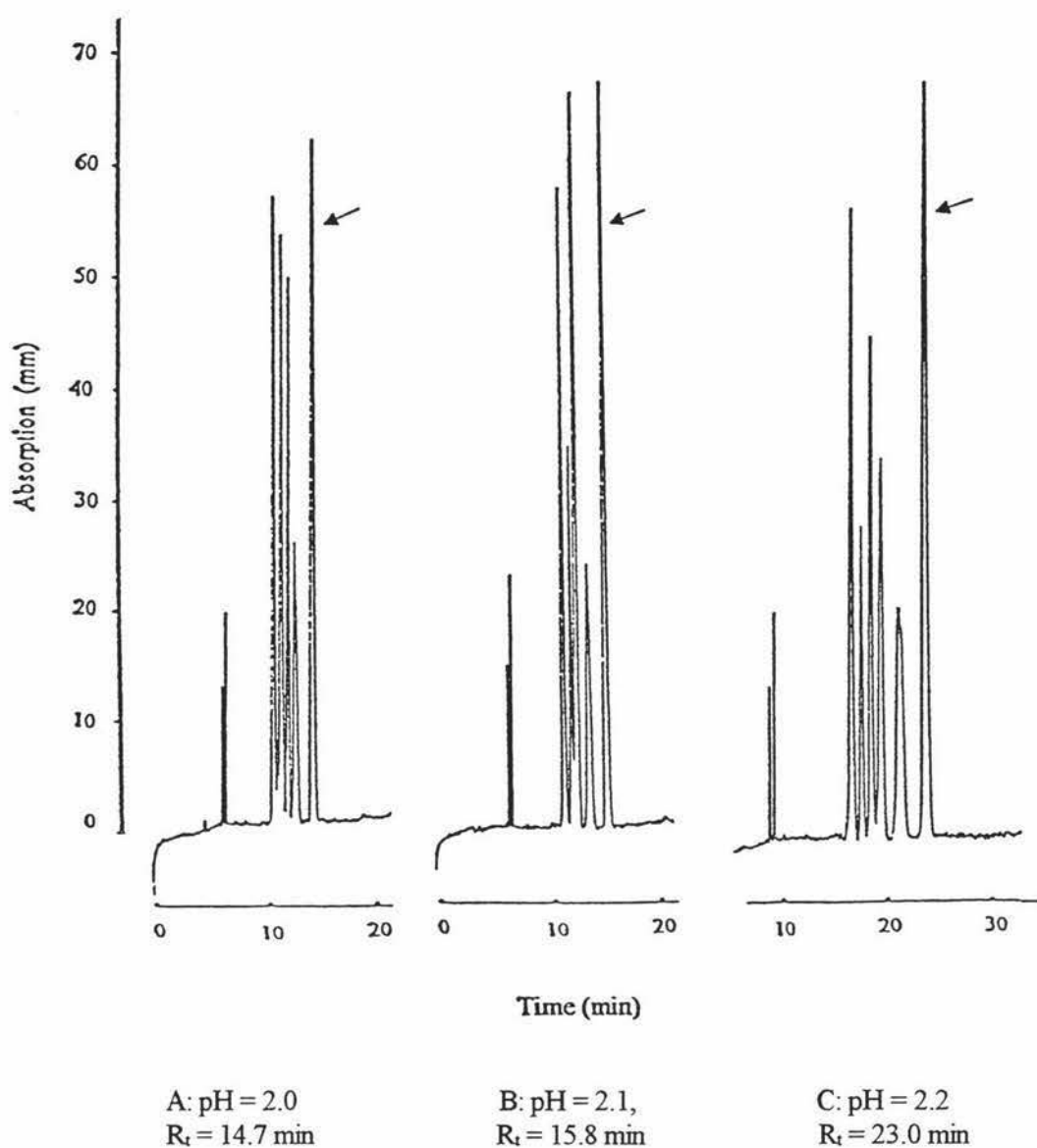


Figure 3.3

Effect of buffer pH to the separation of felinine

Conditions: 20 mM NaH₂PO₄ + H₃PO₄ buffer; voltage, 10 kV; injection time, 1 sec; vacuum: temperature, 35°C; capillary length, 47 cm (25cm to detector), i.d., 70 μm; detection, 200 nm. Samples: standard amino acid mixture, L-methionine, L-methionine sulfoxide, L- cysteine and 0.5 mg/ml felinine

* The felinine peak is indicated by an arrow.

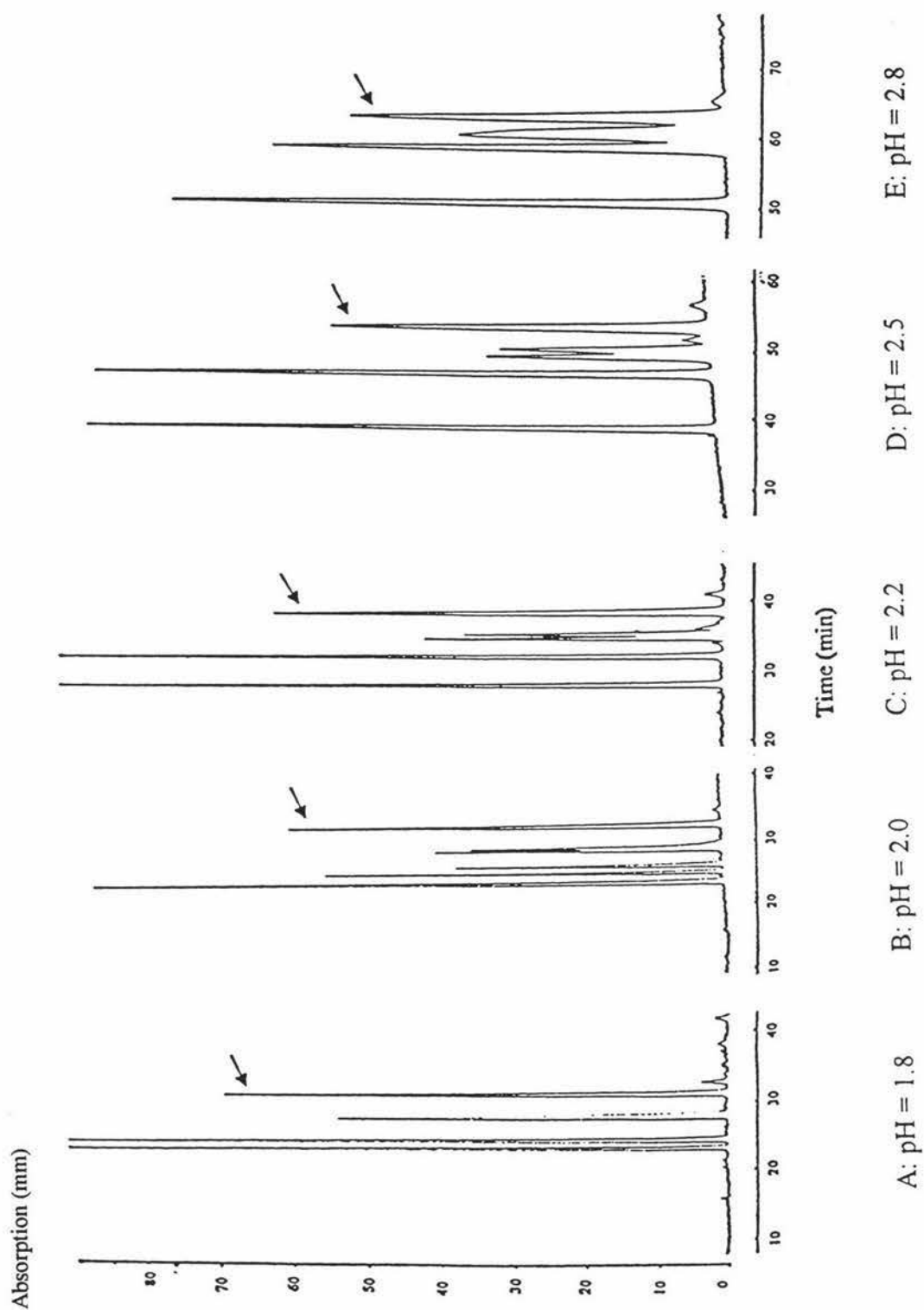


Figure 3.4

Separation of felinine at different pH

Conditions: buffer, 20 mM NaH_2PO_4 ; applied voltage, 12 kV; capillary, $75\ \mu\text{m} \times 50\ \text{cm}$

Sample: L-methionine, L-methionine sulfoxide, L-cysteine and felinine

* The felinine peak is indicated by an arrow.

Buffer concentration

Concentration and ionic strength of the buffer both have significant effects on solute mobilities and separation efficiency. It was well known that the buffer concentration must be sufficiently high to maintain a constant conductivity and pH within the analyte zone to prevent band broadening. Buffer concentration at least 100 times that of the sample may be necessary to meet this requirement. However, high concentrations of ionic buffers produce high conductivity, and therefore high currents and high heat loads. The typical buffer concentrations used for small molecule separations range from 15 to 25 mM. These buffer concentrations have sufficient buffering capacity yet minimal conductivity, which means Joule heating is usually not a problem. Higher buffer concentrations can be utilized, but often the electroosmotic flow is suppressed. This feature can be used advantageously to adjust the selectivity of the separation. The higher concentration buffers tend to give greater linear dynamic range and peak symmetry at the expense of high conductivity (Grossman and Colburn, 1992). When the capillary temperature is controlled, increasing the buffer concentration will lower the electroosmotic flow. If the temperature is not controlled, increasing concentration may cause an increase in electroosmotic flow because it increases the current and, consequently, the temperature, which lowers the viscosity (Baker, 1995).

The electrophoretic mobility of an ion (μ_{EP}) has been discussed in Chapter One (Part II), As Eq. 1.5 shows, the electrophoretic mobility is also given as follows:

$$\mu_{EP} = q / 6 \pi \eta r \quad (3.3)$$

where q is the effective charge of the ion, η is the viscosity of the solution, and r is hydrodynamic radius of the ion. μ_{EP} is also given by

$$\mu_{EP} = \varepsilon \zeta / 6 \pi \eta \quad (3.4)$$

where ε is the dielectric constant and ζ is the zeta potential, which is directly proportional to the

thickness of the double layer:

$$\zeta = 4 \pi \delta e / \varepsilon \quad (3.5)$$

where e is the excess charge in solution per unit area and δ is the thickness of the double layer, which is proportional to the inverse of the square root of the buffer concentration.

It is expected from Eq. 3.3 that electrophoretic mobility of a solute depends mainly on the charge-to-radius ratio. Nevertheless, the effective charge of the ions may be affected by the type and concentration of the buffer, as a result of the effect of the buffer on the double layer. This is because μ_{EP} depends on the zeta potential (Eq. 3.4), which is in turn dependent upon the total excess charge in the buffer solution (Eq. 3.5).

In general, it has been observed that mobility depends inversely on buffer concentration. An expression has been given for the dependence of electrophoretic mobility on concentration:

$$\mu_{EP} \cong e / 3 \times 10^7 |Z| \eta C^{1/2} \quad (3.6)$$

where C is the concentration of the buffer (Li, 1992).

Observations were made by Rasmussen and McNair (1990) in their study on the effects of buffer concentration, capillary internal diameter and electric field strength on the coefficient of electroosmotic flow. With a capillary of 50 μm I.D., μ_{∞} decreased when more concentrated phosphate buffers were used (**Figure 3.5**). In a 100 μm capillary, the same relationship between buffer concentration was observed at lower electric field strengths. However, Joule heating became more pronounced in the larger capillary, especially when concentrated buffers were employed. μ_{∞} was found to increase markedly with electric field strength (**Figure 3.6**).

In my study, felinine migration time (R_t) increased when more concentrated NaH_2PO_4 was used, especially when 50 mM buffers was employed (**Figure 3.7** and **Table 3.2**). From the above discussion and experiments, 20 mM NaH_2PO_4 buffer with pH 2.0 was the best choice for the

separation of felinine since it met all of the above criteria and gave reproducible electropherograms.

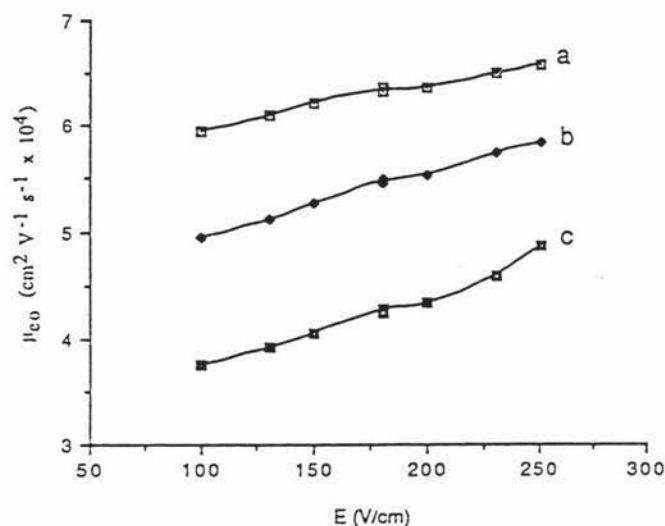


Figure 3.5 Influence of buffer concentration and electric field strength (E) on the coefficient of electro-osmotic flow (μ_{eo}) in 50 μm capillaries. NaH_2PO_4 concentrations: a = 0.01 M; b = 0.02 M; c = 0.05 M (Rasmussen and McNair, 1990).

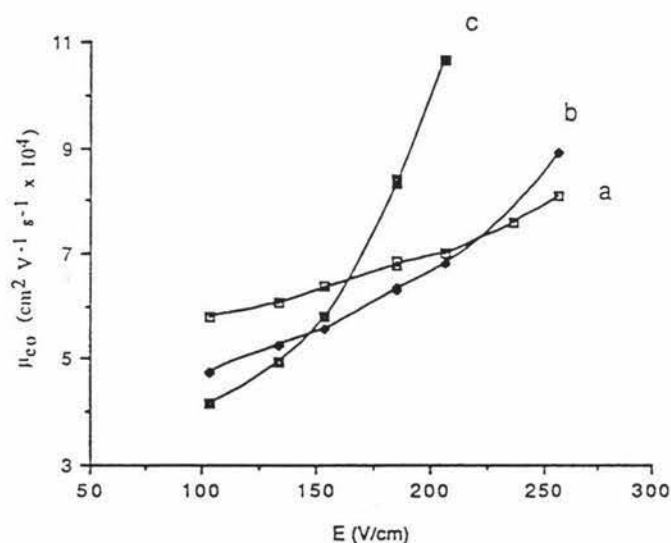


Figure 3.6 Influence of buffer concentration and electric field strength (E) on the coefficient of electro-osmotic flow (μ_{eo}) in 100 μm capillaries. NaH_2PO_4 concentrations: a = 0.01 M; b = 0.02 M; c = 0.05 M (Rasmussen and McNair, 1990).

Table 3.2 Felinine migration time at different buffer concentration

NaH ₂ PO ₄ concentration	Felinine migration time (R _f)
20 mM	13.0 min
30 mM	14.4 min
40 mM	16.0 min
50 mM	18.0 min

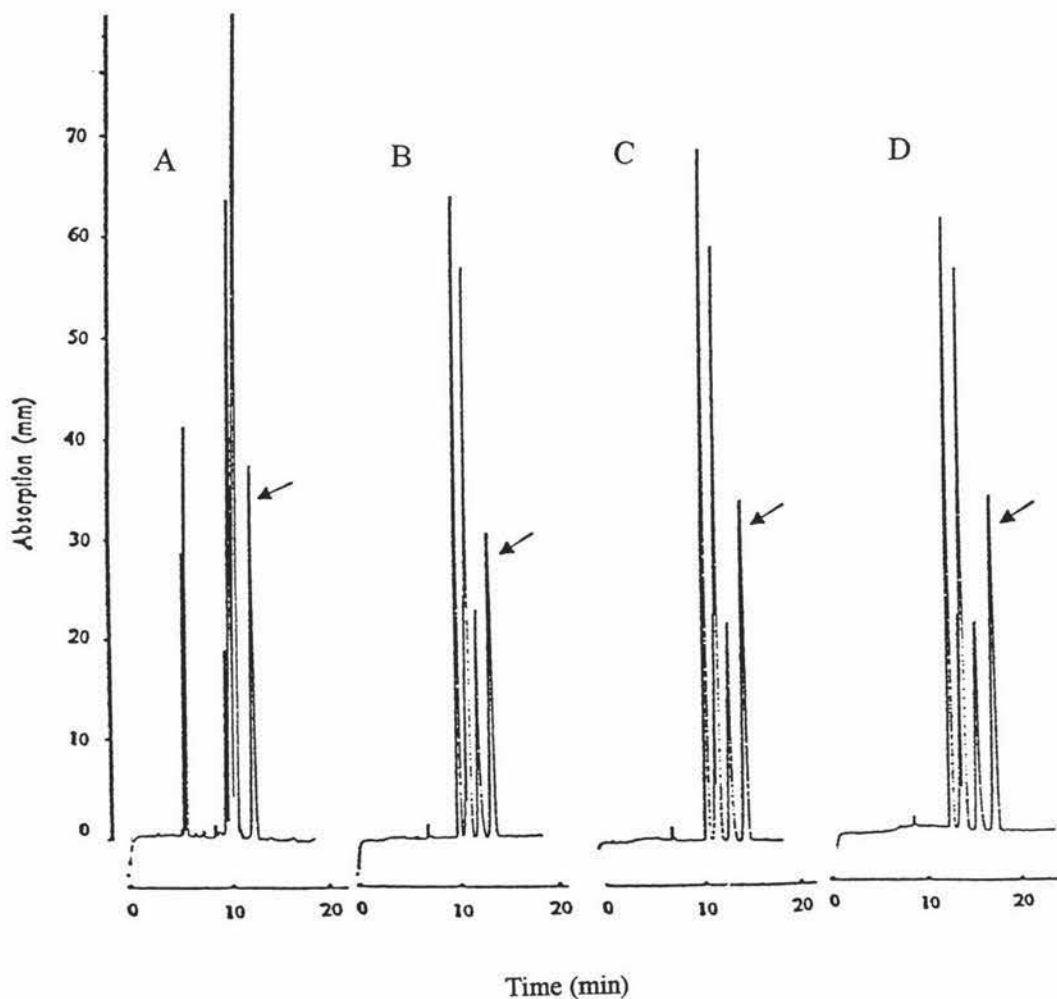


Figure 3.7 Influence of buffer concentration on the migration time of felinine
 Sample: felinine and standard amino acid mixture (A); felinine and L-methionine, L-methionine sulfoxide, L-cystine and L-cysteine (B, C and D).
 Condition: 10 kV voltage; 25 cm length capillary; 1 sec injection; vacuum injection;
 NaH₂PO₄ concentration: A = 20 mM; B = 30 mM; C = 40 mM; D = 50 mM. pH 2.0.
 * The felinine peak is indicated by an arrow.

3.2.5 Voltage selection

For rapid separation of analytes, the application of a high voltage is desirable. High voltages give faster separation with higher efficiencies and resolutions. However, the applications of ever increasing voltage is limited by the Joule heat. In capillary zone electrophoresis, the migration time for a solute is given by

$$t = L^2 / \mu V \quad (3.7)$$

where t is a solute migration time, L is the capillary length, μ is the solute electrophoretic mobility, and V is the applied voltage. The separation efficiency, in terms of the total number of theoretical plates, N , is

$$N = \mu V / 2 D \quad (3.8)$$

where D is the solute diffusion coefficient.

From these two equations we can see first, high separation efficiencies are best achieved through the use of high voltages. Second, capillary length has a profound influence on migration times and hence analysis times. It appears that the ideal situation is to apply as high a voltage as is available to capillaries which are as short as possible to yield the highest separation efficiency in the shortest possible time. There are practical limits to this approach. As voltage is increased, there is more Joule heat generated in the capillary, which can be detrimental to the separation. Moreover, if the capillary is made shorter the amount of Joule heat that must be dissipated increases because of the decreasing electrical resistance of the capillary. At the same time, surface area available for heat dissipation is decreasing (Jorgenson, and Lukacs, 1983). If the heat is effectively removed, then higher voltages can be used.

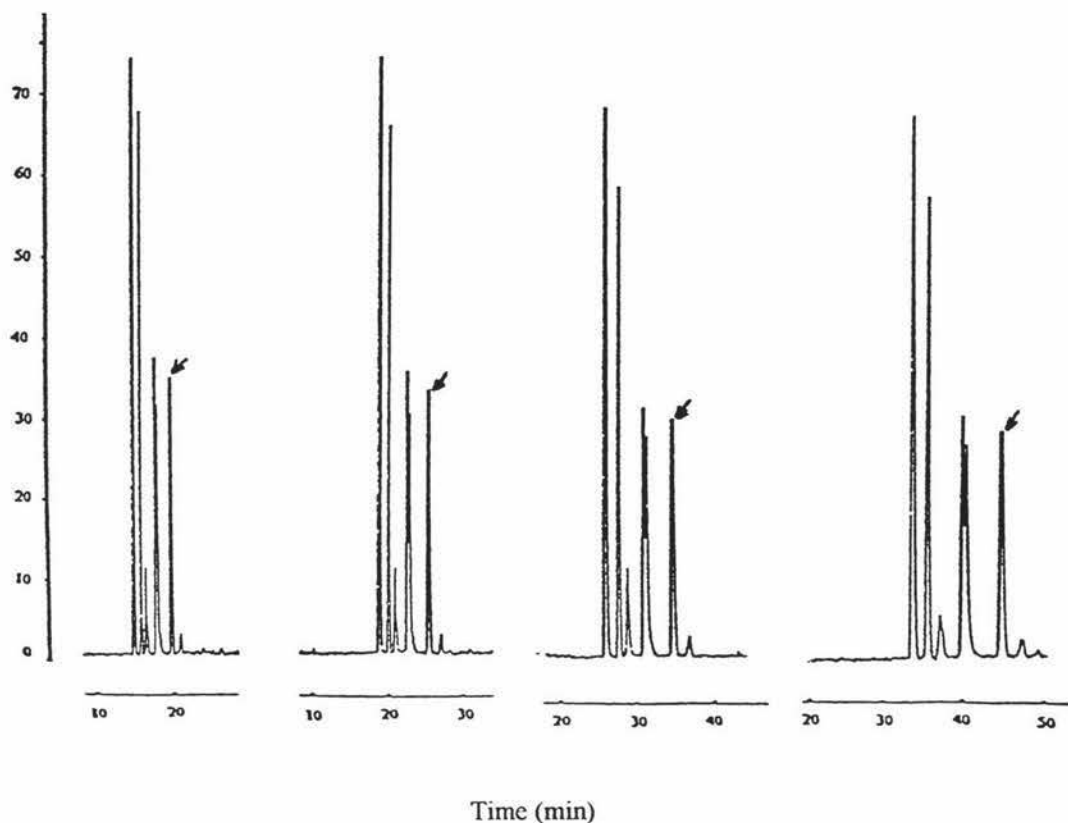
The separation of felinine from other sulphur containing compounds at different voltages (10 kV, 12 kV, 15 kV and 18 kV) using 50cm length (from inlet to detector) capillary was demonstrated in **Figure 3.8** while other conditions were kept constant. **Table 3.3** shows the migration time (R_t) and the current generated. As the applied voltage is increased, felinine

migration times decrease rapidly, presumably because the increased temperature in the capillary at the higher applied voltages reduces the buffer viscosity and thus increases felinine mobility. Although the 18 kV voltage gave the shortest time for the separation, the highest current was accompanied by the disadvantage of more Joule heat generated. Quantitative precision was probably worse under conditions of higher current levels (Schwartz et al., 1989). Moreover, increasing voltage decreased the sensitivity. The 10 kV voltage gave the best sensitivity and the lowest current generated, but felinine migration time was as long as 43.8 min. Voltages of 12 kV and 15 kV gave relatively shorter migration times (35.5 min and 26.0 min) and reasonable smaller currents generated (50 μ A and 62 μ A), which can avoid more Joule heat generated. In this study, conditions with voltage at 12 kV and 15 kV were used in order to avoid more Joule heat generated and yet produce reasonable fast separations.

Table 3.3 **Felinine migration time and current generated at different voltages**
Conditions as Figure 3.8

Voltage	Migration time (R_t)	Current (μ A)
10 KV	43.8 mins	40 μ A
12 KV	34.5 mins	50 μ A
15 KV	26.0 mins	62 μ A
18 KV	20.0 mins	78 μ A

Absorption (mm)



A: 18 kV

B: 15 kV

C: 12 kV

D: 10 kV

Figure 3.8 Influence of voltage on the separation of felinine (capillary 50cm to detector)
 Conditions: 20 mM NaH_2PO_4 , pH 2.0; injection time, 1 sec; vacuum injection
 Sample: felinine, L-met, L-met sulfone, L-met sulfoxide, L-cystine and L-cysteine.
 * The felinine peak is indicated by an arrow.

