Amino Acids (2004) 27: 49–55
DOI 10.1007/s00726-004-0095-3

Amino Acids
Printed in Austria

Use of capillary (zone) electrophoresis for determining felinine and it's application to investigate the stability of felinine

S. M. Rutherfurd[1], **F. Zhang**[2], **D. R. K. Harding**[3], **A. D. Woolhouse**[4], and **W. H. Hendriks**[1]

[1] Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand
[2] Fonterra Research Centre, Palmerston North, New Zealand
[3] Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand
[4] Industrial Research Ltd., Lower Hutt, New Zealand

Received January 6, 2004
Accepted February 25, 2004
Published online June 4, 2004;

Summary. A rapid capillary electrophoresis method was established to quantify felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid) in cat urine and used to investigate felinine stability. Synthetic felinine was stable in the presence of oxygen while 11% of the natural felinine in urine disappeared after 4 days exposure to air. Both synthetic felinine and the natural felinine (in urine) were stable for up to 3 months when stored at −5°C and 20°C. Thirty percent of the synthetic felinine was lost after 5 hours at 100°C while 95% of the natural felinine disappeared after only 2 hours at the same temperature. The recovery of felinine under certain conditions was greater than 100%. It is possible that acetyl-felinine may be present in the urine and that it is deacetylated during incubation. Overall synthetic felinine was found to be stable but the felinine in cat urine much less so. Other compounds present in the urine may contribute to the decomposition of felinine.

Keywords: Felinine – Capillary electrophoresis – Cats – Urine

Introduction

Felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid) ($HOOCCH(NH_2)CH_2SC(CH_3)_2CH_2CH_2OH$) is an important sulphur containing amino acid known to be present in large amounts in the urine of entire male cats. It has been postulated that felinine plays a role as a male pheromone designed to attract female cats (Hendriks et al., 1995b). Indeed, 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 when odourless synthetic felinine and isolated ''natural'' felinine was stored at 4°C and at room temperature, they developed the distinctive tom cat urine smell, which they postulated, was most likely derived from the degradation of felinine. Working with this amino acid has proved difficult over the years, with most of the work conducted using methods such as paper chromatography with which to characterise felinine. Indeed studies of the oxidation, degradation and acid hydrolysis of felinine have only been carried out using such simple techniques (Westall, 1953). The first quantitative method used for determining felinine was ion exchange chromatography, however, no quantitative assessment of the stability of felinine has been conducted to date.

Studies have been carried out on its characterisation, synthesis, excretion levels and biosynthesis (Westall, 1953; Trippett, 1957; Datta and Harris, 1953; Roberts, 1963; Avizonis and Wriston, 1959; Hendriks et al., 1995, 1995a). Published data on levels of felinine in urine have been obtained using paper chromatography (Westall, 1953; Roberts, 1963) and ion exchange chromatographic methods (Hendriks et al., 1995; Tallan et al., 1954; Avizonis and Wriston, 1959; Shapiro, 1962). However, these methods are either not quantitative (paper chromatography) or quite lengthy in their analysis times (ion exchange chromatography). Capillary electrophoresis (CE), a relatively new technique, has the advantages of having rapid runtimes and not requiring derivatisation of felinine. Felinine is a free amino acid when present in urine, and as such hydrolysis of urine samples (normally required to release amino acids from protein) is not required. Consequently, application of this method for determining felinine would advance the investigation of felinine metabolism and biochemistry. This

paper describes the use of CE for quantifying felinine from cat urine and subsequent studies carried out on the stability of felinine. It also describes preliminary work investigating 3-mercapto-3-methyl-l-butanol, the main postulated breakdown products of felinine.

Materials and methods

Acetonitrile, and hydrochloric acid (both HPLC grade), acetic acid (analytical grade reagent), were from BDH Laboratory Supplies, Poole, England; sodium dihydrogen phosphate (analytical grade reagent) was from Riedel-de Haen, Seize, AG, Germany. All other reagents were analytical grade wherever possible. Water was Milli-Q grade. Cat urine and plasma were obtained from cats in the Centre for Feline Nutrition at Massey University, Palmerston North. Human plasma and serum were from the Nutrition Laboratory, Institute of Food Nutrition & Human Health, Massey University. Amino acid standard mixtures were from Pierce, Rockford, Illinois 61105, U.S.A. Synthetic felinine was synthesised by Dr. Tony Woolhouse using the procedure of Hendriks et al. (1995b). 3-mercapto-3-methyl-l-butanol was synthesised by Dr. Tony Woolhouse, the procedure for which is described below.