When a 25 cm (from inlet to detector) capillary was used for the separation, the migration time was much shorter and the peak separation was much less as capillary length affects both peak spacing and separation time (**Figure 3.9**). Migration times are proportional to $(l/L)^{1/2}$, where l is the capillary length to the detector and L is the total capillary length. At voltage 8 kV, felinine migration time was as short as 20mins, but the current generated was about 70 μA . Under the same conditions, a shorter capillary allowed use of a lower voltage.

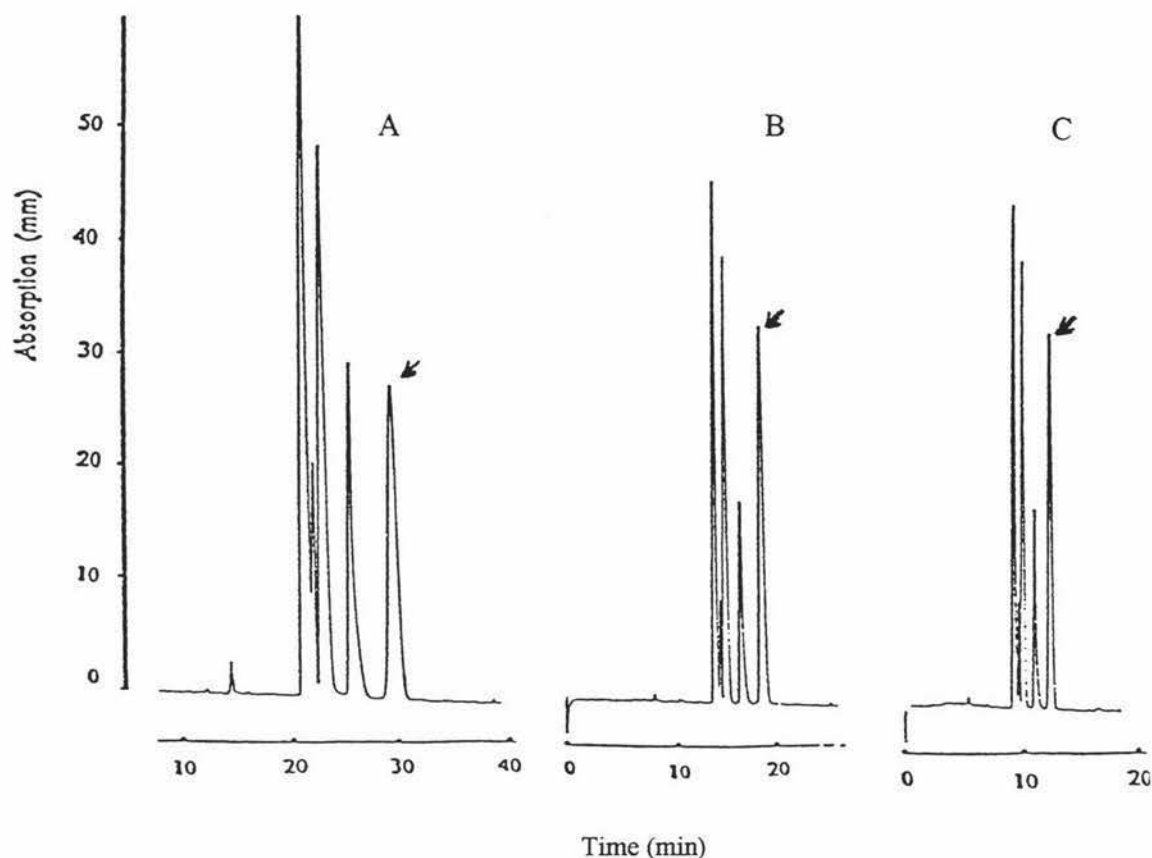


Figure 3.9 Influence of voltage on the separation of felinine (capillary length 25 cm to detector). A: 6 kV, B: 8 kV, C: 10 kV. Other conditions as **Figure 3.8**
 * The felinine peak is indicated by an arrow.

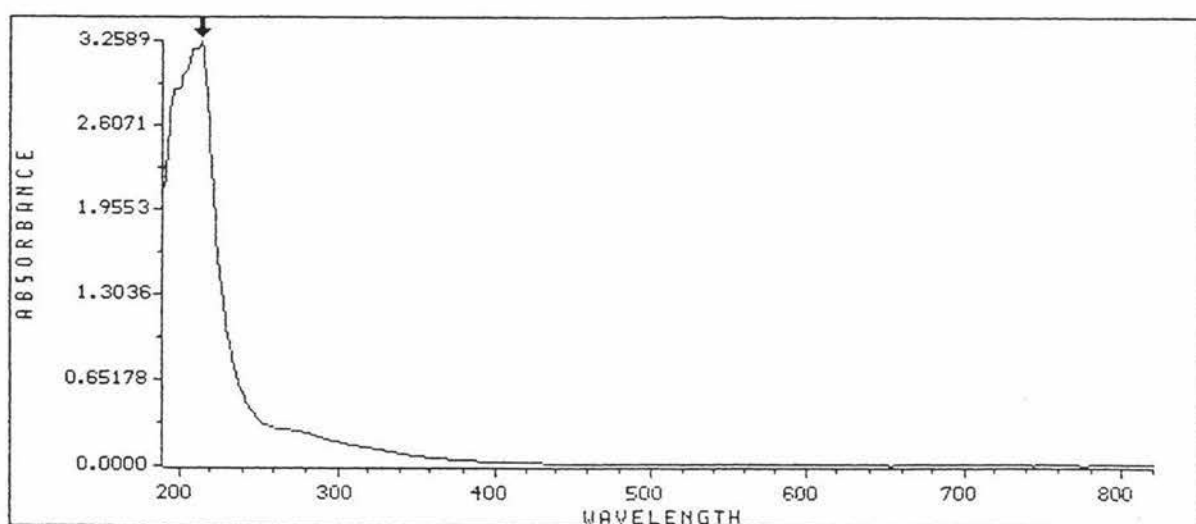
3.2.6 Selection of wavelength

A variety of detectors have been used in capillary electrophoresis. Detection based on absorbance of ultraviolet or visible (UV/Vis) light is most widely used because of its sensitivity to a wide range of compounds and functional groups. The principle of UV/Vis absorbance detection is based on Beer's law where absorbance, A is given by

$$A = \log 1 / T = a b C \quad (3.9)$$

where T is the transmittance, a is the absorptivity, b is the path length of light in the sample compartment in cm, and C is the solute concentration.

The best wavelength is not necessarily the wavelength of maximum absorbance. Selecting a wavelength for optimum sensitivity should take into account both the absorbance spectrum of the analyte and the noise characteristics of the detector cell. **Figure 3.10** shows the absorbance spectrum for 1mg/ml felinine. The absorbance has a maximum in the vicinity of 214 nm. The Model 270A CE which was used in this study is approximately five times more sensitive for proteins at 200 nm than at 214 nm the wavelength typically used for HPLC (**Figure 3.11**). **Figure 3.12 A and B** also show that felinine has a much higher absorbency peak at 200 nm than that at 214 nm. Wavelength 200 nm was then used for the analysis of felinine throughout this study.



Annotated Wavelengths:

1 : Wavelength = 214 Result = 3.258911

Figure 3.10 Felinine (1mg/ml) UV absorbance
solvent: water; blank: water

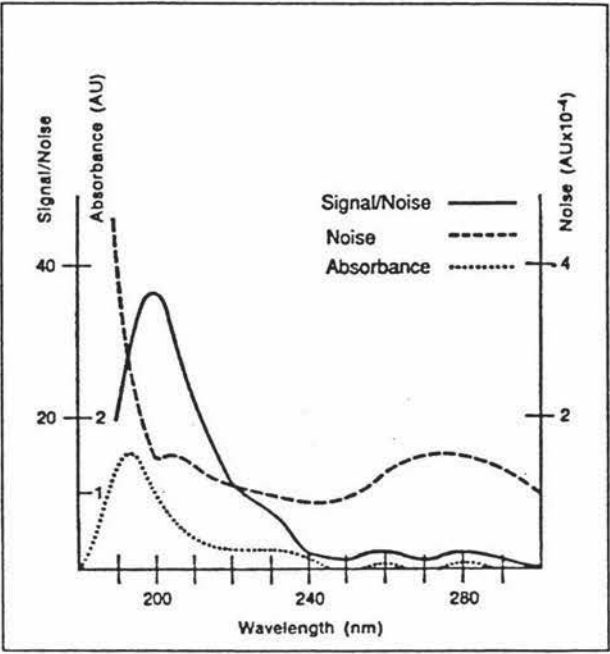


Figure 3.11 The absorbency spectrum for Bovine Serum Albumin (Applied Biosystems). AU = Absorbance Units

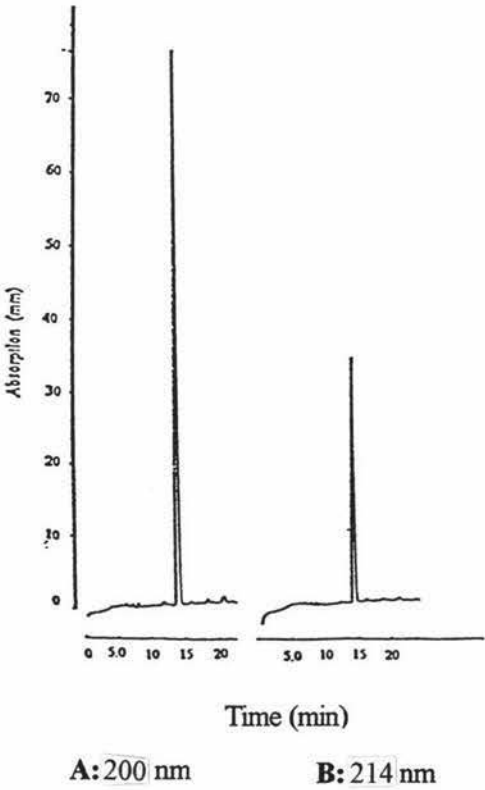


Figure 3.12 Detection of felinine at wavelength 214 nm and 200 nm. Felinine concentration 0.5 mg/ml, 20 mM NaH₂PO₄ buffer, pH 2.0.

3.2.7 Temperature setting

Capillary temperature control is very important in capillary electrophoresis. Changes in capillary temperature can cause variation in efficiency, migration times, injection volumes, and detector response. Also, elevated temperatures may cause sample decomposition with resulting changes in molecular configuration of solutes. If the capillary temperature changes during or between separations, qualitative and/or quantitative errors result. Temperature changes may affect a myriad of physical parameters that can alter the migration velocities of analytes through alteration of the electrophoretic mobility (increasing at a rate of ca. 2%/°C) of the analytes, the electro-osmotic flow rate, or both. The buffer pH, dielectric constant, and viscosity (factors which strongly influence analyte mobility), are dependent on temperature. Analyte adsorption on the capillary walls, dissociation of ionizable groups on the analyte and capillary walls, and conformation of biomolecules are also dependent on temperature. Therefore, changes in capillary temperature will cause changes in migration times and peak areas.

The temperature at which a separation is performed is determined by both the ambient level (or pre-set temperature of the temperature control system) and the temperature generated by the Joule heating from the applied potential. Given the influence that temperature has on a separation, it is important that the capillary temperature be maintained accurately and precisely for good reproducibility within and between laboratories. This means in order to have a stable migration time for identification, the temperature must be constant during the analysis. The CE apparatus can only keep the temperature steady when the run temperature is at least 5°C higher than the room temperature (Guo, 1998). **Figure 3.13** illustrates felinine separation at temperature 30°C and 35°C. The migration times of felinine decreased about 1 min when the temperature was changed from 30°C to 35°C. Since felinine separation electropherograms at temperature 30°C and 35°C had no significant difference, the operating temperature was set at 35°C as it leads to shorter migration time and improves the separation sensitivity.

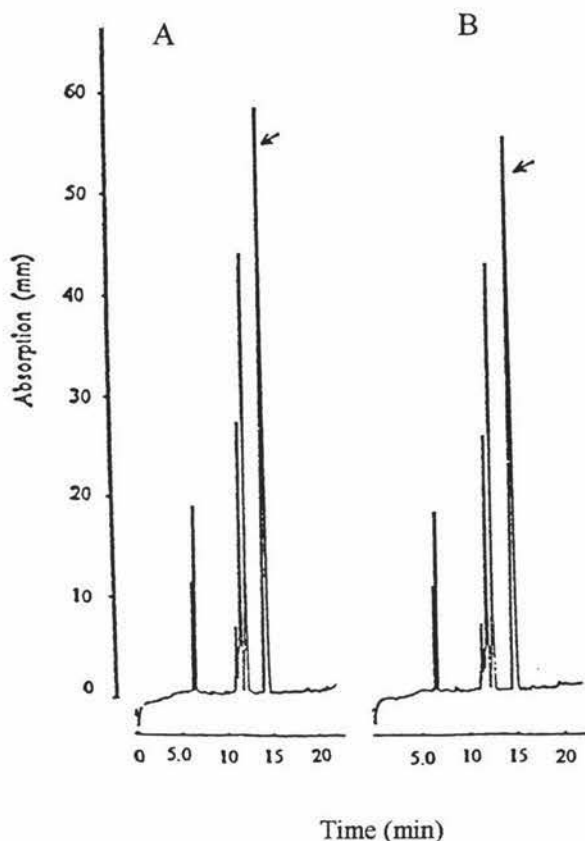


Figure 3.13 Felinine separation at temperature 35 °C (A) and 30 °C (B)

* The peak of felinine is indicated by an arrow.

3.2.8 Reproducibility of the electropherograms

Reproducibility of migration time

In order for any analytical technique to be useful, the results obtained must be reproducible. The migration reproducibility may depend on several operational factors: ionic strength of the buffer, age of the capillary, previous capillary treatments, frequency of capillary treatments, applied voltage and external capillary temperature (Smith, 1991). The easiest measurement to make in capillary electrophoresis is simply the migration time for each peak. Vacuum injection has been shown to offer the greatest reproducibility, due to the fact that better control over physical parameters can be maintained. Good reproducibility of peak shape was observed (Figure 3.14). The felinine shape was reproducible over eleven runs, with washing between

runs with 0.1 M sodium hydroxide solution.

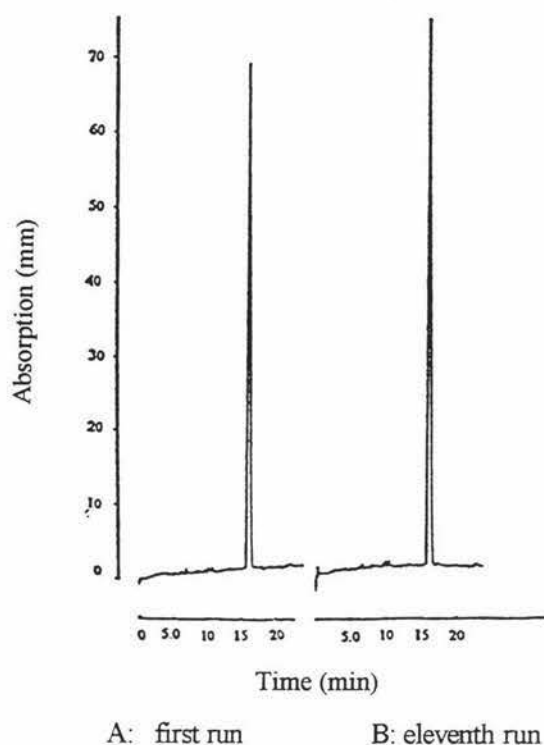


Figure 3.14 Feline electropherograms from first and last run of reproducibility. Other conditions as **Table 3.4**

Migration times, standard deviations (S.D.) and CV for the feline are listed in **Table 3.4**. Because a coated capillary was used, migration times were not influenced by electro-osmotic flow. Hence, electrophoretic mobilities μ_{EP} could be directly calculated by Eq. 3.10 (Takigiku, and Schneider, 1991) and were included in Table 3.4.

$$\mu_{EP} = L_d L_t / t V \quad (3.10)$$

Here L_d is the length of the capillary from the injection end to the detector, L_t is the total length of the capillary, t is the migration time and V is the applied voltage.

Table 3.4 Reproducibility of migration time and mobility of felinine

Felinine	Migration time (min)	S.D	CV	Mobility
0.5 mg/ml	16.5	-0.02	-0.12%	7.12
0.5 mg/ml	16.2	-0.32	-1.94%	7.25
0.5 mg/ml	16.2	-0.32	-1.94%	7.25
0.5 mg/ml	16.5	-0.02	-0.12%	7.12
0.5 mg/ml	16.8	0.28	1.69%	6.99
0.5 mg/ml	16.5	-0.02	-0.12%	7.12
0.5 mg/ml	16.5	-0.02	-0.12%	7.12
0.5 mg/ml	16.8	0.28	1.69%	6.99
0.5 mg/ml	16.6	0.08	0.48%	7.08
0.5 mg/ml	16.6	0.08	0.48%	7.08
0.5 mg/ml	16.5	-0.02	-0.12%	7.12

Conditions: sample, 0.5 mg/ml felinine; run buffer, 20mM NaH₂PO₄ (pH 2.0); capillary 47 cm × 75 µm i.d., length to detector 25 cm; vacuum injection for 1 sec; 10 kV applied voltage; wavelength 200 nm. Coefficient of variation CV = [S.D./mean] × 100. Mean migration time is 16.52 min; mean standard deviations (S.D.) is ± 0.13 and mean CV (%) is 0.79.

Mobility is primarily influenced by analyte size and charge (characteristics which can be influenced by solvent properties such as pH and viscosity), but hydrophobicity and molecular conformation can also affect mobility. Mobilities are also influenced by temperature fluctuations. Good reproducibility can only be achieved by rigorous temperature control. Felinine showed relatively good reproducibility over 11 runs as the Model 270A CE provides precise control of temperature (+/- 0.1 °C) of the capillary to ensure reproducible mobilities and migration times.

Reproducibility of peak height and area

Peak height and peak area are usually used for precision studies. Some studies have noted the advantages of measuring peak areas. Peak area values showed less variation than peak heights (Snyder et al., 1979; Kenney, 1991 and Honda et al., 1989). Both felinine peak height and peak area were measured in my study. It was found that eleven repetitions of an injection on the

same felinine sample gave more consistent result with peak area (R.S.D. ± 2.15 , CV 1.7%) than peak height (R.S.D. ± 4.56 , CV 3.0%) (Table 3.5 and Fig. 3.15).

Table 3.5 Reproducibility of peak height and peak area.
All conditions are as Table 3.4

Number of runs	Height (mm)	R.S.D.	CV	Area (mm ²)	R.S.D.	CV
1	146.5	-5.7	-3.74	124.5	-0.9	-0.72
2	147.0	-5.2	-3.42	125.0	-0.4	-0.32
3	148.0	-4.2	-2.76	118.4	-7.0	-5.58
4	147.0	-5.2	-3.42	125.0	-0.4	-0.32
5	148.0	-4.2	-2.76	125.8	0.4	0.32
6	153.0	0.8	0.53	122.4	-3.0	-2.39
7	151.5	-0.7	-0.46	128.8	3.4	2.71
8	155.0	2.8	1.84	131.8	6.4	5.06
9	157.0	4.8	3.15	125.6	0.2	0.16
10	162.5	10.3	6.77	125.6	0.2	0.16
11	158.5	6.3	4.14	126.8	1.4	1.12
Mean	152.2	± 4.56	3.0%	125.4	± 2.15	1.7%

As an integrator was not attached to the recorder, peak heights were measured from the recorder trace, and relative peak areas were calculated from

$$A = D_{1/2} \times H \tag{3.11}$$

where A is area, $D_{1/2}$ is the peak half-height width and H is peak height. $D_{1/2}$ and H were measured from the peak.

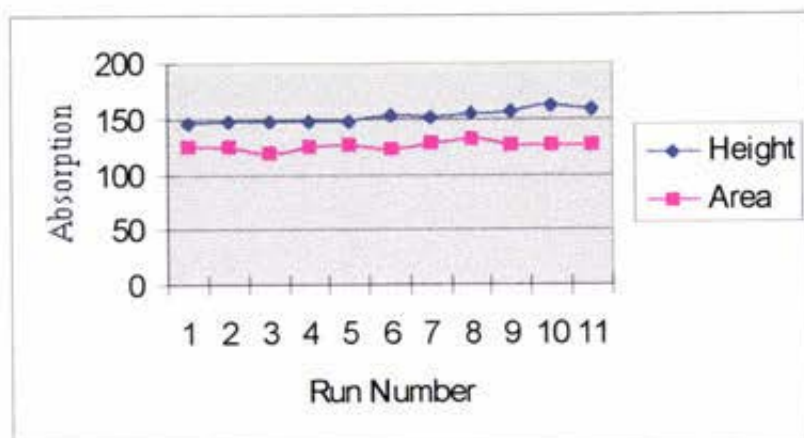


Figure 3.15 Comparison of precision: peak height versus peak area measurements on a single felinine sample. Conditions as **Table 3.14**

3.3 Conclusion

In the present study, a simple, fast, quantitative, and reliable CZE analytical method to separate, identify, and measure felinine has been developed, and analytical conditions have been validated. Under the conditions selected, felinine can be separated from standard amino acid mixture and sulphur containing analytes in a simple and rapid manner. The good reproducibility of migration time, peak height and peak area allows the quantitative analysis of felinine to be performed directly on CZE.

CHAPTER FOUR

DETERMINATION OF FELININE IN CAT URINE

4.1 Introduction

Published quantitative data on levels of felinine in urine have been obtained using paper chromatography (Westall, 1953; Roberts, 1963) and ion exchange chromatographic methods (Tallan et al., 1954; Avizonis and Wriston, 1959; Shapiro, 1962). Earlier study showed that the amount of felinine excreted in the urine of the domestic cat is sex dependent (Roberts, 1963). Adult entire male cats had higher urinary concentrations of felinine than adult female cats. This study was based on measurements using paper chromatography. The work of Roberts (1963) was extended by Hendriks et al. (1995a), who assayed 24 hr urinary felinine excretion in a total of 28 cats including entire female, entire and castrated male cats using HPLC analysis and a synthetic felinine standard of known purity. The average felinine concentration in the urine of entire male cats was found to be 2.0 g/l (range 0.4-3.6 g/l) with entire females excreting on average 0.3 g/l.

Over 380 publications have addressed the wide use of capillary electrophoresis in clinical chemical analysis (Perrett, 1999). It was demonstrated that proteins in human urine and plasma/serum have been separated and quantitatively analysed by CZE (Guzman, et al., 1990; Jenkins, 1997; Gordon, et al., 1991; Dolnik, 1995).

This primary study of the separation of felinine using capillary zone electrophoresis has been discussed earlier and an analytical method established in Chapter Three. Determination of felinine concentrations in male, castrated male and female cat urine using this CZE analytical method will be further developed and demonstrated in this Chapter. Moreover, HPLC and TLC methods will be compared with CZE method for the determination and separation of felinine. It was expected that CE method will give more accurate, simple and fast measurement of this sulphur containing amino acid.

4.2 Results and Discussion

4.2.1 Separation and identification of felinine in cat urine

The key for any separation technique is found in its flexibility towards adjustment of conditions to optimize a specific analysis. Because CE is still in the relatively early stages of development, systematic approaches for optimizing experimental conditions are not available. For separations of ionic species in CE, a largely empirical approach is often necessary. Variables, such as buffer type, buffer ionic strength, buffer pH, column diameter, and operating voltage which affect both efficiency and selectivity, must be considered (O'Neill et al, 1994). In Chapter Three, the analytical conditions of CE for the separation of felinine from mixture of standard amino acids and some sulphur containing chemicals were discussed. That method will be used here for the determination of felinine in cat urine.

Since the composition of cat urine is complex, felinine peak identification is desirable. To ascertain if a compound is present in a sample, a "spiking" method can be used. It can be done by injecting the sample mixture, obtain an electropherogram, then "spiking" the mixture with the compound of interest, inject the spiked mixture, and see if one of the peaks gets bigger. Peak identification can also be assigned by matching the electrophoretic mobility with that obtained for a standard. Felinine peak identities in cat urine were accomplished by both comparing electrophoretic mobility and spiking with a pure felinine standard.

Spiking of felinine is illustrated in **Figure 4.1**, which represents electropherograms of a typical male cat urine sample diluted 10 times (A), felinine only (B) and the same urine spiked with felinine (C). As we can see from above experiment, the last large sharp peak of urine with migration time at 32.2 minutes (A) was increased in size (C) when the urine was spiked with felinine (B). Moreover, the migration time of this peak (32.2 minutes) is the same as standard felinine, which means the peaks have the same mobility ($\mu_{EP} = L_d L_t / tV$, see Chapter Three). Therefore, the last peak, which has a migration time at 32.2 minutes, represents felinine peak under these analytical conditions.