Synthesis of 3-mercapto-3-methyl-l-butanol

A solution of methyl 3-methyl-2-butenoate (5.0 g, 44 mmol) in a mixture of ethanol (36 ml) and triethylamine (46 ml) contained in a thick-walled glass tube (ca 250 ml) was cooled to −70°C and then saturated with hydrogen sulphide before being sealed and then heated at 70°C overnight. The tube was recooled to −70°C, opened and the contents vented before being concentrated to an oil and then dissolved in ether (100 ml). The ethereal phase was washed once with dilute hydrochloric acid (20 ml) and then once with water and then dried and concentrated to a light yellow oil. This mercapto ester was dissolved in dry ether (40 ml) and added dropwise to a suspension of lithium aluminium hydride (1.3 g) in ether. After stirring for 2 hours, the mixture was quenched with saturated aqueous ammonium chloride and the ethereal phase dried and concentrated to give a homogeneous, mobile liquid. Bulb-to-bulb (Kugelrohr) distillation (ca 90°C/0.5 mm) furnished the mercaptobutanol as a colourless liquid (3.6 g).

Instrumentation

Capillary electrophoresis system

An Applied Biosystems (Foster City, Ca, U.S.A.) 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 4 ml 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. Injection was achieved using vacuum injection for 1 sec and the felinine was detected at 200 nm and the sample run in a 20 mM NaH_2PO_4 buffer (pH 2.0) with an applied voltage of 12 kV. The electropherogram was recorded and evaluated on a Kipp and Zonen (Bohemia, NY, U.S.A.) DB 40 recorder. Detection at 200 nm with 0.02 absorbance units full scale (AUFS) was used.

Mass spectrometry

Samples (200 mL of the standard and 1000 mL of urine) were pipetted into 20 mL glass vials and the vials closed with a aluminium foil cap. They were then incubated at 60°C for 20 min prior to the headspace analysis. The urine sample was analysed twice so the total incubation time for the second analysis was about 60 min. The headspace was sampled for 20 min using a Carboxen-Polydimethylsiloxane solid phase micro-extraction fibre (Supleco, Inc, Bellefonte, PA, U.S.A.). The volatiles were analysed on an Agilent 6890 GC with 5793 mass detector (Agilent Technologies, Palo Alto, Ca, U.S.A.). The GC method was as follows. The solid phase micro-extract was desorbed at 300°C for 3 min onto a Zebron-ZB 5 (5%-phenyl-95%-dimethylpolysiloxane) (30 m, 0.25 mm i.d., 0.25 μm film) column (Phenomenex, Torrance, Ca, U.S.A.). The inlet was pulsed splitless (18 N/cm^2) for 2 min and the column headpressure was 11 N/cm^2. The column was held at 35°C for 3 min and then ramped at 8°C/min to 300°C. The column was connected to a 5973 mass detector with a transfer line temperature of 280°C and source temperature of 220°C with the quads at 180°C. Data were collected using Chemstation software in the total ion mode with the following settings: low mass 40, high mass 550 and threshold 150. 3-mercapto-3-methyl-l-butanol was tentatively identified using it's relative retention times and it's fragmentation mass spectra which was compared to fragmentation mass spectra from the Wiley MS database (Wiley Publishers, Hoboken, NJ, U.S.A.).

Urine collection

Cat urine samples were collected from both entire and castrated adult male cats according to the method of Hendriks et al. (1999). The cats were housed at the Best Friend Feline Nutrition Research Unit at Massey University and urine samples were stored at −20°C after collection and prior to further use. Urine samples were filtered again just prior to loading on the CE systems.

Felinine stability studies

For hydrolysis of the urine and felinine, 100 μl urine or felinine solution (1 mg/ml) was taken and placed into a side-arm glass tube followed by 1 ml 6 M HC1 (with 1 mg/ml phenol). The sample was degassed under vacuum, sealed and heated at 110°C for 24 hrs, after which they were filtered through a 0.45 μm filter before injection on the CE.

Cat urine and felinine were hydrolysed in different HCl concentrations (6 M, 4 M, 3 M, 1 M and 0.5 M) at room temperature. One drop of β-mercaptoethanol was added to protect the thiol group. Hydrolysis was conducted for different times, after which the samples were filtered before injection on the CE.

Results

A series of experiments were conducted to determine the capillary electrophoresis (CE) conditions required to achieve a separation for felinine in which felinine eluted as a single peak at an acceptable retention time. While the actual experiments will not be described here, the final conditions that were decided upon are presented in the Materials and methods. A typical separation of cat urine using these conditions is shown in Fig. 1. A further series of experiments were conduct to validate the method. The linearity of the absorbance response to the increasing amounts of felinine injected onto the CE system (linear range) using the newly developed CE method was determined over a concentration range of 0–1 mg/ml of synthetic felinine (Fig. 2). The absorbance response was linear ($R^2 = 0.999$) over the concentration range tested.

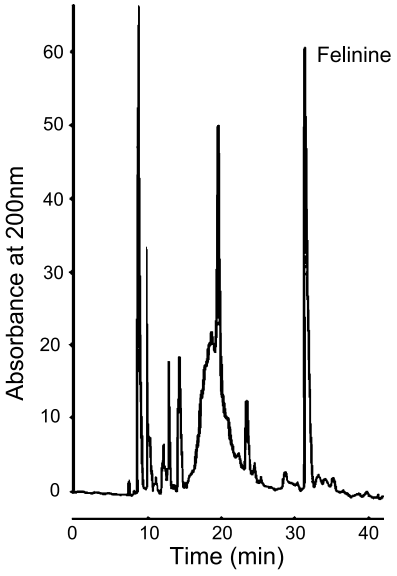

Fig. 1. Typical chromatogram for entire male cat urine separated using CZE

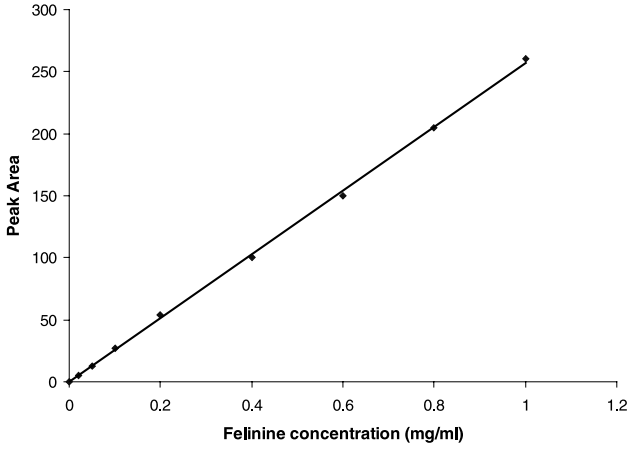

Fig. 2. Linear response for felinine using CZE

The reproducibility of the retention time and absorbance response for felinine was also examined. Six consecutive injections of a synthetic felinine standard solution were made using the CE instrument. The retention time and peak areas were recorded and compared between runs. The mean retention time was 32.4 ± 0.12 min and the mean peak area was 120.2 ± 0.77 units. Overall the reproducibility for the retention time and absorbance response for felinine was excellent and boded well for the possible use of CE as a rapid and simple method to determine felinine.

The recovery of felinine from a urine sample spiked with varying amounts of synthetic felinine was also determined. 0.05–0.5 mg of synthetic felinine was added to 1 ml of urine collected from castrated male cats. Using the newly developed CE method, the amount of felinine in the urine was determined (Table 1). Recovery of felinine was calculated as follows:

$$\text{Recovery}(\%) = C_x/(C_o + C_s) \times 100$$

Where C_x is the concentration of felinine determined by CE after addition of synthetic felinine, C_o is the felinine concentration measured by CE without addition of synthetic felinine, and C_s is the concentration of synthetic felinine added (Che, 1995).