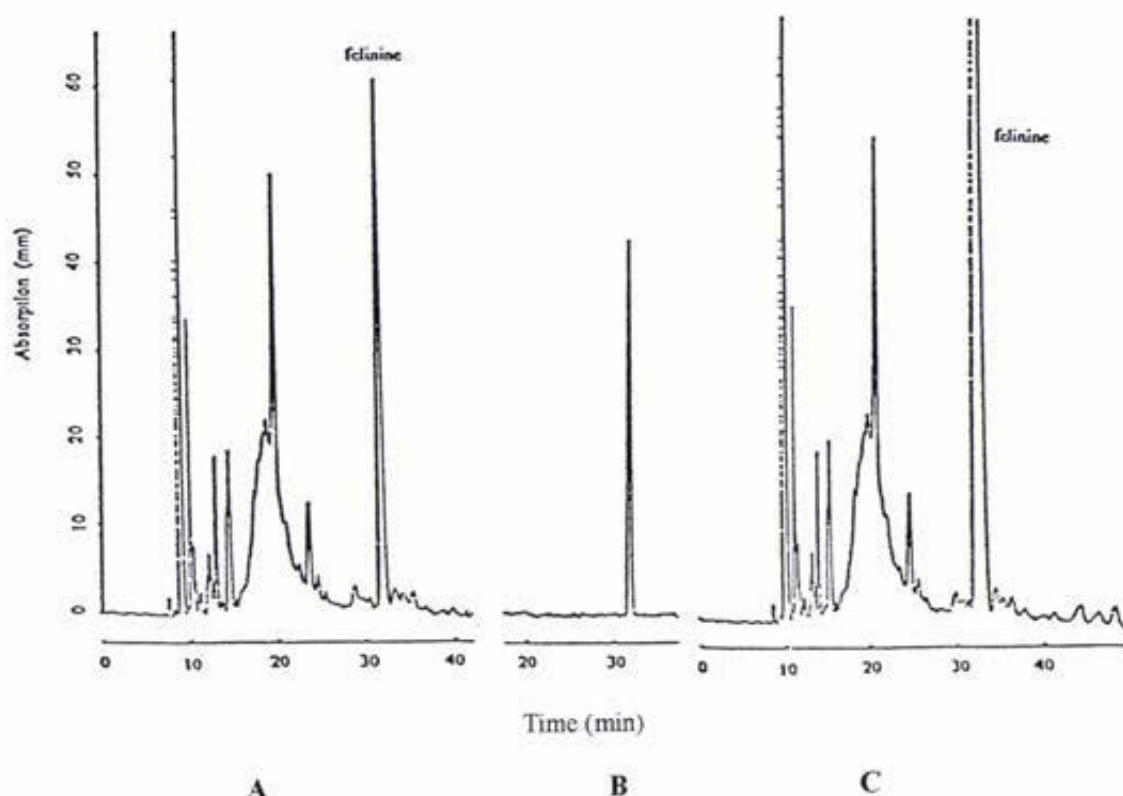


Figure 4.1 Spiking felinine
 Electropherogram of A: entire male cat urine; B: felinine; C: spiked felinine
 Conditions: capillary: 75 $\mu\text{m} \times 75$ cm (50 cm to detector); buffer: 20 mM NaH_2PO_4 (pH 2.0); applied voltage: 12 kV; vacuum injection for 1 sec; 200 nm wavelength; recorder range 0.02, rise time 2.00.

4.2.2 Quantitative methods

The quantitative information from an electropherogram is reported as the area under a peak. With most detectors that use linearizing electronics, peak area is directly proportional to the sample mass or concentration for a fixed volume of sample. In some cases the peak height can be more accurately correlated to a physical property of a molecular substance. The use of peak area, however, is the preferred method for quantitative analysis because it provides a greater linear response with respect to sample amount than peak height (Moring, 1992).

The task of quantitative determination requires instrument calibration with a series of standards having a known concentration range. Proper calibration requires the preparation

of standards that are in a solvent or buffer compatible with the separation method and whose concentration is accurately measured (Moring, 1992). The steps in developing a quantitative methods are: choose either an external standard or internal standard calibration method; calibrate with either peak height or peak area; inject standards of known concentration or amount and construct a calibration plot; then inject samples and determine the solutes concentrations from the calibration plot (Snyder and Kirkland, 1979).

Calibration method

The most common calibration methods use either an *external standard* or an *internal standard*. For the external standardization (ESTD) method of calibration, a standard solution of the solutes of interest is prepared in known concentrations and then injected, and the peak heights or areas of each solutes are measured. A calibration plot of peak height, peak area, or peak area divided by migration time vs. solute concentration is then prepared. For internal standard (ISTD) calibration, a known amount of a compound that is never present in the sample is added to the standards, and the standards are injected. Peak heights and peak areas of each of the standards and the internal standard are measured. A calibration plot is constructed for each solute to be quantified, as in the ESTD method of calibration. Instead of peak area or peak height vs. concentration as in ESTD, the area ratios of solute-to-internal standard are plotted vs. concentration ratios of solute-to-internal standard (Baker, 1995).

Vacuum injection was used in this study as it gave good reproducible injection volumes. The urine sample was not taken through any pretreatment(s) prior to injection except for filtration. An ESTD method is desirable.

Standard felinine

A synthetic felinine of 80% purity, obtained according to the synthesis procedure described by Hendriks et al. (1995b), was used as the standard felinine. **Figure 4.2** shows the mass spectrum of the felinine.

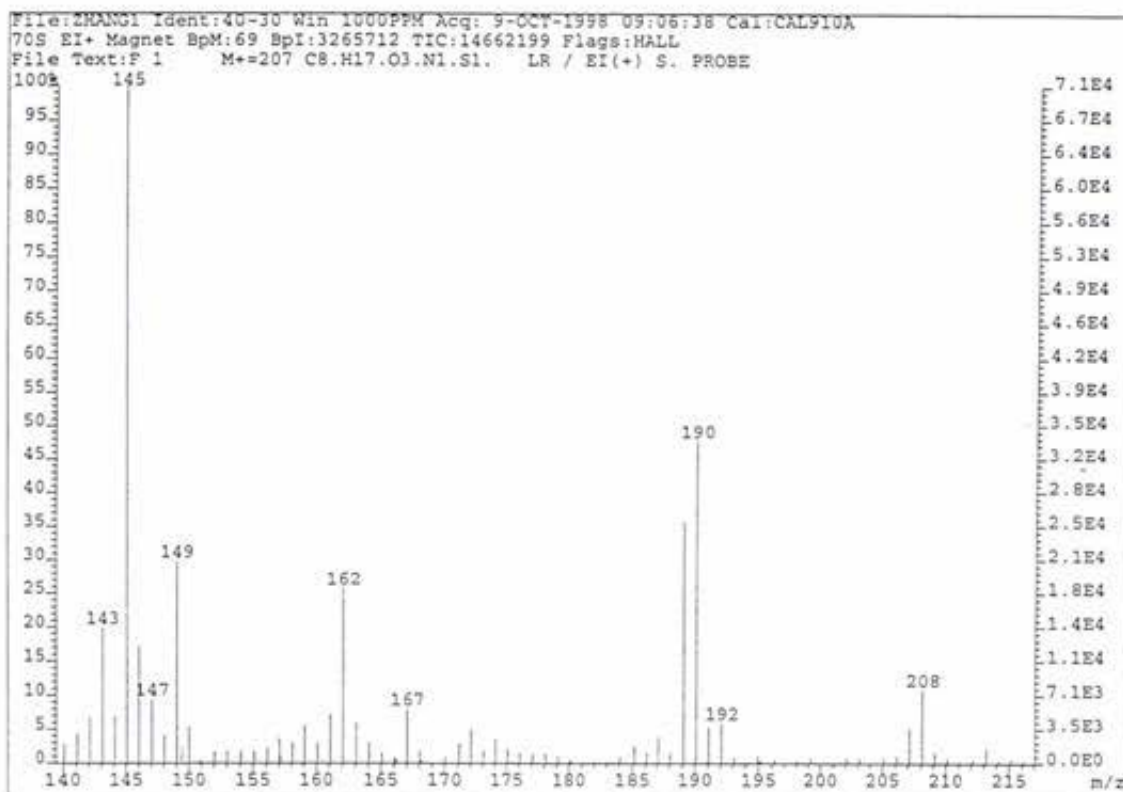
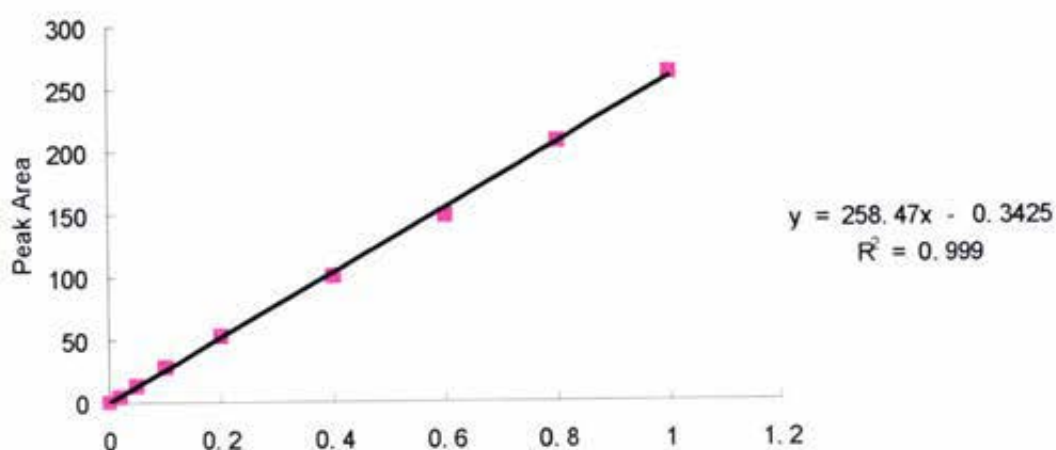


Figure 4.2 Mass spectrometry of synthetic felinine

Standard curves were constructed using the standard solutions of felinine. Two standard felinine calibration plots of peak height and peak area vs. concentration are then prepared as represented in **Figure 4.3 A** and **B**. Calibration will be changed under differing analytical conditions.

The reproducibility of felinine peak height and peak area was discussed in Chapter Three. The precision of peak area is better than peak height. In a standard felinine calibration study, felinine peak area also gave better linearity in the calibration plot, as evidenced by higher correlation coefficient ($R^2 = 0.999$) than peak height calibration ($R^2 = 0.980$). The use of peak height exhibited the poorer linearity (**B**). Therefore, peak area (**A**) provides better quantitative information than peak height. Peak area calibration was then used for felinine quantitative analysis.

A:



B:

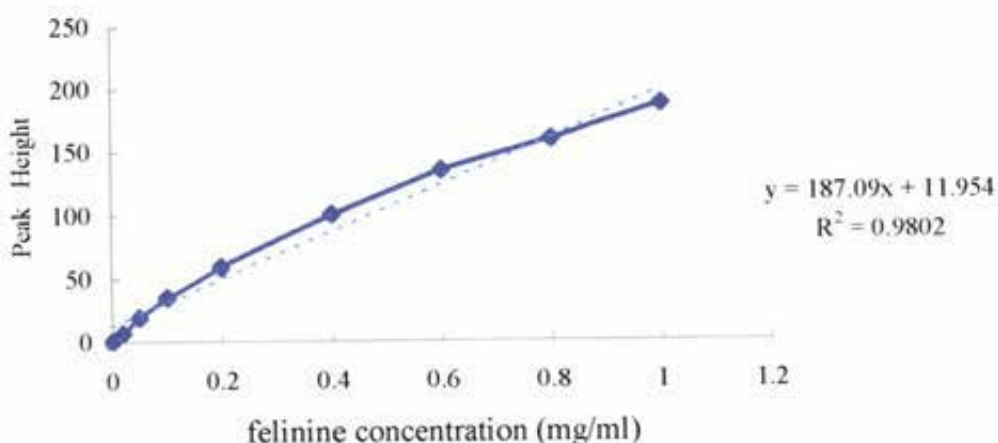


Figure 4.3 Comparison of linearity for standard felinine at 200 nm with measurement of peak area (A) and peak height (B). All conditions as **Figure 4.1** except the apply voltage was 10 kV.

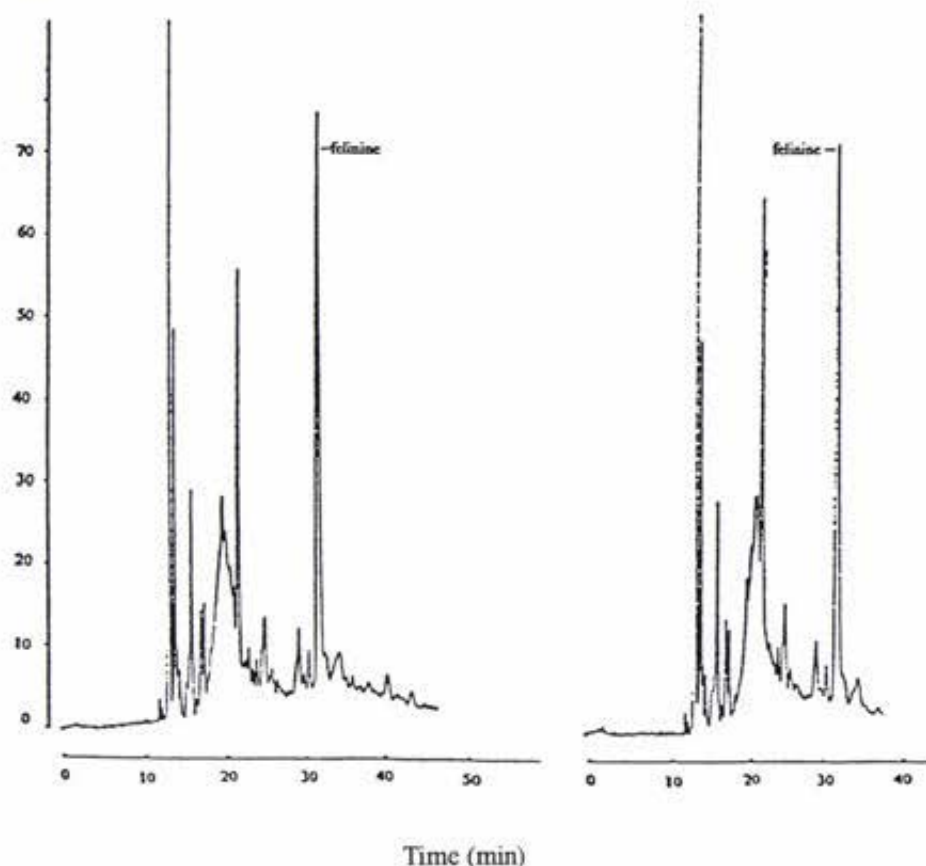
4.2.3 Reproducibility

4.2.3.1 Felinine peak area and migration time

In order to examine the reproducibility of peak areas and migration times, the mean values, the standard deviation (S.D.) and the coefficient of variation (C.V.) of peak area and migration time were calculated from electropherograms obtained by six repeated

injections of a sample of male cat urine. Good reproducibility of peak shape (**Figure 4.4**), peak area and migration time (**Table 4.1**) was observed. All peaks in urine were reproducible over six consecutive runs, with washing between runs with 0.1 M sodium hydroxide solution. Vacuum injection has been shown to offer the greatest reproducibility, due to the fact that better control over physical parameters can be maintained. The provision for a capillary wash at the beginning of every run minimizes migration time variability.

Absorption (mm)



A: first run

B: sixth run

Figure 4.4 Reproducibility of felinine in cat urine. First run and sixth run. Sample: No. 3 urine dilute 10-fold. Conditions: 20 mM NaH_2PO_4 (pH 2.0); 12 kV applied voltage; 75cm \times 75 μm i.d.(50 cm to detector) capillary; vacuum injection for 1sec; 200 nm wavelength; range 0.02; rise time 2.00

Table 4.1 Reproducibility of migration time and peak area of felinine in cat urine

No. of run	Migration time (min)	Mean \pm S.D. (min)	C.V. (%)	Peak area	Mean \pm S.D. (min)	C.V. (%)
1	32.2	32.37 \pm 0.12	0.35	120.7	120.23 \pm 0.77	0.64
2	32.3			121.3		
3	32.2			120.2		
4	32.4			118.5		
5	32.5			121.0		
6	32.5			119.7		

4.2.3.2 Standard felinine calibration plot

Quantitative reproducibility depends on the constancy of the calibration. When a quantitative method is first put into practice, the calibration should be repeated frequently. The calibration can be checked by measuring the slopes and Y -intercepts of the calibration plots and calculating the CV (%) of the slopes and Y -intercepts for several calibrations obtained over a period of time. Day-to-day precision was tested to check both the constancy of instrumental response to a given analyte and the concentration and migration time reproducibility, because the latter is a key parameter for peak assignment. For this purpose, standard felinine calibration was repeated six times over three months. The results show in **Table 4.2**, were used to determine the concentrations of felinine. The concentration range of felinine was from 0.05 mg/ml to 0.6-0.8 mg/ml. Felinine concentrations in female, castrated male and entire male urine diluted 10-fold were within this concentration range. Linear relationships between peak area and concentration of felinine were found for all plots tested within the concentration range of concern ($R^2 > 0.99$). The CV of the slopes and Y -intercepts were 2.32% and 24.8%, respectively.

As these calibrations had some variation at different periods of time. Felinine concentration in cat urine will be calculated with the calibration equations measured in the same period, and *standard felinine purity was assumed to be 100%*.

Table 4.2 Reproducibility of standard feline calibration plots
Conditions as Figure 4.5

Number of calibration	Regression equation	R ²	Average slope ± S.D.	Average Y- intercept ± S.D.
1	$y = 283.2x + 1.4788$	0.9934	280.30 ± 6.49	1.7017 ± 0.422
2	$y = 285.5x + 2.266$	0.9979		
3	$y = 265.8x + 1.6609$	0.9971	Slope CV (%)	Y-intercept CV (%)
4	$y = 289.05x + 1.900$	0.9981	2.32	24.8
5	$y = 284.33x + 0.6981$	0.9976		
6	$y = 273.93x + 2.2061$	0.9958		

4.2.4 Feline concentration in cat urine

Fresh cat urine was collected from different gender cats over a period of time. The urine was injected directly into the CE after filtration. Entire male urine was diluted 10-fold prior to injection. Using the calibrations shown above, feline concentrations were calculated and the results were showed in Table 4.3.

Table 4.3 Feline concentrations in cat urine

Urine sample	Gender of cat	Feline concentration (mg/ml)
1	Entire male*	4.36
2	Entire male*	2.75
3	Entire male*	4.54
4	Entire female	0.637
5	Castrated male	None
6	Castrated male	0.939
7	Castrated male	0.911
8	Castrated male	0.798

* same cat, urine collected at different times

It can be clearly seen from **Table 4.3** that there is a significant difference between entire males and castrated males but no much difference between castrated males and the female. This result is similar with that obtained by Hendriks (1995), who analysed 24 hour urinary excretion of felinine in a total of 28 entire and castrated cats with an HPLC method. The amount of felinine excretion from the same entire cat varied at different times, as the level of felinine depends upon the diet, the water intake and the period within the sexual cycle of the cat. Samples 1, 2 and 3 were from the same cat, but the levels of felinine were different as the urine was collected at different times. Three castrated males excreted similar amounts of felinine (0.798, 0.911 and 0.939mg/ml). Their urine also had similar CE electropherograms, as showed in **Figure 4.5A**. This result may prove that dietary and water intake factors have some influence on the urinary excretion of felinine as the three cats were fed with the same food in the period that the urine was collected. Another castrated male, who did not excrete felinine, had a different CE electropherogram. Spiked felinine was used to verify the absent of felinine in the urine (**Figure 4.5B**).

4.2.5 Recovery

Recovery is related to specificity and is determined by adding known amounts of an analyte to a real specimen and determining what percentage of the added analyte is detected (recovered). This should be as close as possible to 100 % (King, 1998). Recovery is an important issue for an analytical method as complete recovery is essential to the accuracy of the analysis and reproducibility of the method. In these experiments, a standard felinine calibration curve was used to quantify the concentration of felinine, and a standard addition method of felinine was used to verify the accuracy of the method and to calculate the relative recovery. The relative recovery of felinine was calculated by:

$$\text{Recovery \%} = C_x / C_0 + C_s \cdot 100 \quad (4.1)$$

where C_x is the concentration of felinine determined by CE after standard addition, C_0 is the felinine concentration measured by CE without standard addition, and C_s is the felinine concentration added (Che, 1995).

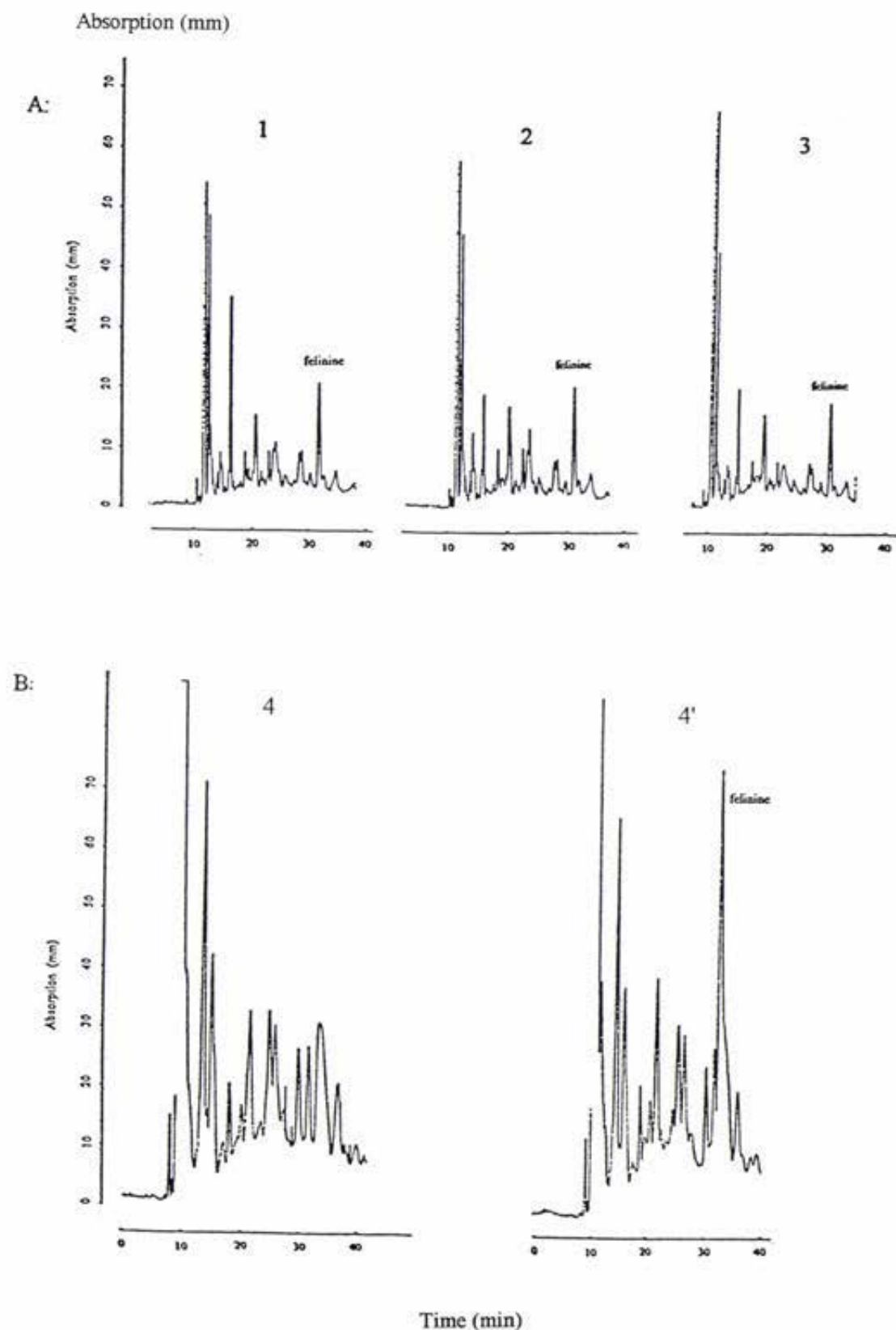


Figure 4.5 Electropherograms of castrated male cat urine.
 1- cat No.6; 2- cat No.7; 3- cat No.8; 4- cat No.5; 4'- cat No.5 spiked with feline.
 Other conditions as **Figure 4.1**

Number 2 standard felinine calibration and felinine recovery are showed in **Figure 4.6** and **Table 4.4**. The calibration equation was used as the recovery was verified at the same period of time. The mean recovery was 95.9% ($n = 6$, S.D. = ± 2.42 and C.V. = 2.54%), indicating that, within the limits of experimental error, full felinine recovery was attained.