The mean recovery of spiked felinine was $95.9 \pm 2.42\%$, and based on this recovery it was deemed that the CE method used to determine felinine was acceptable for routine determination of felinine in cat urine.

Table 1. Recovery[1] (%) of felinine spiked into a urine sample

Amount of felinine added (mg/ml)	Recovery of spiked felinine (%)
0.05	100
0.1	91.5
0.2	92.4
0.3	94.5
0.4	94.3
0.5	97.2

[1] Recovery (%) $= C_x/(C_o + C_s) \times 100$; where C_x is the concentration of felinine determined by CE after standard addition, C_o is the felinine concentration measured by CE without synthetic felinine addition, and C_s is the felinine concentration added

Stability of felinine

Several qualitative studies have been conducted on the stability of felinine (Westall, 1953; Joulain and Laurent, 1989; Hendriks et al., 1995b), but a quantitative description of felinine stability has essentially yet to be made. In this study several conditions were examined in order to characterise felinine stability in a physiologically relevant manner.

Oxidation of felinine

The oxidation of felinine has been examined using powerful oxidising agents such as hydrogen peroxide with which to carry out the oxidation. Paper chromatography was employed to measure the extent of oxidation (Westall, 1953). Westall (1953) also postulated that oxidation also occurs after exposure to air. However, no quantitative data has been produced to verify this hypothesis. This study aimed to examine the susceptibility of felinine to

Table 2. Recovery (%) of synthetic felinine and natural felinine in entire male cat urine after exposure the oxygen

Exposure time (days)	Synthetic felinine[1]	Felinine in cat urine[2]
0	100	100
1	98.9	98.4
2	99.5	–
5	–	89.0

[1] Synthetic felinine was exposed to oxygen by bubbling pure oxygen through a solution of felinine. [2] Felinine in cat urine was exposed to air only

Table 3. Recovery of felinine (%) in entire tomcat urine after storage at different temperatures[1]

Storage time (days)	Storage temperature (°C)	
	−20	5
0	100	100
2		119
17	105	128
24		120
35	103	107
60	102	110
80	96	105

[1] Felinine was stored in sealed containers at −20°C, 5°C for up to 80 days

oxidation by using both synthetic felinine and entire male cat urine. The oxidation of synthetic felinine was studied by continually passing oxygen through a capillary into a solution of synthetic felinine over a three day period. During that time the synthetic felinine assumed the odour of cat urine. The recovery of synthetic felinine was determined each day using the CE method and the results are shown in Table 2. Only approximately 1% of the synthetic feline disappeared over the 2 day period suggesting that the felinine itself is resistant to oxidation. The second part of this experiment was to expose entire male cat urine, which contained felinine, to the atmosphere at room temperature for a period of five days. The recovery of felinine relative to the felinine in the original urine samples was determined using CE. The results are showed in Table 2. Overnight, the urine dried out and each morning deionised water was added to dissolve the remnants. After 1 day only 1.5% of the felinine in the urine had disappeared, this increased to 11% after a period of 5 days.

Stability of felinine during storage

The stability of felinine at low temperatures, which are typically used to store felinine, was examined. After collection and filtration, entire male cat urine samples containing felinine were assayed by CE and then stored in two different temperatures: 5°C and −20°C. Felinine in the stored urine was analysed at various intervals up to 80 days. The recovery of felinine in these urine samples over the three month storage period is shown in Table 3.

The recovery of felinine in the urine when stored at 5°C increased in the first 17 days of storage, it then decreased to close to the initial felinine concentration and remained almost unchanged for the remainder of the storage period. The concentration of felinine stored at 20°C during storage remained similar to the concentration of felinine in the fresh urine. Suggesting little or no degradation of felinine occurs even over long storage periods. Anecdotally, the characteristic tomcat odour was strong in the stored urine samples and did not noticeably diminish even after storage for several months.