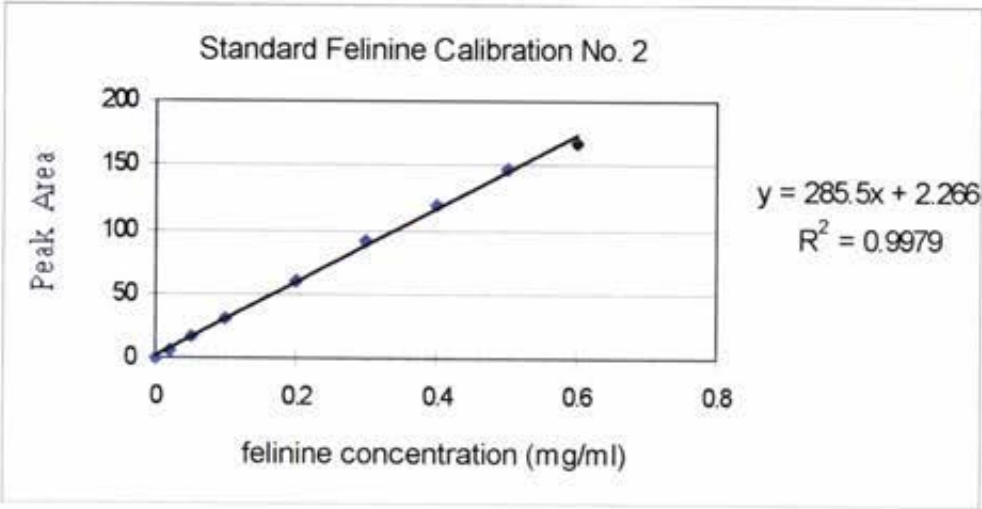


Figure 4.6 Number 2 felinine calibration

Table 4.4 The percent recovery of felinine

A_{fel} in urine (mm ²)	C_{fel} in urine (mg/ml)	standard C_{fel} added (mg/ml)	measured total A_{fel} (mm ²)	measured total C_{fel} (mg/ml)	percent recovery (%)
19.5	0.063	0.05	36.4	0.113	100.0
19.5	0.063	0.1	48.75	0.150	91.5
19.5	0.063	0.2	78.4	0.243	92.4
19.5	0.063	0.3	114.4	0.343	94.5
19.5	0.063	0.4	146.3	0.436	94.3
19.5	0.063	0.5	158.4	0.547	97.2

where A_{fel} is felinine area, and C_{fel} is felinine concentration.

4.2.6 Comparison the CE results with other methods

4.2.6.1 HPLC method

HPLC is widely used in serum and urine analysis and recently has been employed in felinine determination (Hendriks, 1995). As capillary electrophoresis is a new method for the determination of felinine in cat urine, it is necessary to compare the result with that of an established method. For HPLC analyses of felinine, a derivatisation method was deemed essential. There were two felinine derivative methods employed in HPLC analysis. They were the OPA-felinine and the BOC-felinine derivative. The OPA-felinine derivative with fluorescence detection has been used to determine the level of felinine in cat urine (Hendriks, 1995). The analyses were performed and the data reported from another laboratory (see Chapter Two). Another felinine method using the BOC-felinine derivative, was employed and the analyses were performed in our laboratory. The same urine samples were analysed using CE and HPLC at the same time, and the same standard felinine was used. The results of the concentration of felinine in cat urine with CE and HPLC analytical methods are shown in **Table 4.5**.

Table 4.5 Comparison of felinine concentration with CE and HPLC method

Urine sample	Felinine concentration (mg/ml)		
	CE method	HPLC method	
		OPA- felinine	BOC-felinine
No. 3	4.54	3.34	
No. 1	5.39		5.38
No. 4	0.637		0.633
No. 5	~ 0		~ 0

The comparison shows that felinine value in No.3 urine obtained with OPA-felinine method was lower than that obtained with CE method but not significantly different. This is probably because the procedure of making OPA-felinine derivative is both complicated and time consuming, so deterioration may occur. In addition this assumes that the synthesis of the derivative is 100% efficient. It should be noted that this was not a statistical analysis but an observation made on the basis of one measurement for the

comparison. The felinine values in No.1 and No.4 urine were almost the same with both CE and BOC-felinine method. The BOC-derivative method developed in our laboratory is reasonably simple and quantitative. By comparing the BOC-felinine area in urine with that of standard felinine, one can easily calculate the concentration of felinine, see **Figure 4.7**.

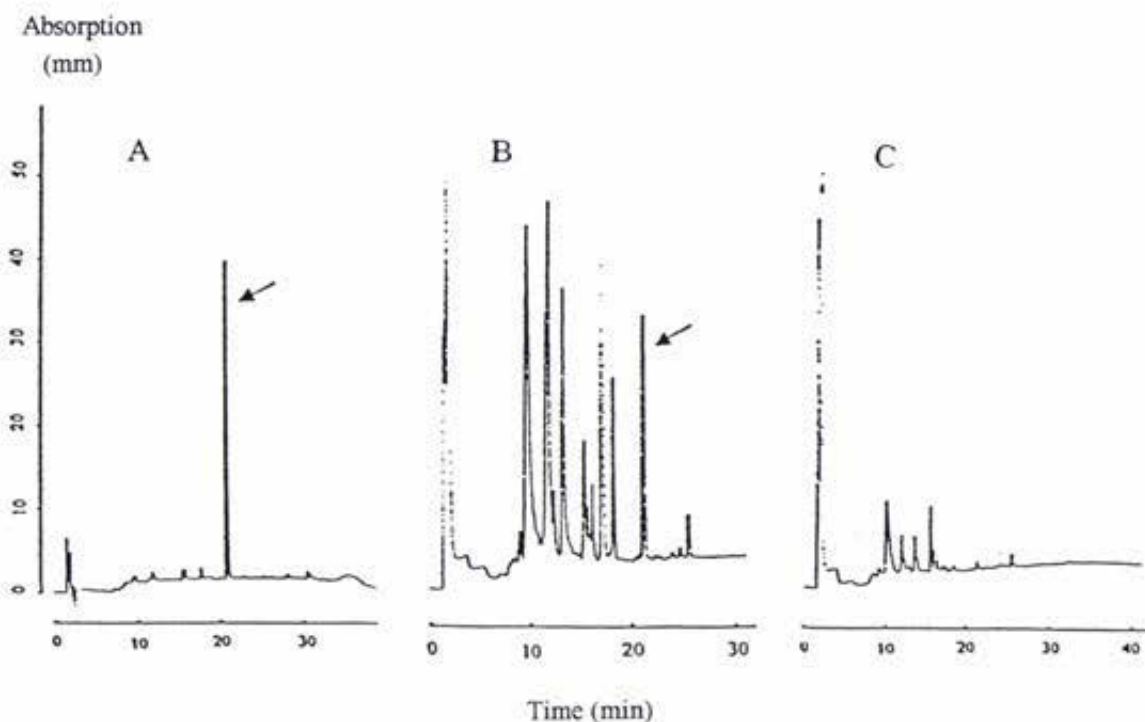


Figure 4.7 HPLC chromatogram of cat urine BOC-felinine derivative
A: 1 mg/ml standard felinine (50 μ l, peak area 27.3 mm^2)
B: No. 1 urine (100 μ l urine made 1ml derivative solution, inject 50 μ l, peak area 14.7 mm^2)
C: No. 5 urine
 * The felinine peak is indicated by an arrow.

The use of CE appears from this study to be readily applicable to the direct determination of felinine in urine. The direct determination of any compound without sample pretreatment is a desirable goal for any analytical technique. One does not need to be concerned with errors occurring during sample preparation. Good reproducibility and repeatability can be easily achieved. Great care must be taken with standard preparation and with dilutions. The comparison of the CE method with the HPLC method of determination of felinine in cat urine proved the reliability and precision of the CE method developed in this study.

4.2.6.2 TLC method

Earlier studies in the determination of felinine in cat urine used a TLC method. The TLC method performed in this study would give further evidence that felinine exists or does not exist in cat urine. As **Figure 4.8A** shows, a ninhydrin colour spot of felinine was separated from cat urine and appeared on the chromatography at a position similar to standard felinine. No ninhydrin colour spot of felinine appeared in **Figure 4.8B**, which indicated that no felinine is found in castrated male cat urine, at least within the limits of the TLC method.

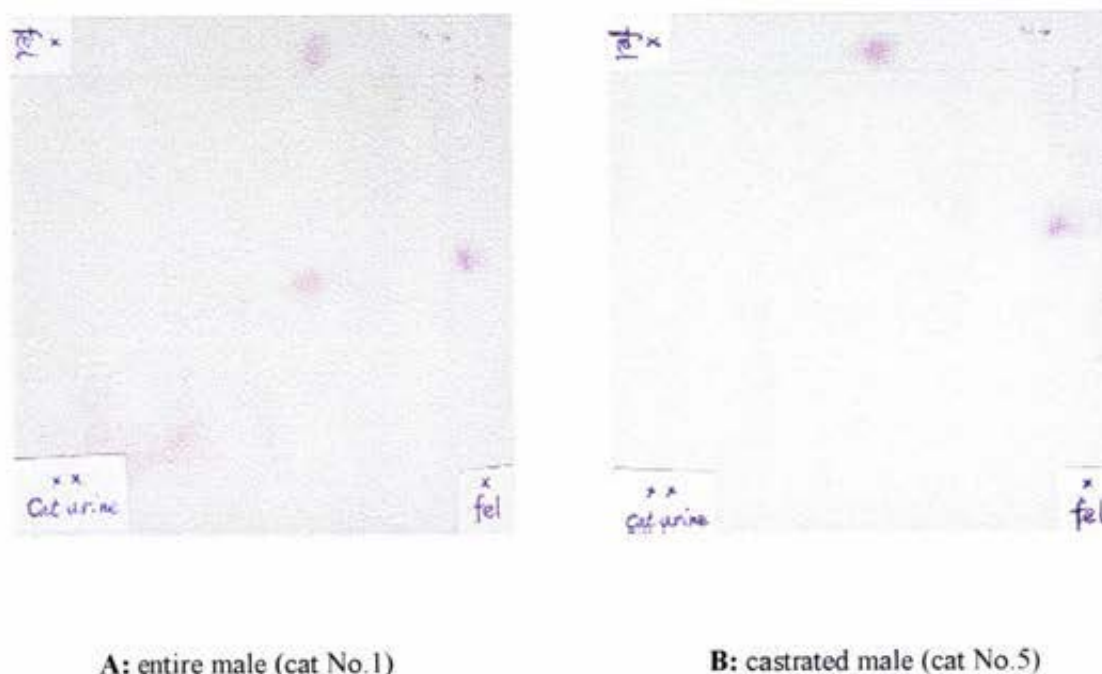


Figure 4.8 Thin layer chromatography of cat urine and felinine

4.2.7 Other experiments

The composition of normal cat urine has been studied by Worden and Waterhouse (1960). The levels of inorganic phosphate, inorganic sulphate, chloride, nitrogen, urea, creatine and creatinine were reported by these authors. Datta and Harris (1953) in no case found both felinine and taurine occurring in quantity in the same urine. Greaves and Scott (1960) found both taurine and felinine may be excreted in cat urine in considerable quantities at

the same time, but that on diets providing less than the minimum concentration of protein necessary to maintain nitrogen equilibrium, feline may be excreted preferentially to taurine.

Since high levels of taurine, urea, creatine and creatinine in cat urine were reported by these authors, some CE experiments were carried out to examine their potential for interference in the quantitation of feline. It was found that taurine and urea were not detectable under the analytical conditions of the CE feline assay. Spiking experiments were carried out with creatinine and creatine. The peaks with migration times at about 14 min and 18 min were found to be creatinine and creatine peaks. As we can see from the electropherogram, a large amount of creatinine existed in the cat urine. Even after the urine was diluted >50 times, creatinine was still detectable (**Figure 4.9**). Creatinine and creatine have no influence on the determination of feline as they elute much earlier than feline. Moreover, it is also possible to estimate creatinine in cat urine samples using this method (Guo, 1998).

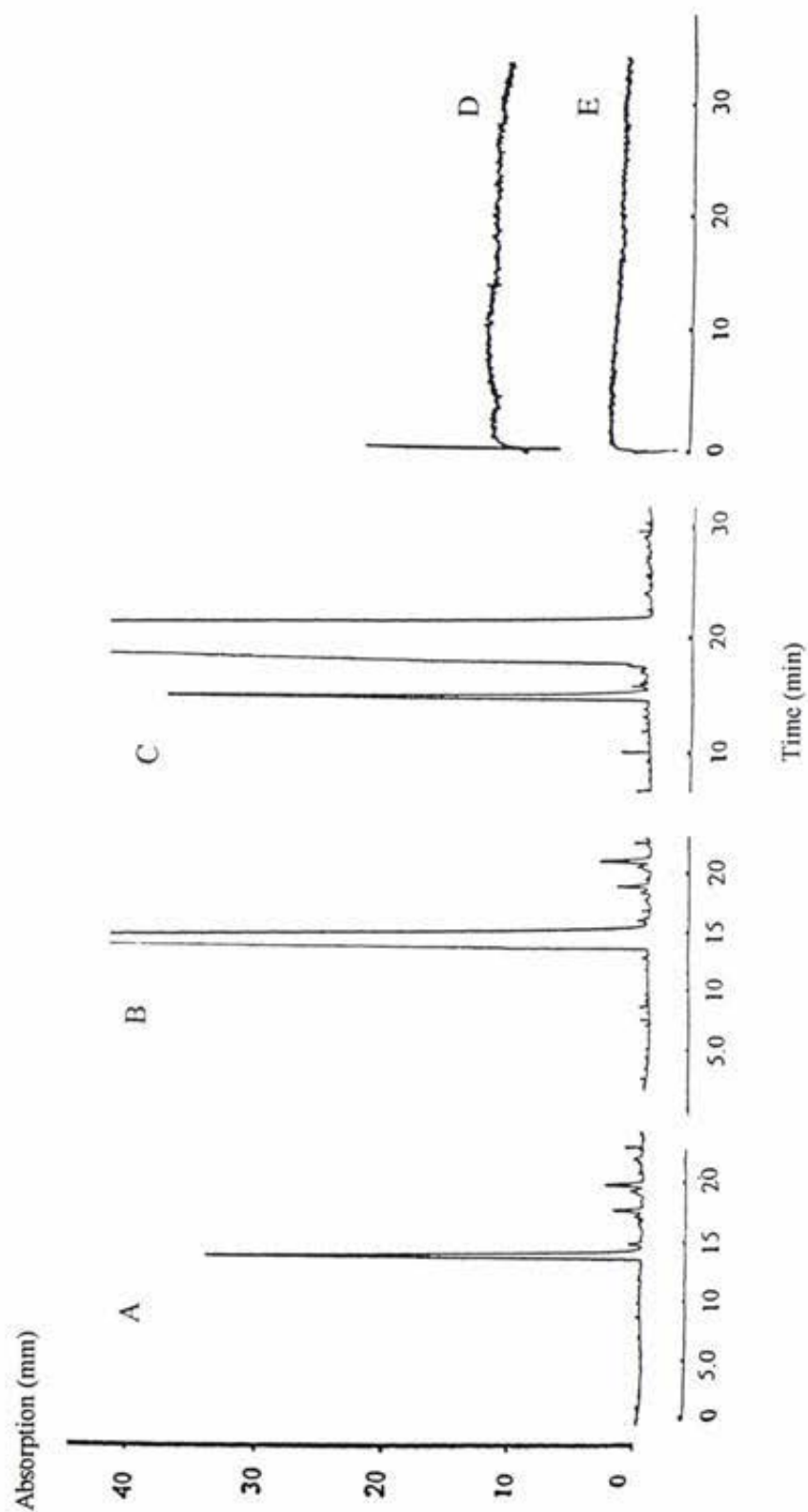


Figure 4.9 Electropherogram of cat urine diluted >50 times (A), cat urine spiked with creatinine (B) and creatine (C), taurine (D) and urea (E). Recorder range 0.10, other conditions as **Figure 4.1**.

4.3 Conclusion

A CZE method for the quantitative determination of felinine in cat urine has been developed. The method is sensitive, accurate and requires only small amounts of urine. No urine pretreatment was required except filtration. The high reproducibility of the felinine peak area, migration time and calibration plot demonstrates conclusively that the CZE method can be used for the routine analysis of felinine. The good recovery of felinine in cat urine further indicated that the method is quantitative. Using the method reported herein, I have been able to quantitatively determine levels of felinine in cat urine. I have reported the differences in concentration of felinine found in samples obtained from different genders of cat. The results obtained by this CE method are comparable with those obtained by HPLC and TLC methodology. This CZE method is ready to quantitatively analyse the levels of felinine in cat urine in any situation.

CHAPTER FIVE

FELININE STABILITY STUDY

5.1 Introduction

An important sulfur containing amino acid felinine, known to be present in large amounts in entire male cat urine, has been proposed as a male pheromone designed to attract the opposite sex (Hendriks et al., 1995b). Studies seem to indicate that felinine appears to attract female cats and also is used to mark the territory of a male cat. Some studies have been carried out on its characterisation, synthesis, excretion levels and biosynthesis etc (Westall, 1953; Trippett, 1957; Datta and Harris, 1953; Roberts, 1963; Avizonis and Wreston 1959; Hendriks et al., 1995). Oxidation, degradation and acid hydrolysis of felinine have been studied by Westall (1953) using paper chromatography. Joulain and Laurent (1989) observed that odourless “fresh” cat urine developed an odour upon “aging” due to the decomposition of felinine. It was also noted by Hendriks et al. (1995b) that odourless synthetic felinine degrades to another compound or other compounds to give rise to a distinct tom cat urine smell when felinine stored as a lyophilisate at -20°C and room temperature. Further study on the stability of felinine has not been carried out. More evidence is needed to show how felinine is converted to the pheromone.

In Chapter Four, a rapid, simple and accurate CE method was developed to quantify the level of felinine in cat urine. This method showed advantages above HPLC and other methods used by earlier researches and allows for further study on this amino acid. The aim of the study in this Chapter is to further research the stability of felinine using the CE method. The results obtained here may aid future researchers who are interested in felinine.

5.2 Results and discussion

5.2.1 Oxidation of felinine

Westall (1952) found that the position of felinine ninhydrin spot changed on the paper chromatogram after treatment with hydrogen peroxide due to oxidation of sulphur atom. He also found that felinine was partly oxidized by air during chromatography, and faint spots

were frequently observed on the chromatograms after treatment with ninhydrin.

In my study, oxidation of felinine was carried out by passing oxygen continually through a capillary into a solution of synthetic felinine for three days. The synthetic felinine assumed the odour of cat urine. Previous studies (Hendriks, 1995b) indicated that at least part of felinine had degraded. Felinine concentration was determined by the CE method every day. As **Figure 5.1** shows the peak area of felinine remained unchanged after three days, that is, felinine had not degraded significantly when contacted with oxygen. It therefore seems that very little material is needed to give the characteristic smell of tom cat urine.

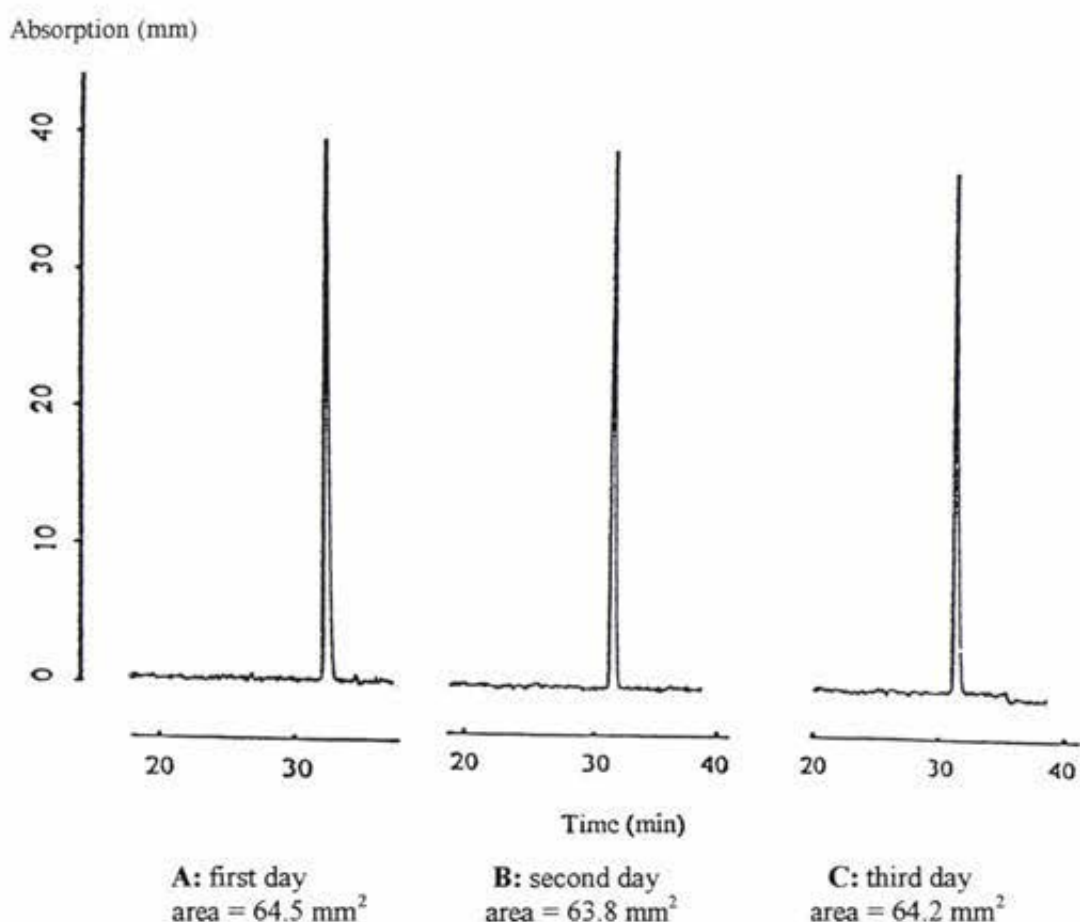


Figure 5.1 Synthetic felinine in oxygen
Conditions (These conditions apply throughout this study unless indicated otherwise): Buffer, 20mM NaH₂PO₄ (pH 2.0); voltage, 12 kV; wavelength, 200nm; capillary length, 75cm (50cm to detector); vacuum injection for 1 sec; recorder rise time, 2.00 and range, 0.02.

Cat urine containing felinine was also exposed to the atmosphere for five days and its stability examined. The urine dried out over night. Water was added to dissolve the

remnants to the starting volume. The sample was then analysed by CE. The same sample was kept contact with air, and the analysis experiment was repeated in the fifth day. The results were showed in **Figure 5.2**

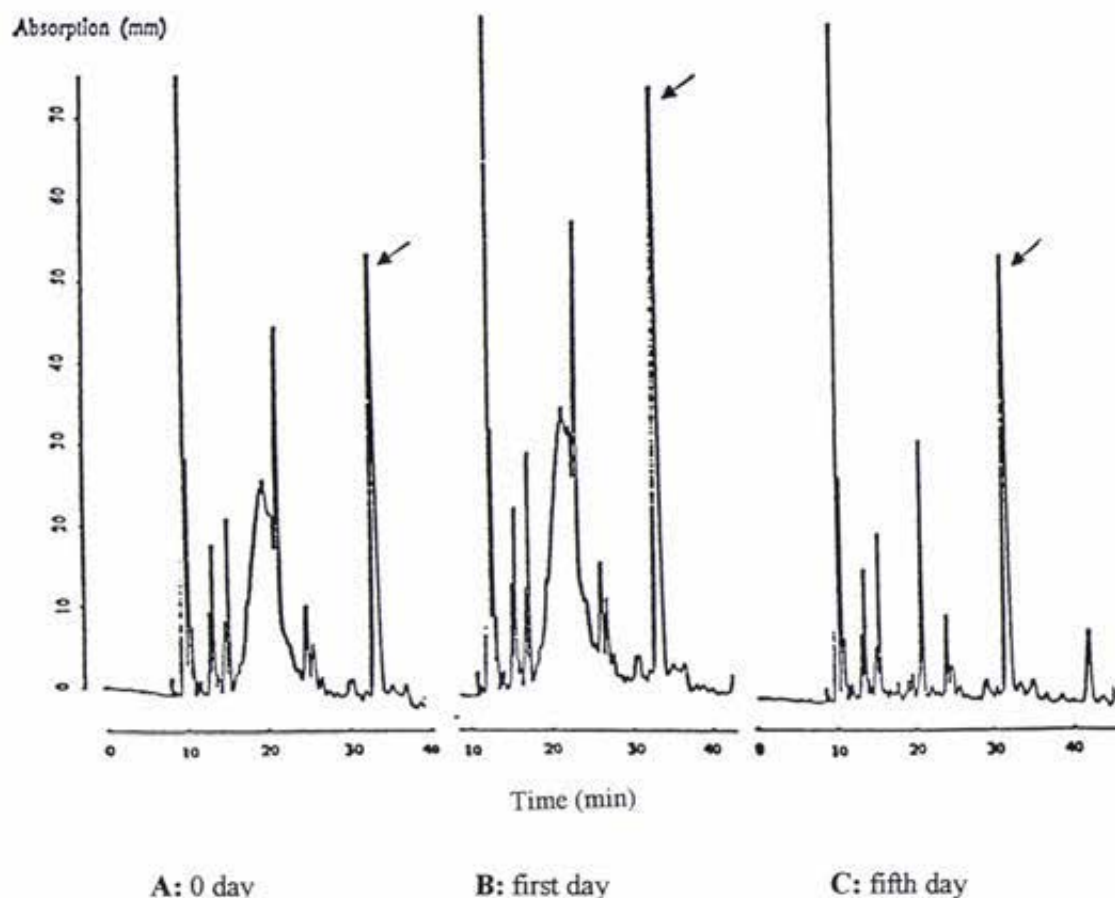


Figure 5.2 Electropherogram of urine exposed to the atmosphere
* The peak of felinine is indicated by an arrow.

From **Figure 5.2** the felinine concentrations in the urine seems to have decreased somewhat. They were 5.12 mg/ml in A, 5.04 mg/ml in B and 4.56 mg/ml in C. Many peaks in the urine were changed during the experiment especially after the fifth day, but felinine remained the same peak shape and migration time (32.5 min). Before the CE analysis was carried out on the fifth day, the urine sample had been dried three days but kept in the atmosphere. The concentration of felinine did not change significantly. This result shows that felinine in cat urine has very high stability. Exposure of the urine to the air at room temperature does not have much influence of felinine stability. There are at least three possible reasons. One is that felinine does not degrade significantly in oxygen and atmosphere. In other words, felinine is more stable than previously thought (Hendriks,

1995). A second reason might be that if the odour does come from felinine, the decomposition is very slow. In other words the decomposition is not readily seen by HPLC and CE under the conditions of my study. A third reason could be that the “felinine” odour in cat urine is not due to felinine at all but some other minor component in impure felinine (e.g. when in urine). The urine sample used in the experiment had been collected and stored in freezer for several days. The results obtained here were similar with those observed by Joulain and Laurent (1989), i.e. “fresh” cat urine was mostly odourless but upon “aging” an odour developed reaching a peak at ~ 20-24 hours. These authors suggested that two volatile compounds, 3-mercapto-3-methyl-1-butanol and 3-methyl-3-methylthio-1-butanol, found in cat urine, might be responsible for the cat urine odour. These two compounds originate from decomposition of felinine due to microbial activity and/or oxidation in air. There was no direct evidence presented to show that felinine degrades to these two compounds. The same result was found in synthetic felinine. After storage, some of the synthetic felinine degraded and a characteristic “catty” odour developed (Hendriks, 1995b). Perhaps once again the decomposition was due to microbial activity?

5.2.2 Storage stability

After collection and filtration, cat urine samples containing felinine were assayed by CE and the samples were then stored in three different ways: room temperature (about 20°C), refrigerator (about 5°C) and freezer (-20°C). Felinine in the stored urine was analysed at various intervals. Frozen urine was thawed before analysis. Filtration of the urine was needed once as precipitation had occurred. The odour of “felinine” was strong and did not disappear ever after storage for several months. The concentrations of felinine, which were detected and calculated by the CE method and felinine calibration curves described in Chapter Four, in these urine samples over three months time are shown in **Table 5.1A** (urine No.1) and **B** (urine No. 2)

Table 5.1 Felinine concentrations in male cat urine in different storage time and storage temperature

A: Urine No. 1

Store time (day)	Felinine concentration (mg/ml)	
	Store at 5°C	Store at -20°C
0	4.36	4.36
5	5.17	
17	5.56	4.56
24	5.23	
35	4.66	4.51
60	4.79	4.45
80	4.56	4.18
90	5.02	4.65

B: Urine No. 2

Store time (day)	Felinine concentration (mg/ml)	
	Store at 20°C	Store at 5°C
0	2.75	
6	2.17	2.53
8		2.67
11	2.29	
25	2.38	2.63
30	2.55	2.86
40	2.10	2.20
70	2.42	2.32
Overall mean	2.38	2.54
S.D.	±0.166	±0.185
C.V. (%)	7.0	7.3

Storage time

Some interesting results are seen in **Table 5.1A**. Firstly, the concentration of felinine in urine No. 1 increased in the first half month to 20 days after collection, then decreased to a

certain concentration and remained almost unchanged when kept at 5°C. One reason for the initial increase might be that there was some felinine existing in combination with other chemicals in the urine that CE could not determine. It has been suggested (Tallan et al., 1954) that felinine may be formed in the cat kidney, where it may exist in a bound form, and be excreted directly. After storage, free felinine is released from the bound form and is revealed as an increase in felinine concentration by CE. Another possible reason is that there were some chemicals, which can produce felinine, excreted in the urine and the reaction occurred to give more felinine after storage. This result was not found in urine No. 2. The level of felinine in urine No. 2 kept almost constant at 20°C and 5°C for 70 days after collection. The average of felinine in urine No. 2 for 70 days was 2.38 (20°C) and 2.54 (5°C) mg/ml, standard deviation (S.D.) was ± 0.166 (20°C) and ± 0.185 (5°C), C.V. (%) was 7.0 (20°C) and 7.3 (5°C).

Figure 5.3 shows the electropherograms of urine No.1 at different storage times. As we can see from these electropherograms changes occurred obviously due to the changing of other substances in the urine after storage for some time, but the peak shape of felinine remained the same. It was also found that during the storage, some cotton-like precipitate (perhaps mould) occurred in the urine over a period of time. No further experiments were done in my study to check what the substances were produced by the mould.

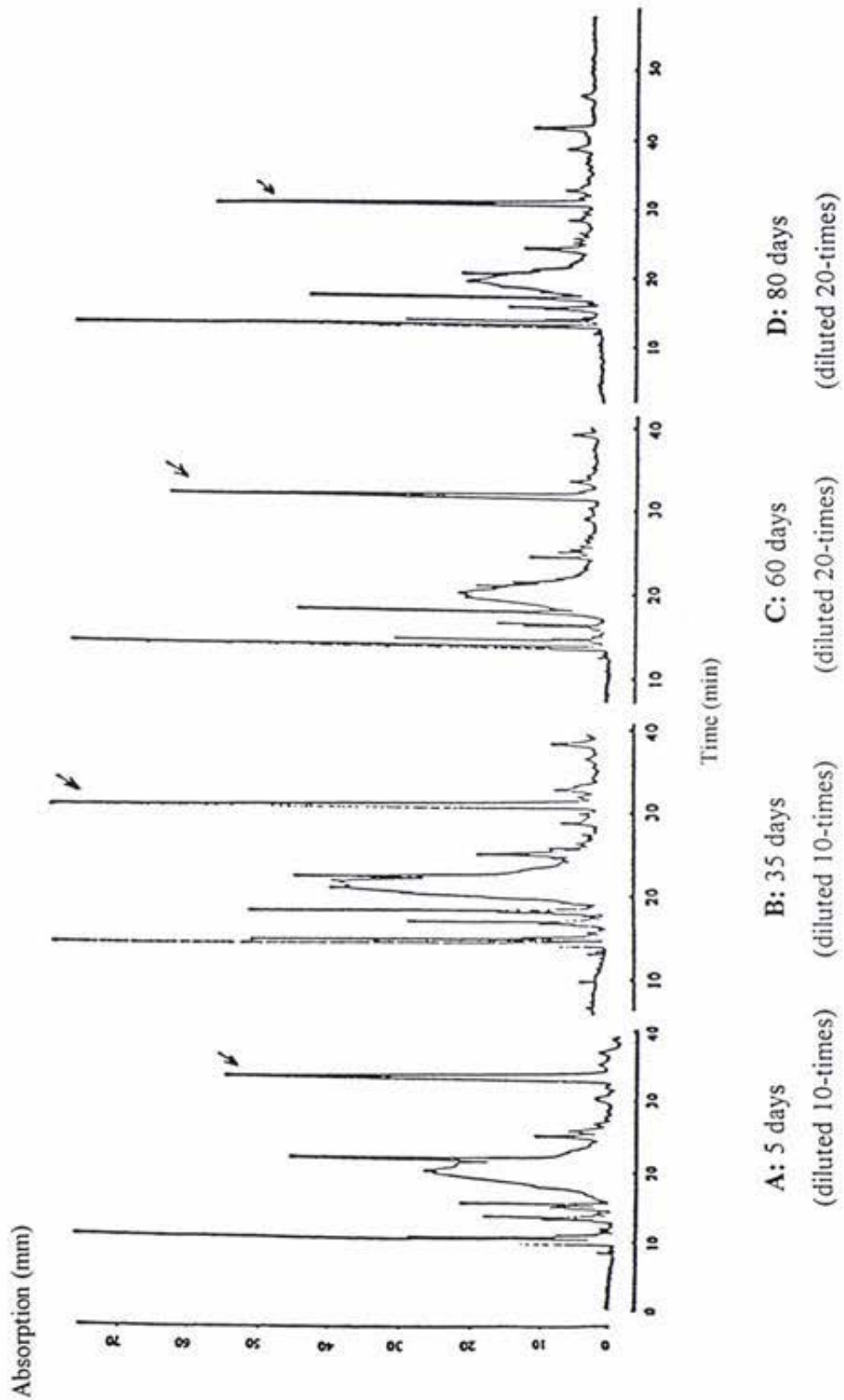


Figure 5.3 Urine electropherograms after storage
 Sample: urine No. 1; storage temperature, 5°C; other conditions as **Figure 5.1**
 * The peak of felinine is indicated by an arrow.

Storage temperature

Storage temperature appears to have no significant influence on felinine stability. Felinine concentrations in urine No.2 were almost the same when kept at 20°C and 5°C. The same result was found in urine No.1 after storage at 5°C and -20°C for 35 days. Minor differences between felinine levels at different storage temperatures were probably caused by sample dilution and peak area measurements. Unfortunately, the level of felinine in urine No.1 stored at -20°C can not be compared with that at 5°C before 35 days, as it was only determined once. **Figure 5.4** shows the different changes of felinine in urine No.1 at two different store temperatures. The influence of temperature on felinine will be further discussed next.

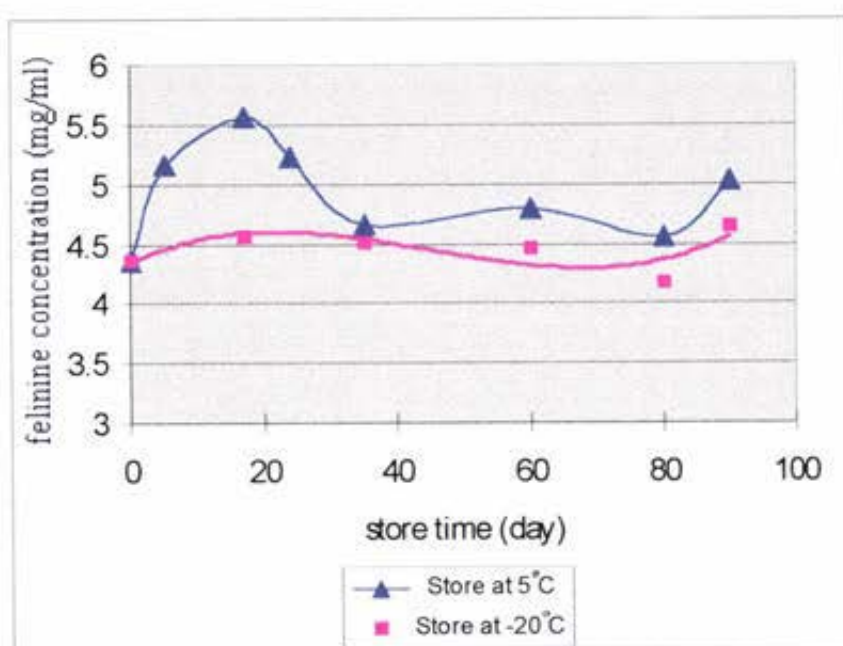


Figure 5.4 The changes in felinine concentration in cat urine No.1 at two different storage temperatures

5.2.3 Influence of temperature on felinine stability

5.2.3.1 Felinine in cat urine

As temperature could influence the stability of felinine in urine, the determination of felinine at different temperatures was deemed necessary. The experiments were carried by

heating the urine at different temperatures (55°C, 70°C, 85°C and 100°C). N-(NO₂)arg was chosen as the internal standard. The same amount of N-(NO₂)arg was added to the cooled solutions after the heating had ceased. The concentrations of felinine in the urine were analysed and showed in **Table 5.2** and **Figure 5.5**. The area of internal standard N-(NO₂)arg ($C = 0.1\text{mg/ml}$) was measured and represented as the ratio of $A_{\text{fel}} / A_{\text{N-(NO}_2\text{)arg}}$ in **Table 5.2**.

Table 5.2 Felinine concentrations at different heating temperature and time

Heating Time (hour)	55°C heating	70°C heating		85°C heating		100°C heating	
	C_{fel} (mg/ml)	C_{fel} (mg/ml)	$\frac{A_{\text{fel}}}{A_{\text{N-(NO}_2\text{)arg}}}$	C_{fel} (mg/ml)	$\frac{A_{\text{fel}}}{A_{\text{N-(NO}_2\text{)arg}}}$	C_{fel} (mg/ml)	$\frac{A_{\text{fel}}}{A_{\text{N-(NO}_2\text{)arg}}}$
0	0.387	0.267	1.02	0.359	1.42	0.253	0.764
0.5		0.353	1.28	0.335	1.38	0.180	0.592
1.0	0.497	0.335	1.25	0.252	1.16	0.059	0.196
1.5				0.243	0.917	0.027	0.089
2.0	0.489	0.319	1.16	0.182	0.688	0.018	0.057
3.0	0.457	0.309	1.16	0.086	0.375		
4.0		0.262	1.01	0.050	0.200		
5.0	0.446	0.244	0.98	0.024	0.145		
7.0	0.373						
23.0	0.333						
24.0		0.057	0.29				

where C_{fel} is the concentration of felinine, A_{fel} is the peak area of felinine, $A_{\text{N-(NO}_2\text{)arg}}$ is the peak area of N-(NO₂)arg. Urine No.1 sample was heated at 55°C, urine No.2 was heated at the other temperatures.

A similar result was found from urine heated at 70°C and 55°C. The felinine concentration increased after heated half to one hour, and then decreased. It took 1 hour to reach the peak at 55°C but only 0.5 hour at 70°C. In the storage temperature study above, a wide peak was also found in urine No.1 stored at 5°C after 15-20 days. It seems likely that higher temperatures can lower the time needed to reach the maximum felinine concentration (for example, see **Figure 5.5**, 55°C trace), that is, bound felinine (if it exists) releases faster at

higher temperatures as might be expected.

High temperature increases the degradation of felinine. Felinine concentration decreased dramatically when it was heated at 100°C. It was almost below the detection limit after 2 hours. Felinine concentration dropped to a low level in 7 hours at 85°C and 24 hours at 70°C. The ratio of $A_{\text{fel}} / A_{\text{N-(NO}_2\text{)arg}}$ showed the same increase and decrease as the felinine concentrations,

The temperature of 55°C did not have any significant influence on the degradation of felinine over the time period studied. After 22-hours of heating, the concentration of felinine changed from 0.497 to 0.333 mg/ml. Most of the felinine remained unchanged, only small amount of felinine had degraded over this period.

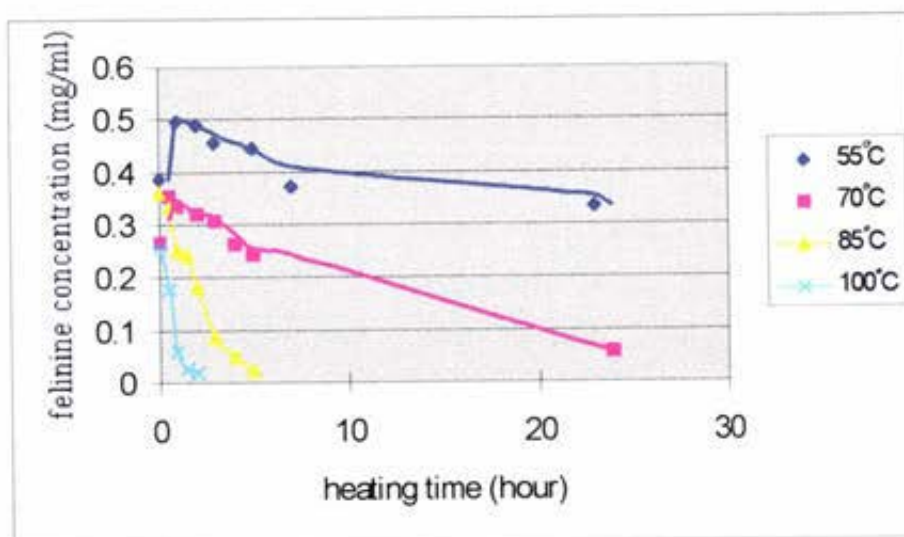


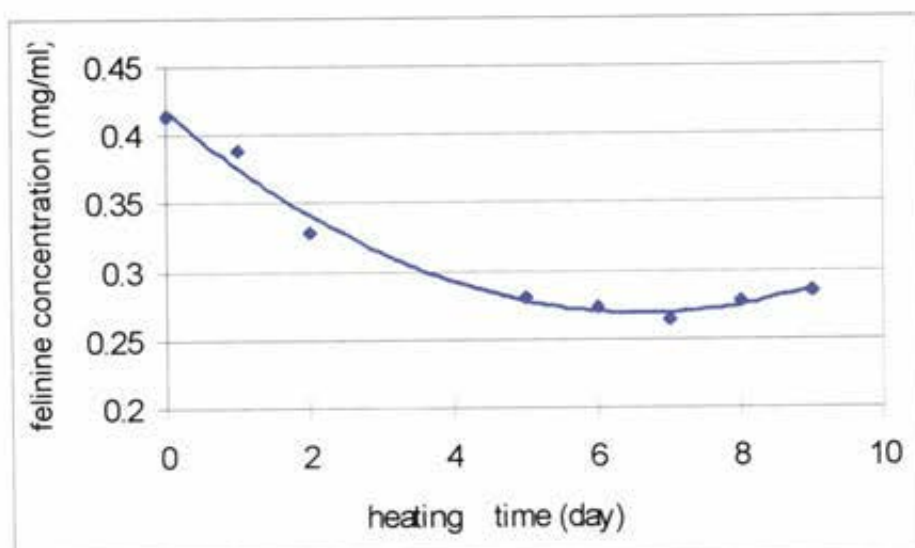
Figure 5.5 Cat urine heated at different temperatures

5.2.3.2 Synthetic felinine

Synthetic felinine was also heated at 100°C to examine its stability. The result is shown in **Table 5.3** and **Figure 5.6**

Table 5.3 100°C heating, synthesis felinine standard only

Heating Time (day)	0	1.0	2.0	5.0	6.0	7.0	8.0	9.0
Felinine Concentration (mg/ml)	0.414	0.389	0.329	0.280	0.273	0.265	0.278	0.285
$A_{\text{fel}} / A_{\text{N-(NO}_2\text{)arg}}$	1.680	1.587	1.576	1.075	0.985	1.258	1.282	1.263

**Figure 5.6** Synthetic felinine standard dissolved in water only heated at 100°C

There was a significant difference observed between synthetic felinine only and felinine in cat urine for the stability study at 100°C. When cat urine was heated at 100°C, most of the felinine was degraded in one hour, but the concentration of synthetic felinine changed only a small amount (0.025 mg/m) after heating at 100°C for 24 hours. The reason might be that the felinine reacted fast with other chemicals in cat urine at high temperature that sped up the degradation. In contrast, the degradation of synthetic felinine alone was much slower when it was heated at 100°C. In this latter study the solution contained only water, felinine and any degradation products as formed.

The concentration of felinine had dropped to the lowest level by the seventh day, then it increased slightly in the eighth and ninth day (**Figure 5.6**). The reason is probably due to experimental variation. Some solvent might have evaporated from the solution when heated for long time, resulting in a more concentrated felinine solution. The ratio of the

changing felinine peak area to N-(NO₂)arg peak area also showed the same result.

5.2.4 Degradation of felinine

5.2.4.1 Felinine in acidic conditions

The pH of cat urine is normally around 6.5 (pH 6.2 for urine No.6 and 6.5 for urine No.2). Most of felinine is stable in these pH values (see 5.2.2). Degradation of felinine can occur at certain concentrations of acid. It was suggested that in the hydrolysis of urine, a large amount of cysteine comes from the decomposition of felinine (Tallan, Moore and Stein, 1954). In an attempt to explain the series of degradations which occurred when felinine was hydrolysed with acid, Westall (1953) suggested that under the treatment with acid the felinine broke down with the liberation of free cysteine. The cysteine then condensed with the unchanged felinine eliminating water and forming a di-cysteine derivative.

In my study, different hydrolysis conditions were applied to cat urine and synthetic felinine to exam the degradation of felinine.

110°C hydrolysis

Treatment of cat urine No.1 and synthetic felinine with 6M HCl and phenol at 110°C, 24 hours (standard amino acid analysis conditions) destroyed the felinine and no felinine remained which would give a peak on CE electropherogram (**Figure 5.7**).

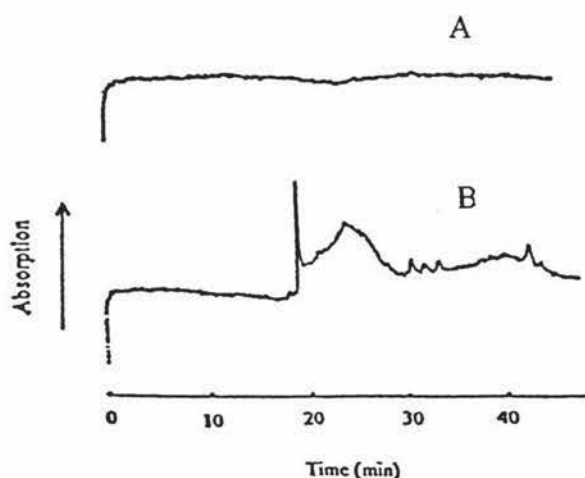


Figure 5.7 Synthetic felinine (A) and cat urine (B) hydrolysed at 110°C, 24 hours

In my previous study (unpublished), cysteine and L-cystine were detectable under the conditions of the CE experiment while cysteic acid hydrate did not give any peak. It was more likely that under high temperatures and concentrated acid, the felinine decomposed to cysteine and cysteine then was further oxidized to cysteic acid. Tallan et al. (1954) have confirmed the existence of cysteic acid when felinine was hydrolysed.

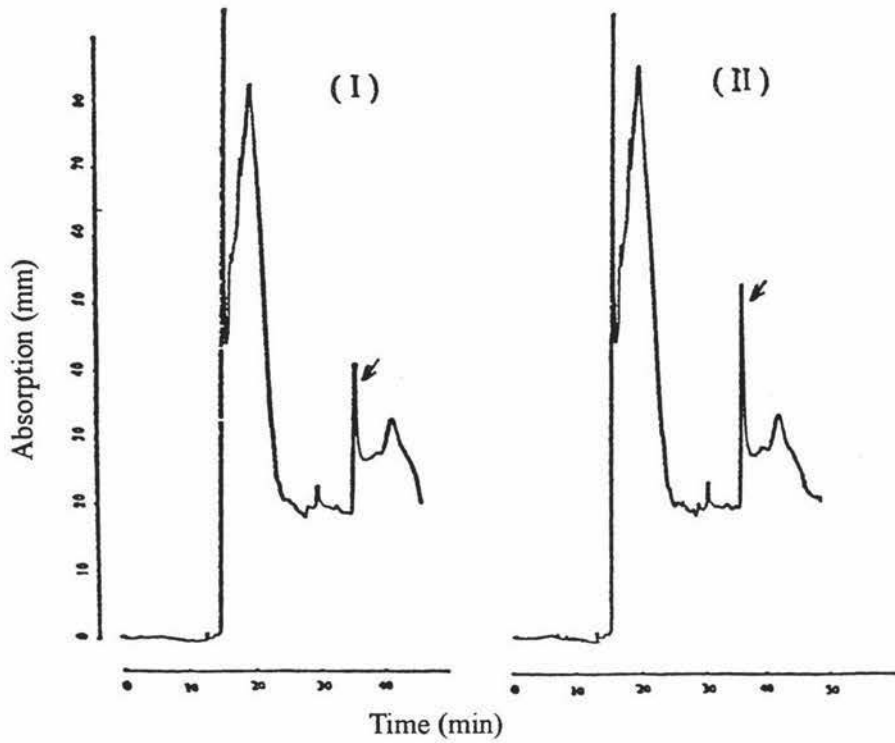
Room temperature hydrolysis

Hydrolysis of urine No.1 was performed with 3 M, 4 M and 6 M HCl at room temperature. Treatment of cat urine No.6 and synthetic felinine with 1 M or 0.5 M HCl was also carried out. **Table 5.4** shows the results of these hydrolyses.

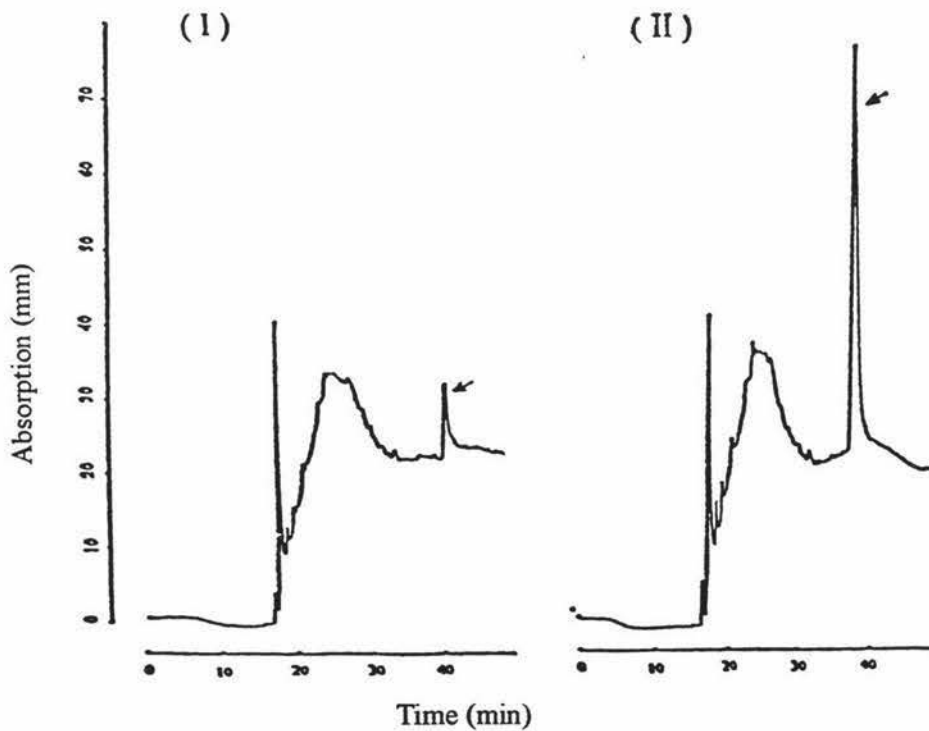
Table 5.4 Conditions of urine hydrolysis (with added phenol and β -ME)

Total volume (μ l)	No. of urine	Amount of urine (μ l)	Amount of felinine (mg)	HCl (37%) (μ l)	HCl (M)	H ₂ O (μ l)
1000	1	500	~2.5	500	6	0
750	1	500	~2.5	250	4	0
600	1	300	~1.5	150	3	150
1000	6	500	~0.3	250	3	250
1000	6	500	~0.3	83.5	1	416.5
1000	6	500	~0.3	42.0	0.5	458.0

Felinine in urine No.1 was not degraded completely even at the stronger HCl concentrations, The sharp non-symmetrical felinine peak with migration time 35.0 to 40.0min was identified by spiking with synthetic felinine (**Figure 5.8**). The migration time of felinine was longer after the hydrolysis. The typical felinine peak was not obtained for urine No.6, with a wide non-symmetrical peak instead appearing after hydrolysis (**Figure 5.9**). This peak was similar with the one from hydrolysis of synthetic felinine under 0.5 M HCl condition (**Figure 5.10**).



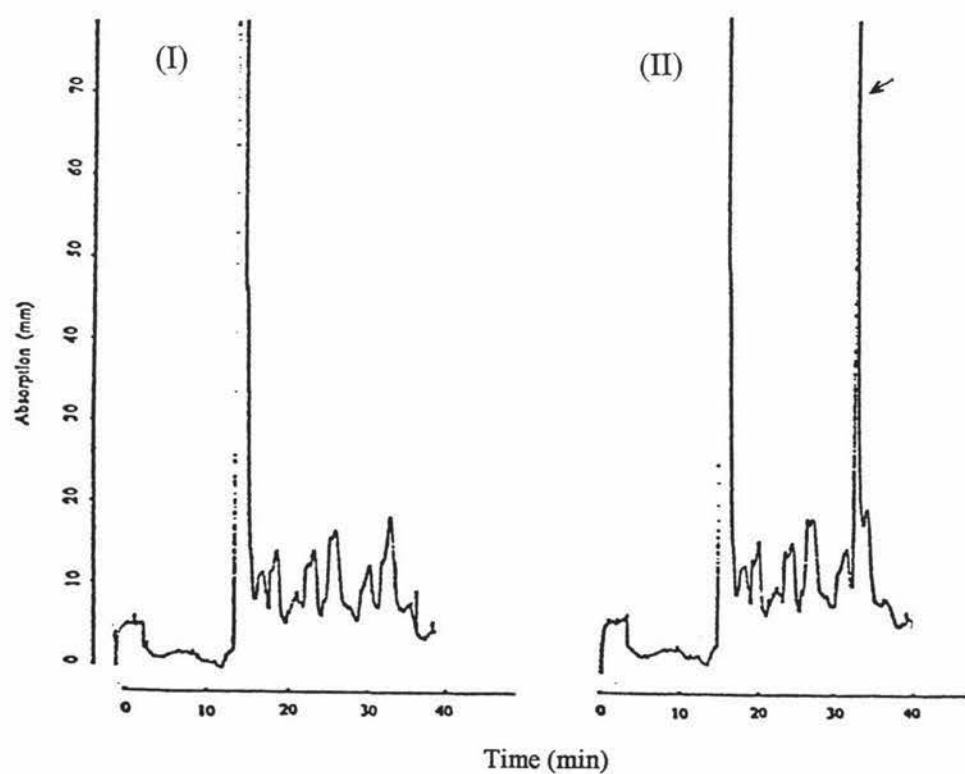
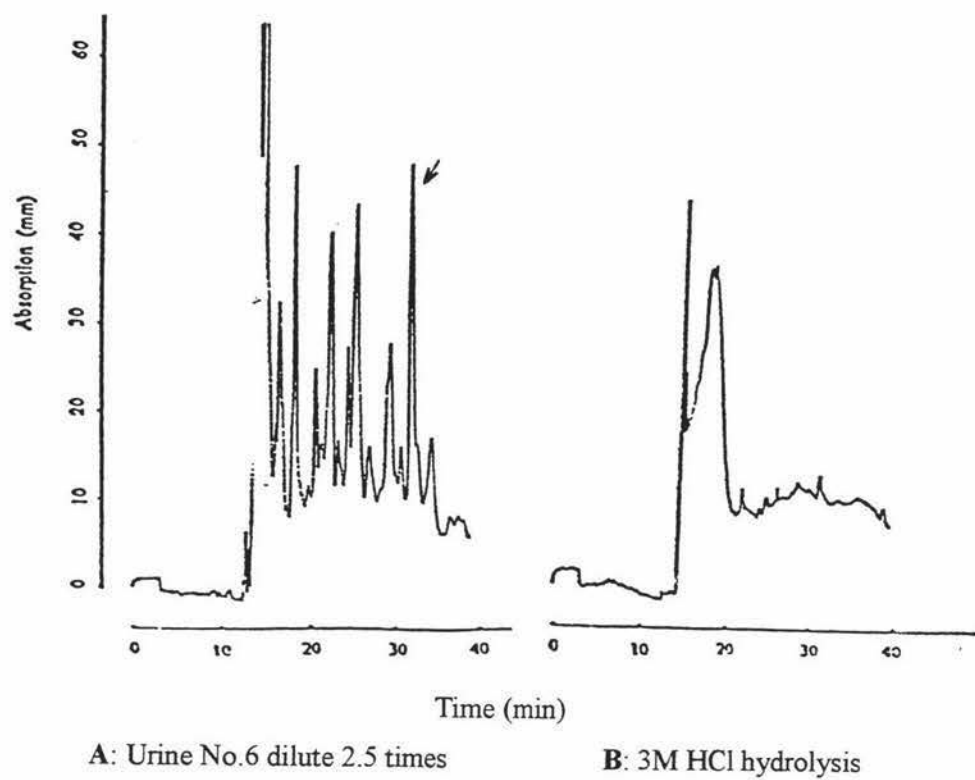
A: 4M HCl (I) and spiking with felinine (II)



B: 6M HCl (I) and spiking with felinine (II)

Figure 5.8 Electropherogram of hydrolysis urine No.1 in 4M (A) and 6M HCl (B)

*The felinine peak is indicated by an arrow.



C: 0.5 M HCl (I) and spiking with felinine (II)

Figure 5.9 Electropherograms of urine No.6 (A) hydrolysis in 3M (B) and 0.5M HCl (C)

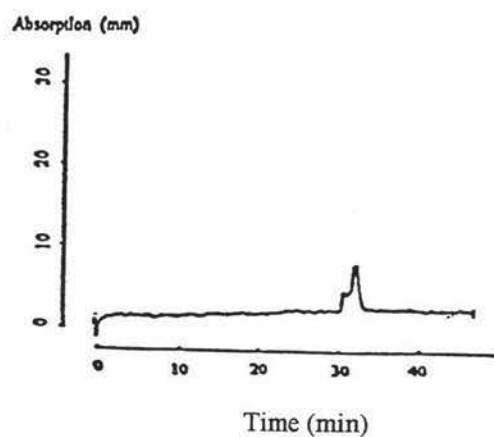


Figure 5.10 Electropherogram of felinine hydrolysis in 0.5M HCl

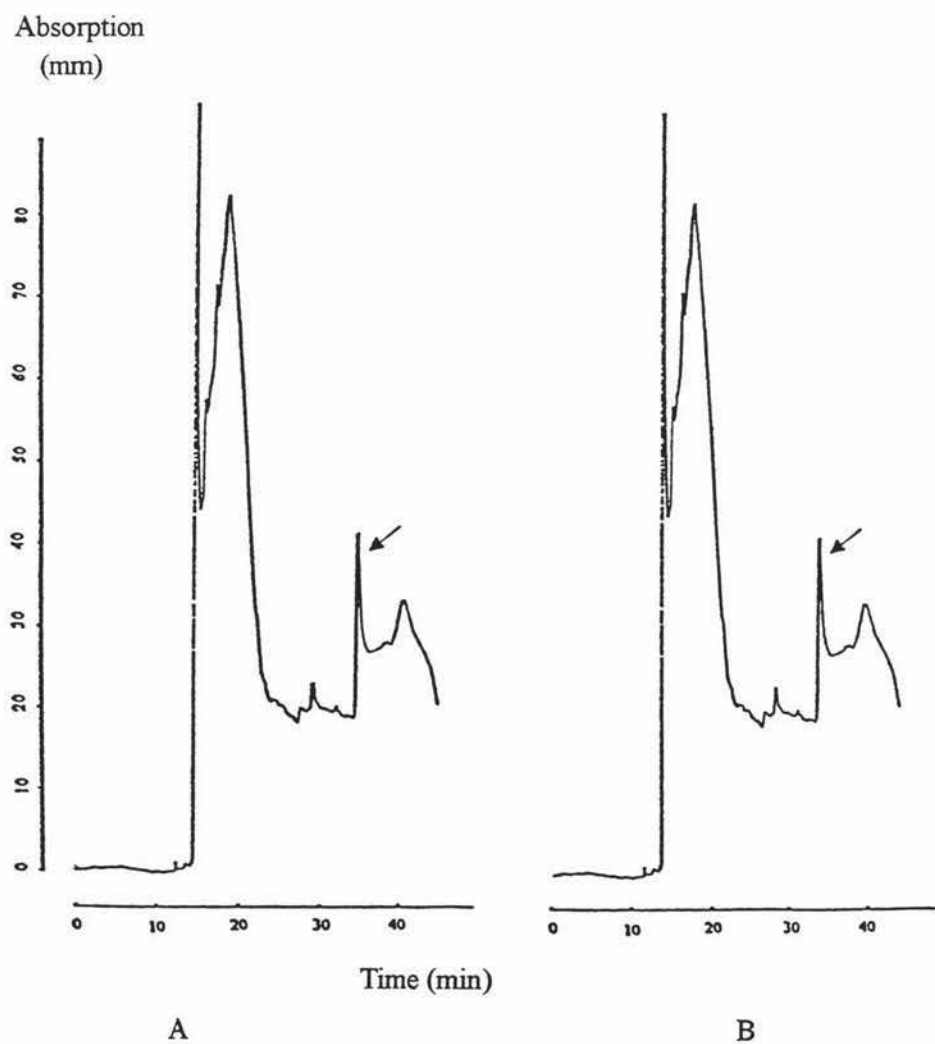


Figure 5.11 Influence of hydrolysis times
Urine No.1 in 4M HCl one day (A) and five days (B)

Experiments have been done to examine the influence of hydrolysis time. It was found that the hydrolysis occurred quickly (less than one day). Samples reacted 2h and 24h; one day, two days or five days gave the same CE electropherogram (**Figure 5.11**):

5.2.4.2 Felinine in alkaline solutions

Felinine can also be degraded under alkaline condition. Westall (1953) found when felinine was treated with 2 M sodium hydroxide at 100°C, the amino acid was destroyed and no substance remained which would yield a ninhydrin positive spot. In my study, moderately alkaline conditions were employed to further examine the stability of synthetic felinine and felinine in cat urine.

Felinine in NH₄OH

Comparison of synthetic felinine in 0.5M NH₄OH (pH \approx 11) and water showed that felinine is stable in this alkaline solution. The amount of NH₄OH does not influence the stability of felinine (**Table 5.5**).

Table 5.5 Stability of synthetic felinine and cat urine in 0.5 M NH₄OH (pH \approx 11)

Amount of urine No.2 (μl)	Amount of synthetic felinine (mg)	Amount of NH ₄ OH (μl)	Amount of H ₂ O (μl)	Felinine migration time (min)	Felinine peak area (mm ²)
0	0.1	0	200	32.5	139.5
0	0.1	200	0	32.2	138.6
0	0.1	0	600	32.2	53.2
0	0.1	600	0	32.5	48.9
0	0.1	0	1000	32.5	33.2
0	0.1	1000	0	32.8	33.6
100	0	0	900	32.5	84.0
100	0	900	0	32.5	84.0

Felinine in NaOH

Treatment of felinine with NaOH solution (pH 7.8 and pH 9.2) at room temperature for 40 hours showed that felinine was still stable. No significant change was observed from the CE electropherogram. 2 M NaOH had some influence on felinine. A wider, lower and non-symmetrical peak was found instead of the usual sharp felinine peak after treatment with 2 M NaOH for 1 hour at room temperature. Spiking with felinine after 2 M NaOH treatment gave a double peak (**Figure 5.12**). There were two possibilities. One was felinine decomposed to another substance, which could not be fully separated by the CE under the analytical conditions. The other was felinine did not degrade in the alkaline condition and the distortion of the peak shape was due to the changing of ionic strength and concentration of the sample at the higher pH.

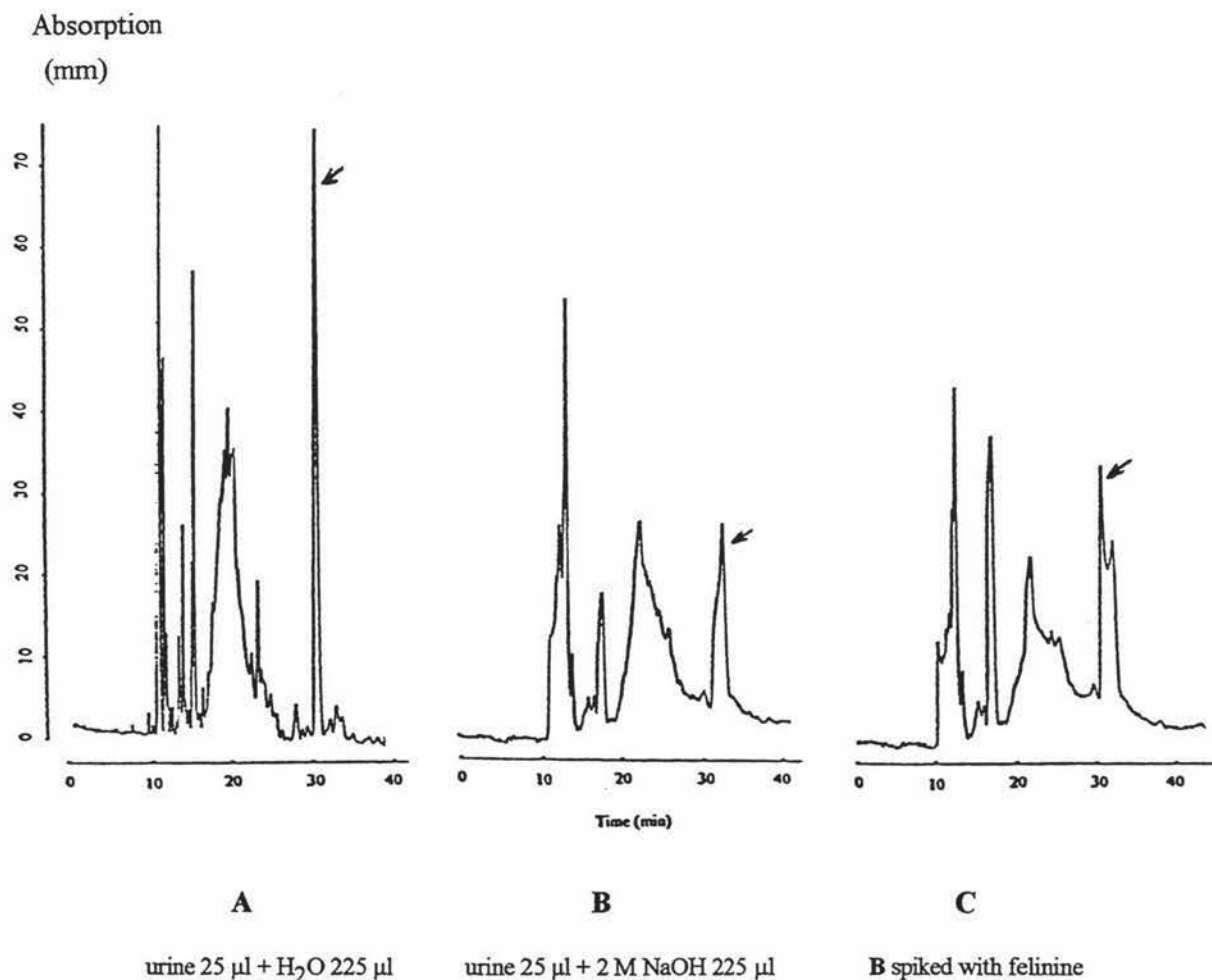


Figure 5.12 Urine No.1 in 2 M NaOH solution

*The felinine peak is indicated by an arrow.

Felinine in cat urine was partly destroyed when treated with 0.5 M and 1M NaOH solution. The felinine peak also became wider after the treatment (**Figure 5.13**). Synthetic felinine seemed stable in 1 M NaOH. The felinine peak area remained almost the same after treatment even though the peak became wider (**Figure 5.14**).

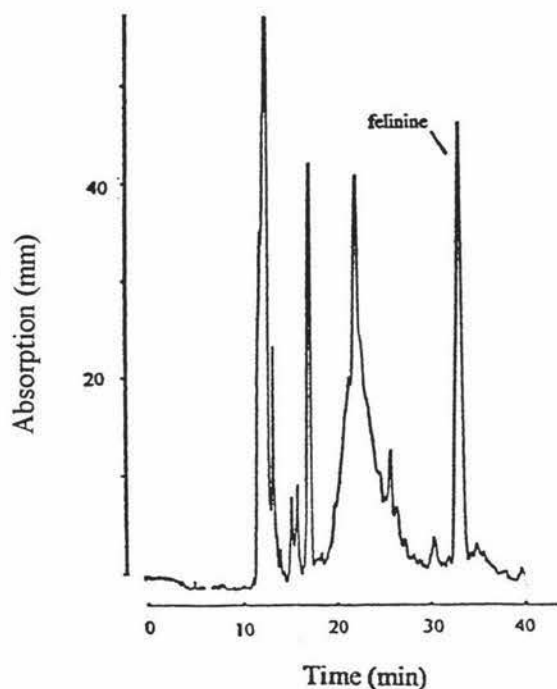


Figure 5.13 Urine No.1 in 1 M NaOH solution (urine 25 μ l + 1M NaOH 225 μ l)

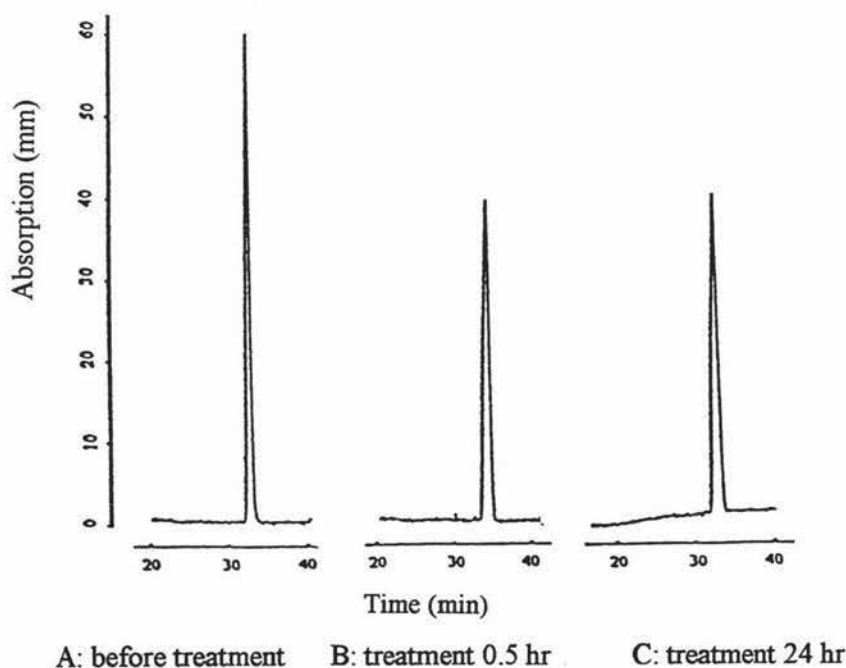


Figure 5.14 Synthetic felinine in 1 M NaOH. Felinine peak areas are A: 145.8mm², B: 147.6 mm² and C: 149.2 mm².

The peak distortion of felinine after treatment with sodium hydroxide was due to the increase of ionic strength and concentration. In Chapter Three, the influence of buffer concentration and ionic strength has been discussed. As pointed out by Mikkers (1979) in early work on capillary electrophoresis, the ionic strength of the sample must be less than 1% of the ionic strength of the separation buffer to avoid distortion of the band shape. This distortion is associated with isotachopheresis; the high ionic strength analyte produces region of high conductivity and low electric field leads to distortion of the peak shape. The distortion of the peak shape depends on the relative mobility of analyte and the buffer components. Analytes that have higher mobility than the separation buffer ions will generate peaks that demonstrate fronting (peak **A** in **Figure 5.15**). Analytes that have the same ionic strength as the buffer will produce symmetrically broadened peak shape (peak **B** in **Figure 5.15**). Analytes with lower mobility than that of the buffer components will demonstrate tailing (peak **C** in **Figure 5.15**).

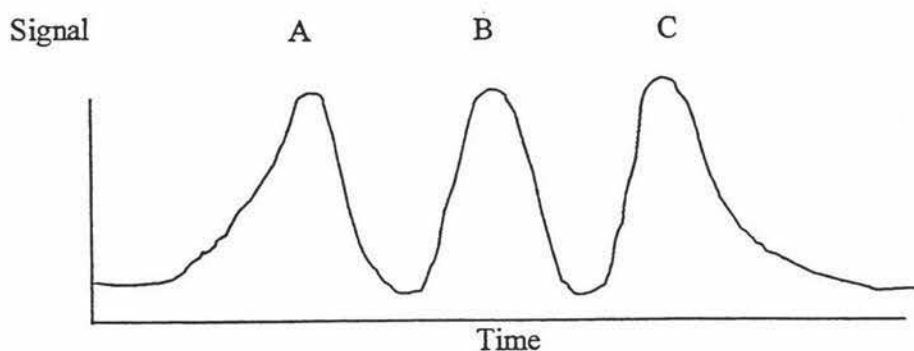


Figure 5.15 Peak distortion at high analyte concentration (Camilleri, 1993).

5.2.5 Felinine in human urine

Mizuhara and Oomori (1960) found an amino acid ($C_8H_{15}O_4NS$) containing cysteine residue which was isolated from the urine of an atherosclerotic patient. The chemical structure of this amino acid is considered to be quite similar to that of felinine, but no evidence was given that felinine exists in the human body. Assuming felinine was unstable in the human body, one would then not be able to find any trace of it in human urine.

Figure 5.16 is the CE electropherogram diluted (1:1) health human urine (pH = 6.2) and added felinine. No typical felinine peak was found in the urine. Felinine was then added into the urine to exam the stability. As for the study of the stability of felinine in cat urine, some similar experiments (storage and heating) were carried out.

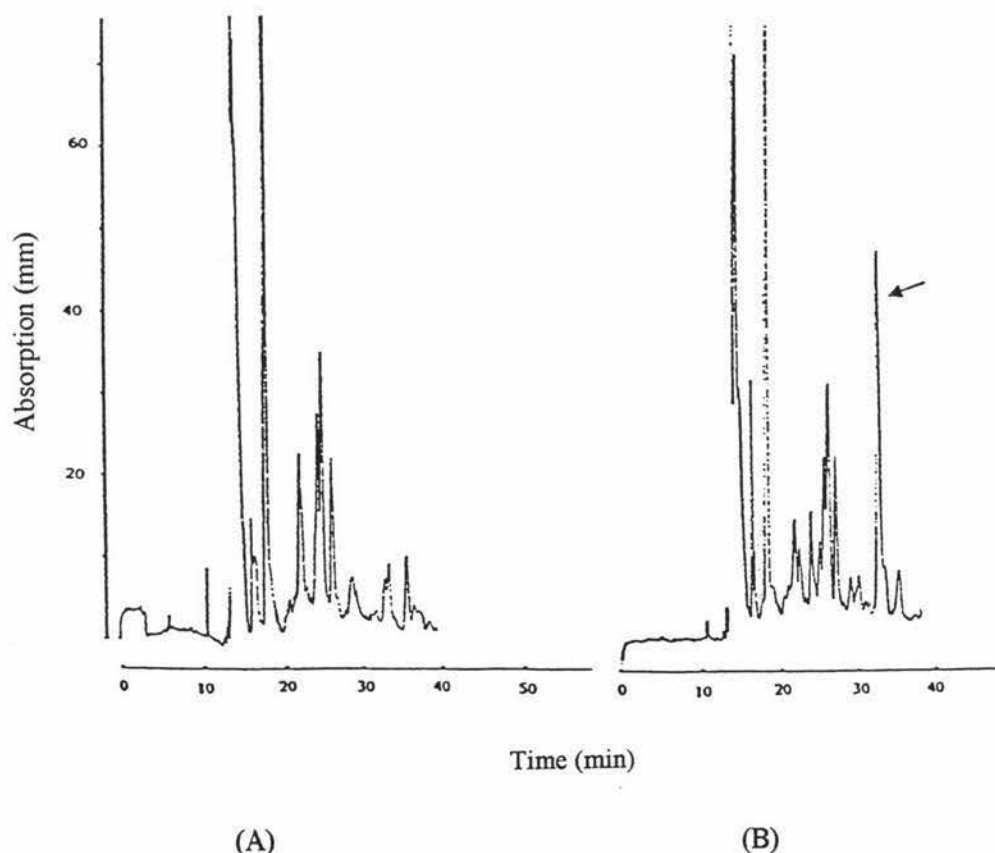


Figure 5.16 Human urine (A) and human urine spiked with felinine (B)

Storage stability

Felinine in human urine was as stable as in cat urine. The concentration, peak shape and migration time of felinine remained unchanged after storage at room temperature for a one-

month period.

100°C heating

Like felinine in cat urine, felinine in human urine was found to be almost completely destroyed in a short time (1.5hr) when heated at 100°C (**Table 5.5**).

Table 5.6 Felinine stability in human urine at 100°C

Heating time (hr)	Felinine concentration (mg/ml)	$A_{\text{fel}}/A_{\text{N-(NO}_2\text{)arg}}$
0	0.301	1.01
0.5	0.102	0.388
1.0	0.023	0.105
1.5	≈ 0	0

Felinine was not found in human urine but nevertheless it had similar stability to that in cat urine at 100 °C.

5.3 Conclusion

The stability of felinine under different conditions has been investigated. The storage temperatures and times had no significant influence on felinine stability. Felinine degradation occurred at higher temperatures. The higher the temperature, the faster the degradation of felinine. The concentration of felinine in cat urine increased and reached a maximum concentration during the storage and heating at low temperatures. The stability of synthetic felinine is much higher than felinine in cat urine. Strong acid and alkaline conditions increase the speed of degradation of felinine while felinine is relatively stable at mild acid and base conditions. No felinine was found in human urine. The stability of synthetic felinine in human urine is similar to that in cat urine.

CHAPTER SIX

FELININE IN PLASMA AND SERUM STUDY

6.1 Introduction

Felinine is a sulfur-containing amino acid excreted in a large amounts in entire male cat urine. It has been suggested by Tallan et al. (1954) that felinine may be formed in the cat kidney and be excreted directly. By using an ion exchange chromatographic method, these authors found only traces of felinine in plasma and bladder tissue, and none in kidney, liver or brain tissue. They found no further evidence for the existence of felinine in cat blood. Westall (1953) was unable to detect any felinine in ultrafiltered blood plasma. Datta and Harris (1953) failed to demonstrate the presence of felinine in blood plasma. Recently, Hendriks (1995) confirmed the findings of Westall (1953), Datta and Harris (1953) by using modern HPLC for the analysis of ultrafiltered tomcat plasma.

An accurate CZE method was developed to determine the level of felinine in urine (Chapter Three and Chapter Four). The aim of the study in this Chapter was to analyse for felinine in blood plasma and serum with the aid of this CZE method and compare the results to an HPLC method. Some further stability studies of felinine in blood plasma and serum were also carried on.

6.2 Results and discussion

6.2.1 CZE method

Small organic molecules, including amino acids, small peptides, bases and water-soluble vitamins, can be separated by capillary zone electrophoresis (CZE) in untreated fused-silica capillaries. When CZE is applied to the separation of multicharged proteins, however, adsorption becomes a serious problem (Lee and Heo, 1991). The adsorption of protein may interfere with the analysis. It was found that plasma and serum concentration has an influence on the elution time and peak shape of felinine and other substances in plasma and serum. When the dilution of plasma was lower, or the concentration of plasma was higher, the elution time of added felinine and other substances in plasma was longer and the

felinine peak shape became lower and wider. To eliminate interference from proteins, a plasma sample should be extensively diluted (normally at least 20 times with water before analysis) or the proteins removed before analysis.

Felinine in cat plasma

Entire male cat and female cat plasma was diluted 20 time and applied directly to the CE. Spiking with felinine was employed to identify felinine peak. As **Figure 6.1** shows, no trace of felinine was found under the analytical conditions developed in Chapter 3 and Chapter 4.

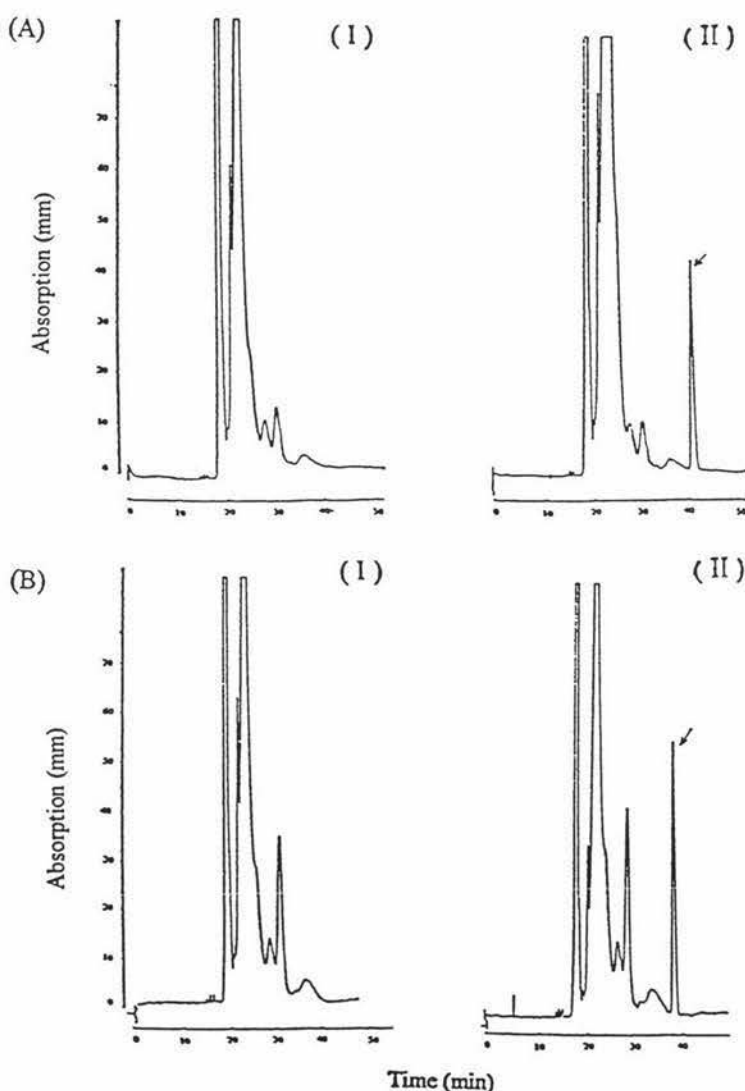


Figure 6.1 CE electropherograms of cat plasma. A: entire male cat plasma (I) and spiked with felinine (II); B: female cat plasma (I) and spiked with felinine (II). Conditions: capillary: 75 μm \times 75 cm (50 cm to detector); buffer: 20 mM NaH_2O_4 (pH 2.0); applied voltage: 12 kV; vacuum injection for 1 sec; 200 nm wavelength; recorder range 0.02, rise time 2.00. (*The peak of felinine is indicated by an arrow.)

Felinine in human plasma and serum

Diluted human plasma and serum with spiked felinine were also analysed on the CE. The result was showed in **Figure 6.2**. No significant difference was found when felinine in cat plasma and human plasma and serum.

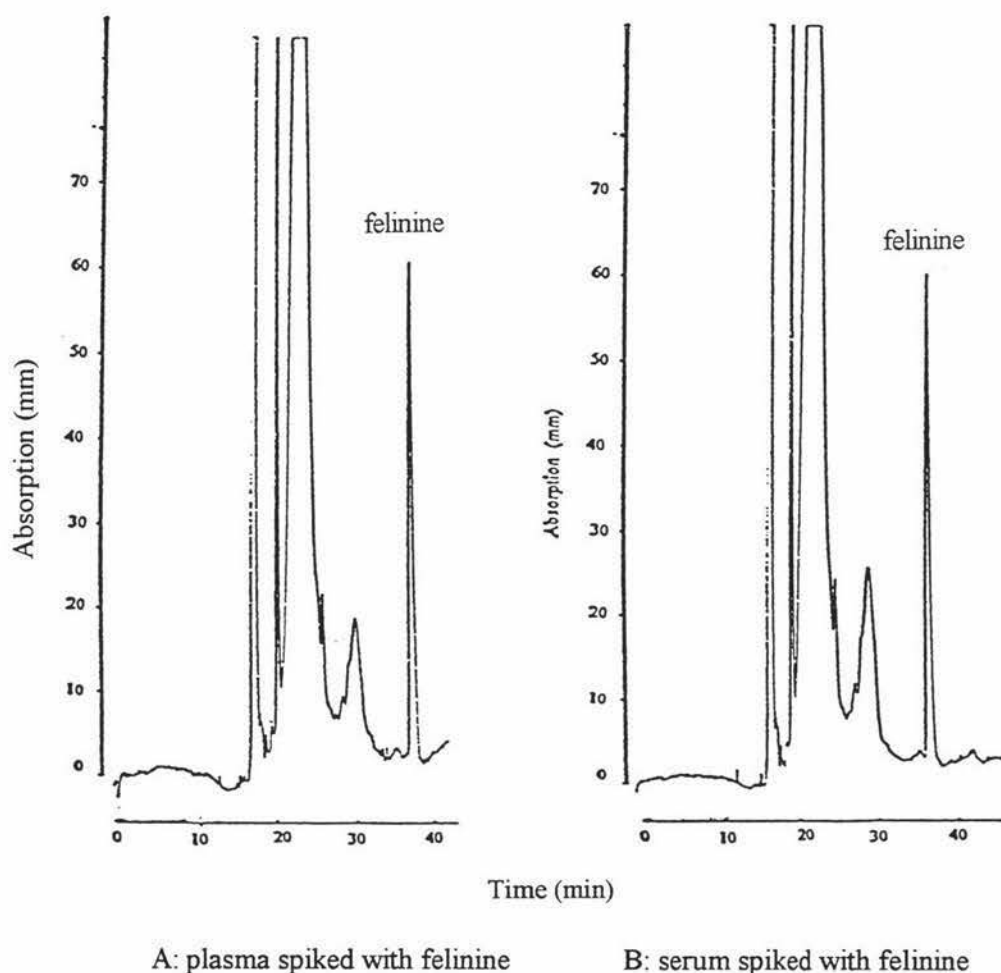


Figure 6.2 CE electropherograms of human plasma (A) and serum (B) spiked with felinine. Plasma diluted 25 times, serum diluted 20 times, other conditions as **Figure 6.1**

Sample stacking

The main limitation of CE is its low sensitivity. One way to enhance the sensitivity of the technique is to concentrate the solutes before electrophoresis. Stacking is a method of sample concentration (Baker, 1995). Stacking in CE is usually carried out with the sample solution having a lower conductivity than the 'running' buffer. Under these circumstances,

a disproportionate percentage of the overall voltage drop occurs across the sample plug. Because the electric field is very high, sample molecules migrate very rapidly until they reach the leading interface between the sample buffer and the 'running' buffer and here the concentration occurs. This means that a large volume (>15 nL) of a dilute solution can be concentrated and detected as a sharp band. As a rule, sample volumes using hydrodynamic injection should be limited to no more than about 2% of the total capillary volume. Attempting to inject larger volumes can cause perturbations of the electric field and result in loss of resolution (Applied Biosystems, 1990).

As cat plasma was diluted more than 20 times with water, its conductivity was relatively low. An injection time of 2 sec was applied to concentrate the sample. As **Figure 6.3** shows, no felinine trace was found.

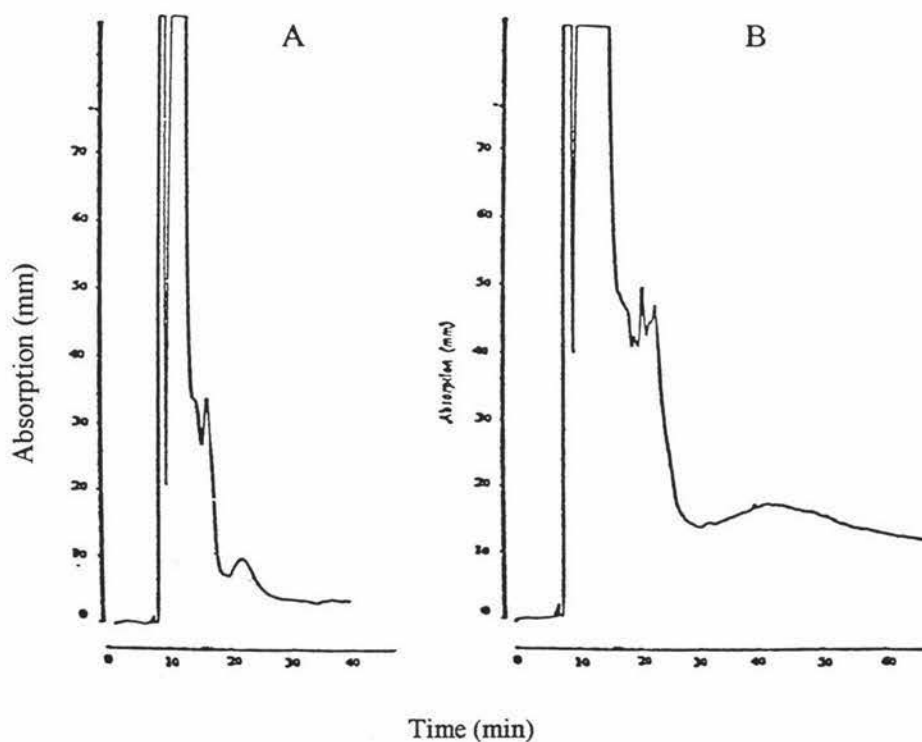


Figure 6.3 Influence of injection time.

Sample: cat plasma. Conditions: capillary 47 cm (30cm to detector), applied voltage 10 kV, injection time: A: 1 sec; B: 2 sec, other conditions as **Figure 6.1**.

6.2.2 HPLC derivative BOC method

The HPLC BOC derivative method has also been employed for the study of felinine in cat plasma. As for cat urine, 100 μ l male and female plasma were taken to make 1 ml of BOC

derivative which was then analysed by HPLC. Injection volumes of 50 μl and 180 μl were compared. The results are shown in **Figure 6.4** for A (male cat plasma) and B (female cat plasma). It is obvious that no felinine exists in cat plasma from a simple comparison with the HPLC chromatogram of felinine-BOC derivative (**Figure 6.4 C**).

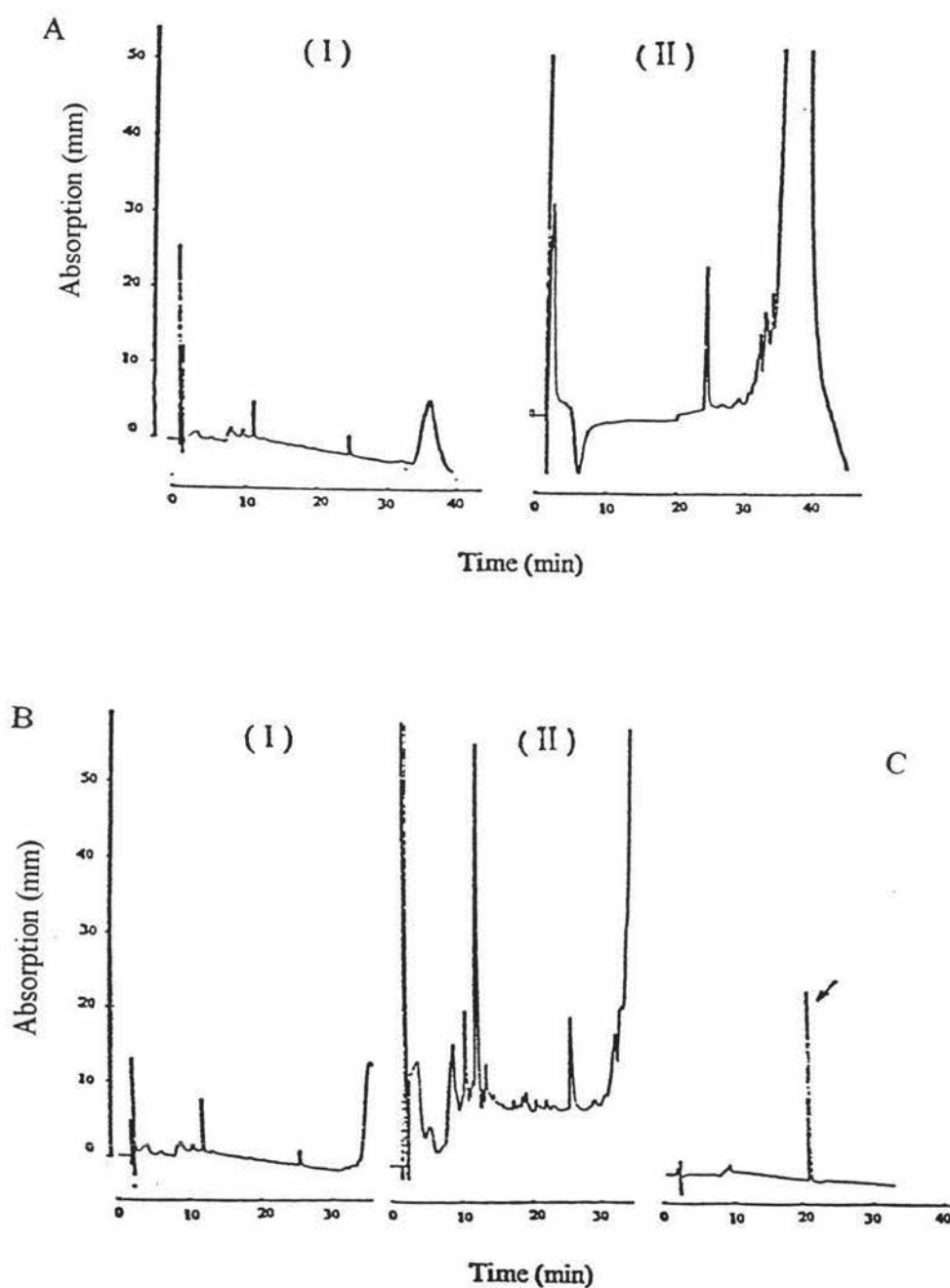


Figure 6.4 HPLC chromatograms of the cat plasma BOC-felinine derivative
 A: male cat plasma, inject 50 μl (I) and 180 μl (II)
 B: female cat plasma, inject 50 μl (I) and 180 μl (II)
 C: 1 mg/ml synthetic felinine, inject 50 μl

6.2.3 Recovery

To examine the accuracy of the CZE method for the determination of felinine in cat plasma, a complete recovery is essential. As for cat urine, a standard felinine calibration curve was used to quantify the concentration of felinine, and a standard addition method of felinine was used to verify the accuracy of the method and to calculate the relative recovery. It was found when the concentration of plasma was higher (diluted 10 times), felinine recovery was not satisfactory. Good recovery (98.8-107.8%) was achieved when plasma was diluted 35 times. The amount of felinine added and the recovery are showed in **Table 6.1**

Table 6.1 The recovery of felinine in male cat plasma

Added amount of standard felinine (mg/ml)	Measure amount of felinine (mg/ml)	Recovery (%)	Mean recovery (%)
0.1	0.0988	98.8	101.7
0.2	0.1945	97.3	
0.3	0.3046	101.5	
0.4	0.4319	107.8	
0.5	0.5145	102.9	

6.2.4 Stability of felinine in plasma and serum

In Chapter Five, the stability of felinine in cat urine at different temperatures was discussed. The stability of felinine in plasma has some similarity with that in cat urine. Spiked cat plasma and human plasma (dilute 20 times) containing 0.5 mg/ml felinine were stored at -20°C and analysed after one week. No significant degradation of felinine was found after the storage. The samples were then heated at 37°C (since this temperature is close to the temperature of human and cat body). Since proteins in plasma and serum influence felinine peak and migration time (see earlier Chapters), a internal standard is desirable. The same amount of internal standard N-(NO₂)arg was added after the heating. The result was then shown by the ratio of felinine peak area to that of N-(NO₂)arg (**Table 6.2**). **Table 6.2** shows that felinine was stable in cat (entire male and female) plasma,

human plasma and serum at 37°C. The ratio of felinine peak area to N-(NO₂)arg peak area remained almost unchanged even after heating for 24 hours and 32 hours.

Table 6.2 The stability of felinine in plasma and serum at 37°C

Heating time (hour)	Felinine peak area / N-(NO ₂)arg peak area			
	In male cat plasma	In female cat plasma	In human plasma	In human serum
0	1.04	1.02	1.17	1.16
1	1.05	1.03	1.16	1.17
2	1.03	1.03	1.18	1.16
3	1.04	1.02	1.18	1.16
4			1.18	1.17
5	1.03	1.03	1.17	1.16
24			1.17	
32				1.17

6.2.5 Plasma (or serum) treatment

An advantage of CE is the compatibility of the running electrolyte with charged bioanalytes. For some assays, no sample preparation at all is required. However, for other analytes, especially those at low concentrations in biological fluids, effort may be required to optimize the sample pre-treatment for CE. This is to avoid effects on migration behavior and interferences in CE from the high ionic strength and protein-rich matrices, especially in plasma and serum (Perrett, 1999).

To remove proteins, the plasma samples with spiked felinine were treated with acetonitrile to precipitation the plasma proteins. In my study, different percentages of acetonitrile were applied to the plasma samples, with the analysis by CE employing the same analytical conditions as above. N-(NO₂)arg was also applied as internal standard. It was found that when the percent of acetonitrile in total sample was 70% or more, felinine and N-(NO₂)arg peaks were not observed at the CE. An injection time of three seconds was compared with an injection time of one second. The results observed were the same (**Figure 6.5**). This result indicated that felinine has been binding with protein in the precipitate after the

treatment. Felinine and N-(NO₂)arg peaks can be observed when less acetonitrile (65%) was used (**Figure 6.6A**). When the percent of acetonitrile was less than 40%, protein precipitation was not be observed. Felinine in a bovine serum albumin (BSA) solution was also treated with 65% acetonitrile (**Figure 6.6B**). No significant difference was found between **Figure 6.6A** and **Figure 6.6B**. The peaks and the migration times of felinine and N-(NO₂)arg in serum and BSA after the treatment were similar with that in water (**Figure 6.6 C**). The ratio of felinine to N-(NO₂)arg was 0.79 for felinine in water (no acetonitrile treatment), 0.78 for felinine in plasma and 0.79 for felinine in BSA, respectively.

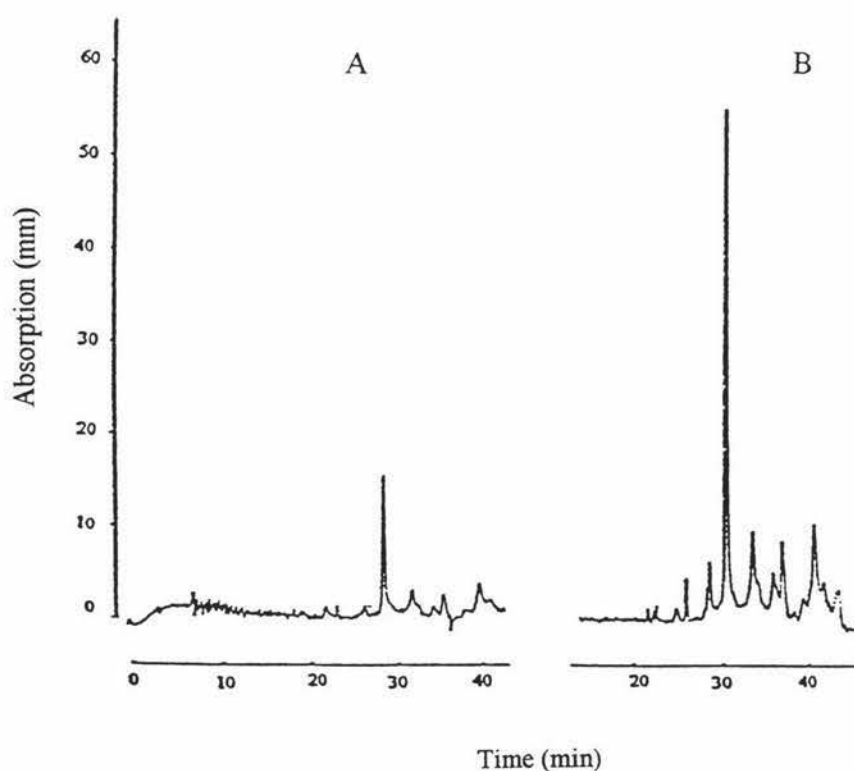


Figure 6.5 CE electropherograms of plasma (73% in acetonitrile) after spiking with felinine. Injection 1 sec (A) and 3 sec (B), other conditions as **Figure 6.1**

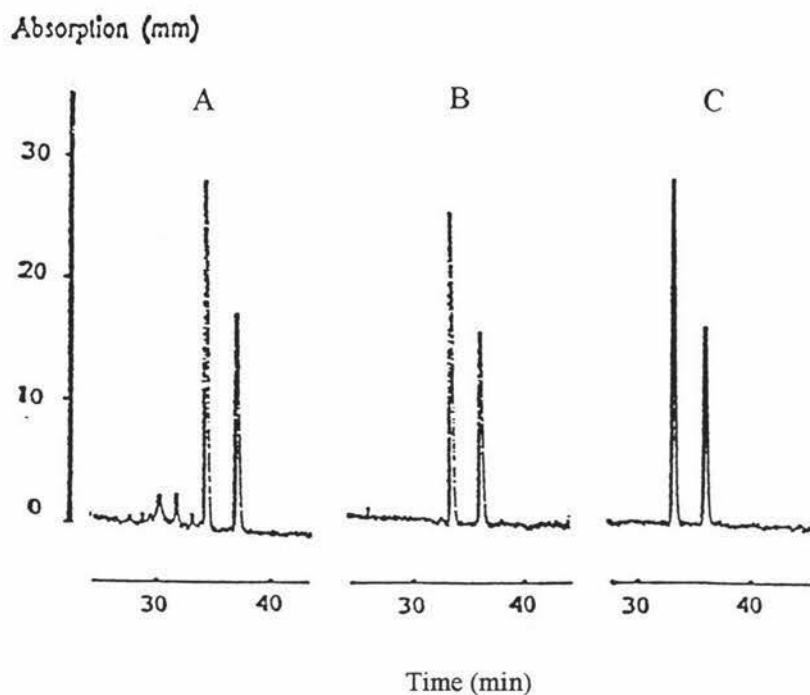


Figure 6.6 CE electropherograms after treatment of plasma with 65% acetonitrile. Conditions as Figure 6.1

A: plasma with spiked felinine and N-(NO₂)arg

B: BSA with spiked felinine and N-(NO₂)arg

C: felinine and N-(NO₂)arg in H₂O (no acetonitrile treatment)

6.3 Conclusion

The CZE method and HPLC BOC derivative method used herein showed that no felinine trace was found in entire male cat and female cat plasma. Protein was removed from plasma by precipitation with acetonitrile. Protein free plasma after addition of felinine (spiking) can be achieved by using certain amounts of acetonitrile. Felinine is stable in cat plasma, human plasma and serum at 37°C. Virtually 100% recovery of felinine in plasma can be achieved in very diluted plasma. The averaged recovery of felinine added to plasma was 101.7%, when the plasma was diluted with water.

CHAPTER SEVEN

CONCLUSION AND FUTURE STUDIES

7.1 Conclusions

The study reported in this thesis investigated the application of a relatively new technique, CZE, to the analysis of the urinary amino acid felinine in urine, plasma and serum.

An Applied Biosystems 270A Capillary Electrophoresis System and fused silica capillaries were used in this study. The basic analytical conditions for the separation of felinine using CZE that evolved were phosphate buffer with concentration 20mM, and pH 2.0. Felinine can be used directly on the CE without making any felinine derivative. Good separation of felinine from an amino acid standard mixture and sulphur containing compounds was achieved with a detection wavelength at 200 nm.

The CZE method developed in this study has been successfully applied to the analysis of felinine in cat urine. The felinine peak in cat urine can be identified and felinine concentration in different cat genders can be quantitatively determined. For better linearity in the standard calibration, felinine peak area is recommended. Reproducibility was noted for the new CZE method's applicability to urinary analyses.

The CZE method was applied to the study of felinine stability. The stability of felinine, either synthetic or natural in cat urine was investigated. Conditions from acidic (HCl) to alkaline (NaOH and NH_4OH), storage temperature from -20°C to 20°C and heating temperature from 55°C to 100°C were investigated. The storage of synthetic felinine and cat urine at relative low temperature is recommended. Felinine peak distortion in stronger acidic and alkaline solutions showed the limitations of the buffer pH.

A similar study of felinine in plasma and serum was carried out. Analysis of plasma and serum samples with or without felinine were directly performed on the CE under the same CZE principles. It was confirmed that no trace of felinine be found in cat plasma at least within the detection limits of this new CZE method. Plasma and serum samples can be

handled efficiently with sufficient dilution. Protein removal may be desirable in some cases.

7.2 Future studies

Further study needs to be carried out to validate the initial in felinine concentration as seen in my storage (**Figure 5.4**) and temperature (**Figure 5.5**) studies.

The peak broadening seen in my stability studies should be further validated with, for example, HPLC and MS. Here the aim is to identify the various breakdown products.

Future studies should focus on the use of the new CZE analytical method to study both precursors and breakdown products of felinine in the cat. A study of the precursors of felinine should reveal further information as to the "real" beginnings of felinine. Studies of the breakdown products (metabolites) will reveal whether felinine or some other agent is the "true" pheromone.

REFERENCES

Applied Biosystems Capillary Electrophoresis. *Application Note 5*.

Avizonis, P. V. and Wriston, J. C. (1959) On the biosynthesis of felinine. *Biochim. Biophys. Acta* **34**, 279.

Baker, Dale R. (1995) Capillary Electrophoresis. *John Wiley and Sons, Inc.*, New York.

Barbas, C., Adeva, N., Aguilar, R., Rosillo, M., Rubio, T. and Castro, M. (1998) Quantitative determination of short-chain organic acids in urine by capillary electrophoresis. *Clin. Chem.* **44**, 1341.

Beaver, B. V. (1992) Feline Behaviour: A Guide for Veterinarians. 1st edn. *WB Saunders Company*, London, England.

Bocek, P., Deml, M., Gebauer, P. and Dolnik, V. (1988) Analytical Isotachophoresis. *VCH Verlagsgesellschaft, Weinheim*, Fed.

Borchers, J., Clauss E., Decker P. and Schoberl A. (1967) The formation and biological role of felinine, a sulfur containing isoprene derivative from cat urine. *Deu. Tierarzt. Wochenschr.* **74**, 532.

Camilleri, Patrick, (1993) Capillary electrophoresis, theory and practice. *CRC Press*, New York.

Che, P., Xu, J., Shi, H. and Ma, Y. (1995) Quantitative determination of serum iron in human blood by high-performance capillary electrophoresis. *J. Chromatog.* **669**, 45.

Cohen, A.S. and Karger, B. L. (1987) High-performance sodium dodecyl sulfate polyacrylamide gel capillary electrophoresis of peptides and proteins. *J. Chromatog.* **397**, 409.

- Cohen, A. S., Paulus, A. and Karger, B. L.** (1987) High-performance capillary electrophoresis using open tubes and gels. *Chromatographia* **24**, 14.
- Datta, S. P. and Harris, H.** (1951) A convenient apparatus for paper chromatography. Results of a survey of the urinary amino patterns of some animals. *J. Physiol.* **114**, 39.
- Datta, S. P. and Harris, H.** (1953) Urinary amino-acid patterns of some mammals. *Ann. Eugen.* **18**, 107.
- Dolnik, V.** (1995) Capillary zone electrophoresis of serum proteins: study of separation variables. *J. Chromatogr. A* **709**, 99.
- Dolnik, V.** (1997) Capillary zone electrophoresis of proteins. *Electrophoresis* **18**, 2353.
- Dulffer, T., Herb, R., Herrmann, H. and Kobold, U.** (1990) Capillary electrophoresis. Basic considerations for industrial application. *Chromatographia* **30**, 675.
- Eggerer, H.** (1962) Synthesis of natural felinine. *Liebigs Ann. Chem.* **657**, 212.
- Fanali, S.** (1997) Controlling enantioselectivity in chiral capillary electrophoresis with inclusion complexation. *J. Chromatogr.* **792**, 227.
- Fukutome, K.** (1961) Biosynthesis of isovalthine in the cat. *J. Biochem.* **49**, 444.
- Gordon, M. J., Lee, K. J., Arias, A. A. and Zare, R. N.** (1991) Protocol for resolving protein mixtures in capillary zone electrophoresis. *Anal. Chem.* **63**, 69.
- Greaves, J. P. and Scott, P. P.** (1960) Urinary amino-acid pattern of cats on diets of varying protein content. *Nature* **187**, 242.
- Grossman, Paul D. and Colburn, Joel C.** (1992) Capillary electrophoresis. Theory and practice. *Academic Press, Inc.* New York.

Grossman, P. D., Wilson, K. J., Petrie, G., and Lauer, H. H. (1988) Effect of buffer pH and peptide composition in the selectivity of peptide separations by capillary zone electrophoresis. *Anal. Biochem.* **173**, 265.

Guo, H. (1998) Determination of creatinine and creatine by capillary electrophoresis. *M. Sc. thesis, Massey University, New Zealand.*

Guzman, N. A., Berck, C. M., Hernandez, L., Advis, J. P. (1990) Capillary electrophoresis as a diagnostic tool. Determination of biological constituents present in urine of normal and pathological individuals. *J. Liq. Chromatogr.* **3**, 3843.

Hage, D. S. (1997) Chiral separations in capillary electrophoresis using proteins as stereoselective binding agents. *Electrophoresis* **18**, 2311.

Harakuwe, A. H. H. (1995) Studies on Separation Selectivity in Capillary Zone Electrophoresis of Inorganic Anions. *Ph. D. thesis, University of Tasmania.*

Harrold, M. P., Wajtusik, Mary Jo, Riviello, John and Henson, Patricia (1993) Parameters influencing separating and detection of anions by capillary electrophoresis. *J. Chromatogr.* **640**, 463.

Hendriks, W. H., Moughan, P. J., Tarttelin, M. F. and Woolhouse, A. D. (1995) Felinine: a urinary amino acid of felidae. *Comp. Biochem. Physiol.* **112B**, 581.

Hendriks, W. H., Tarttelin, M. F. and Moughan P. J. (1995a) Twenty-four hour felinine excretion patterns in entire and castrated cats. *Physiol. Behav.* **58**, 467.

Hendriks, W. H., Woolhouse, A. D., Tarttelin, M. F. and Moughan, P. J. (1995b) Synthesis of felinine, 2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid. *Bioorg. Chem.* **23**, 89.

- Hjerten, S., Elenbring, K., Kilar, F., Liao, J. L., Chen, A. J. C., Siebert, C. J. and Zhu, M. D.** (1987) Carrier-free zone electrophoresis, displacement electrophoresis and isoelectric focusing in a high performance electrophoresis apparatus. *J. Chromatogr.* **403**, 47.
- Hjerten, S. and Zhu, M. D.** (1985) Adaptation of the equipment for high-performance electrophoresis to isoelectric focusing. *J. Chromatogr.* **346**, 265.
- Honda, S., Iwase, S. and Fujiwara, S.** (1987) Evaluation of an automatic siphonic sampler for capillary zone electrophoresis. *J. Chromatogr.* **404**, 313.
- Honda, S., Suzuki, K., and Kakehi, K.** (1989) Simultaneous determination of codate and periodate by capillary zone electrophoresis. *Anal. Biochem.* **177**, 62.
- Huang, X., Coleman, W. and Zare, R.** (1989) Analysis of factors causing peak broadening in capillary zone electrophoresis. *J. Chromatogr.* **480**, 95.
- Jandik, P. and Jones, W. R.** (1991) Optimization of detection sensitivity in the capillary electrophoresis of inorganic anions. *J. Chromatogr.* **546**, 431.
- Jellum, E., Thorsrud, A. K. and Time, E.** (1991) Capillary electrophoresis for diagnosis and studies of human disease, particularly metabolic disorders. *J. Chromatogr.* **559**, 455.
- Jenkins, M. A.** (1997) Clinical application of capillary electrophoresis to unconcentrated human urine proteins. *Electrophoresis* **18**, 1842.
- Jorgenson, J.W. and Lukacs, K. D.** (1981) Free-zone electrophoresis in glass capillaries. *Clin. Chem.* **27**, 1551.
- Jorgenson, J.W. and Lukacs, K. D.** (1983) Capillary zone electrophoresis. *Science* **222**, 266.

Jorgenson, J.W. (1986) Electrophoresis. *Anal. Chem.* **58**, 743.

Joulain, D. and Laurent, R. (1989) The catty odour in black currant extracts versus the black-currant odour in the cat's urine? In *11th International Congress of Essential Oils Fragrances and Flavours* (Edited by Bhattacharyya S. C., Sen N. and Sethi K. L.), Oxford & IBH Publishing Co., New Delhi, India.

Karger, B. L., Cohen, A. S. and Guttman, T. M. (1989) High-performance capillary electrophoresis in the biological sciences. *J. Chromatogr.* **492**, 585.

Kenney, B. F. (1991) Determination of organic acids in food samples by capillary electrophoresis. *J. Chromatogr.* **546**, 423.

King, Roger (1998) Essay validation of new methods and establishing reference ranges. *Clin. Biochem. Paper* **22**, 782, 2.

Knox, J. H. (1988) Thermal effects and band spreading in capillary electrophoresis. *Chromatographia* **26**, 329..

Kuwaki, T., Oomori, S. and Mizuhara, S. (1963) Biosynthesis of isovalthine precursor in liver homogenates. *Biochem. Biophys. Acta* **78**, 553.

Lauer, H. H and McManigill, D. (1986) Capillary zone electrophoresis of protein in untreated fused silica tubing. *Anal. Chem.* **58**, 166.

Lee, K. J., Heo, G. S. and Doh, H. J. (1992) Determination of creatinine in serum by capillary zone electrophoresis. *Clin. Chem.* **38**, 222.

Li, S. F. Y. (1992) Capillary electrophoresis: principles, practice and applications. *J. Chromatogr. Lab.* Amsterdam, New York.

- MacDonald, M. L., Rogers, Q. R. and Morris, J. G.** (1984) Nutrition of the domestic cat, a mammalian carnivore. *Ann. Rev. Nutr.* **4**, 521.
- Mattian, M. J. I., Pignatello, J. J. and Swihart R. K.** (1991) Identification of volatile components of bobcat urine. *J. Chem. Ecol.* **17**, 451.
- McCormick, R. M.** (1988) Capillary zone electrophoresis separation of peptides and proteins using low pH buffers in modified silica capillary. *Anal. Chem.* **60**, 2322.
- Messana, I., Rossetti, D. V., Cassiano, L., Misiti, F., Giardina, B. and Castagnola, M.** (1997) Peptide analysis by capillary (zone) electrophoresis. *J. Chromatogr. B* **699**, 149.
- Mikkers, F. E. P., Everaert, F. M. and Verheggen, T. P. E. M.** (1979) Concentration distributions in free zone electrophoresis. *J. Chromatogr.* **169**, 1.
- Miyake, M., Shibukawa, A. and Nakagawa, T.** (1991) Simultaneous determination of creatinine and uric acid in human plasma and urine by cellular electrokinetic chromatography. *J. High Res. Chromatogr.* **14**, 181.
- Mizuhara, S. and Oomori, S.** (1960) A new sulfur-containing amino acid. *Arch. of Biochem. Biophys.* **92**, 53.
- Moring, Stephen E.** (1992) Quantitative Aspects of Capillary Electrophoresis Analysis. *Capillary Electrophoresis, Academic Press, Inc.*
- Nishi, H.** (1997) Enantioselectivity in chiral capillary electrophoresis with polysaccharides. *J. Chromatog. A* **792**, 327.
- Novotny, M. V., Cobb, K. A. and Liu, J.** (1990) Recent advances in capillary electrophoresis of proteins, peptides and amino acids. *Electrophoresis* **11**, 735.

O'Neill, K., Shao, X., Zhao, Z., Malik, A., and Lee, M. L. (1994) Capillary electrophoresis of nucleotides on ucon-coated fused silica columns. *Anal. Biochem.* **222**, 185.

Perrett, D. (1999) Capillary electrophoresis in clinical chemistry. *Ann. Clin. Biochem.* **36**, 133.

Petucci, C. J., Kantes, H. L., Strein, T., G. and Veening, H., (1995) Capillary electrophoresis as a clinical tool Determination of organic anions in normal and uremic serum using photodiode-array detection. *J. Chromatogr. B* **668**, 241.

Rasmussen, H. T. and McNair, H. M. (1990) Influence of buffer concentration, capillary internal diameter and forced convection on resolution in capillary. *J. Chromatogr.* **516**, 223.

Roberts, R. N. (1963) A study of felinine and its excretion by the cat. *Ph.D. thesis, University of Buffalo, NY.*

Rose, D. J. and Jorgenson, J. W. (1988) Characterization and automation of sample introduction methods for capillary zone electrophoresis. *Anal. Chem.* **60**, 642.

Schoeberl, A., Borchers, J., Grafje, H. and Grewe-Pape C. V. (1966) Synthesis of L-felinine. *Angew. Chem. Internat. Edit.* **5**, 249.

Schoeberl, A., Borchers, J. and Hantzsch, D. (1968) Synthesis of L-felinine. *Chem. Ber.* **101**, 373.

Schoots, A. C., Verheggen, T. P. E. M., De Vries, P. M. J. M., and Everaerts, F. M., (1990) Ultraviolet absorbing organic anion in uremic serum separated by capillary zone electrophoresis, and quantification of hippuric acid. *Clin. Chem.* **36**, 435.

Schwartz, H. E., Melera, M. and Brownlee, R. G. (1989) Performance of an automated injection and replenishment system for capillary electrophoresis. *J. Chromatogr.* **480**, 129.

Schwer, Ch. and Kenndler, E. (1990) Capillary Electrophoresis. *Chromatographia* **30**, 546.

Shapiro I. L. (1962) In vivo studies on the metabolic relationship between felinine and serum cholesterol in the domestic cat. *Ph. D. thesis, University of Delaware.*

Shunzi, Mizuhara and Shinji, Oomori (1961) A New Sulfur-Containing Amino Acid. *Arch. Biochem. and Biophys.* **92**, 53.

Smith, S., Strasters, J. K. and Khaledi, M. G. (1991) Influence of operating parameters on reproducibility in capillary electrophoresis. *J. Chromator.* **559**, 57.

Snyder, L. R. and Kirkland, J. J. (1979) Introduction to Modern Liquid Chromatography. *Wiley*, New York.

Spady, D. K. and Dietschy, J. M. (1983) Serol synthesis in vivo in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster and rat. *J. Lipid Res.* **24**, 303.

Sullivan T. P., Nordstrom L. O. and Sullivan D. S., (1985a) Use of predator odors as repellents to reduce feeding damage by herbivores. I Snow shoe hares. *J. Chem. Ecol.* **11**, 903.

Sullivan T. P., Nordstrom L. O. and Sullivan D. S. (1985b) Use of predator odors as repellents to reduce feeding damage by herbivores. II Black tailed deer. *J. Chem. Ecol.* **11**, 921.

Swihart, R. K., Pignatello J. J. and Mattina M. J. I. (1991) Aversive responses of white tailed deer, *odocoileus virginianus*, to predator urine. *J. Chem. Ecol.* **17**, 767.

Takigiku, R. and Schneider, R. E. (1991) Reproducibility and quantitation of separation for ribonucleoside triphosphates and deoxyribonucleoside triphosphates by capillary zone electrophoresis. *J. Chromator.* **559**, 247.

- Tallan, H. H., Moore, S. and Stein, W. H.** (1954) Studies on the free amino acids and related compounds in the tissues of the cat. *J. Biol. Chem.* **211**, 927.
- Terabe, S., Otsuka, K. and Ando, T.** (1989) Band broadening in electrokinetic chromatography with micellar solutions and open-tubular capillaries. *Anal. Chem.* **61**, 251.
- Terabe, S., Ishihama, Y., Nishi, Y., Fukuyama, T. and Otsuka, K.** (1991) Effect of urea addition in micellar electrokinetic chromatography. *J. Chromatogr.* **545**, 359.
- Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A. and Ando, T.** (1984) Electrokinetic separation with micellar solutions and open-tubular capillaries. *Anal. Chem.* **56**, 111.
- Trippett, S.** (1957) The synthesis of (\pm)-felinine. *J. Chem. Soc.* 1929.
- Wainright, A.,** (1990) Capillary electrophoresis applied to the analysis of pharmaceutical compounds. *J. Microcolumn Sep.* **2**, 166.
- Wang, T. M.** (1964) Further studies on the biosynthesis of felinine. *Ph.D. thesis, University of Delaware.*
- Westall, R. G.** (1951) The amino acids and other ampholytes of urine. 1. A general method of isolation. *Biochem. J.* **52**, 638.
- Westall, R. G.** (1953) The amino acids and other ampholytes of urine. 2. The isolation of a new sulphur-containing amino acid from cat urine. *Biochem. J.* **55**, 244.
- Weinberger, R., Sapp, E. and Moring, S.** (1990) Capillary electrophoresis of urinary porphyrins with absorbance and fluorescence detection. *J. Chromatogr.* **516**, 271.
- Wiktorowicz, J. E. and Colburn, J. C.** (1990) Separation of cationic proteins via charge reversal in capillary electrophoresis. *Electrophoresis* **11**, 769.

Worden, A. N., Waterhouse, C. E. and Sellwood, E. H. B. (1960) Studies on the composition of normal cat urine. *J. Small Ami. Proc.* **1**, 11.

Yashima, T., Tsuchiya, A., Morita, O. and Terabe, S. (1992) Separation of closely related large peptides by micellar electrokinetic chromatography with organic modifiers. *Anal. Chem.* **64**, 2981.

Yu, M. and Dovichi, N. J. (1989) Attomole amino acid determination by capillary zone electrophoresis with thermooptical absorbance detection. *Anal. Chem.* **61**, 37.