Felinine stability at high temperatures

To examine the stability of felinine exposed to high temperatures, both synthetic felinine and entire male cat urine known to contain felinine were incubated at high temperatures for varying lengths of time. In the first study, synthetic felinine was heated to 100°C for 0–9 days and the results are shown in Fig. 3. The recovery of synthetic felinine decreased from 100% to approximately 65% over the first five days of heating, after that there was no further loss of synthetic felinine.

In the second study, entire male cat urine known to contain felinine was heated from 55°C to 100°C for up to 24 hours and the results are shown in Fig. 4. At 55°C

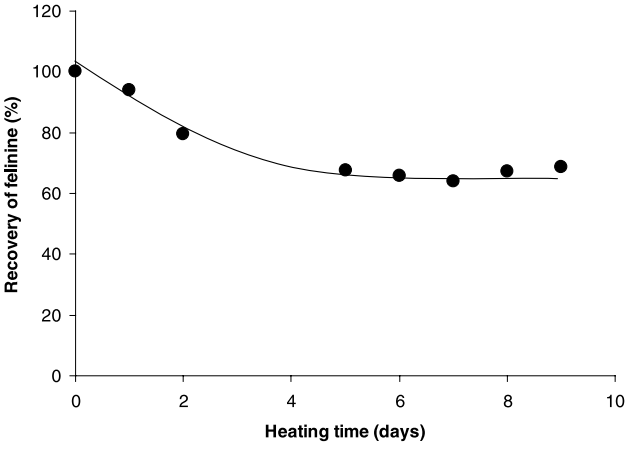

Fig. 3. Recovery of synthetic felinine (%) heated at 100°C for up to 9 days

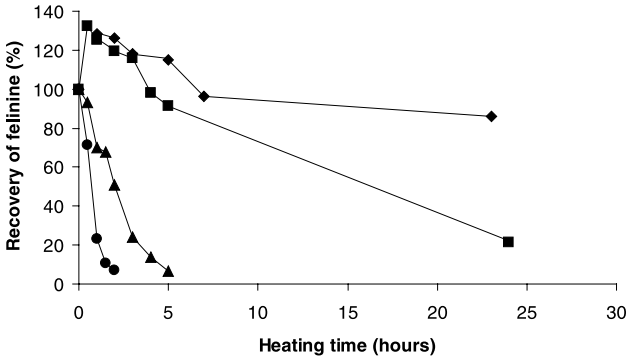

Fig. 4. Recovery of felinine (%) in entire male cat urine heated at various temperatures. The urine was heated at 55°C (◆), 70°C (■), 85°C (▲) and 100°C (●) for 0–24 hours and the levels of felinine remaining in the urine was determined using CE

and 70°C the recovery of felinine increased to approximately 125% before decreasing to 86% and 21% respectively. This increase could be due to deacetylation of felinine in the urine as a significant portion of the felinine present in urine is purported to be acetylated (Hendriks et al., 2004). The felinine levels in the urine heated to 85°C and 100°C decreased to approximately 5% levels within 2 and 5 hours, respectively. If the felinine recovery for the 100°C incubations for the synthetic felinine and the felinine present in the urine are compared, then the synthetic felinine appears to be considerably more stable than the felinine present in urine. Indeed, 95% of the felinine in the urine had disappeared after 2 hours incubation at 100°C, while in the same incubation conditions only 0.5% of the synthetic felinine had disappeared.

Analysis of 3-mercapto-3-methyl-l-butanol in entire male cat urine

3-mercapto-3-methyl-l-butanol was synthesised and used as a standard for chromatographic analysis of entire male cat urine. The identity of 3-mercapto-3-methyl-l-butanol was confirmed using mass spectrometry (Fig. 5). The mass spectra and fragmentation ions observed for 3-mercapto-3-methyl-l-butanol based on the Wiley MS database is very similar to that observed for the standard prepared in our laboratory, clearly confirming the identity of our standard. The urine of an entire male cat was analysed by GCMS for the presence of 3-mercapto-3-methyl-l-butanol. The chromatogram of the 3-mercapto-3-methyl-l-butanol standard shows a major peak at 14.9 min. In contrast, there is no corresponding peak in the chromatogram of the entire male cat urine. Consequently, there was no 3-mercapto-3-methyl-l-butanol found in the urine.

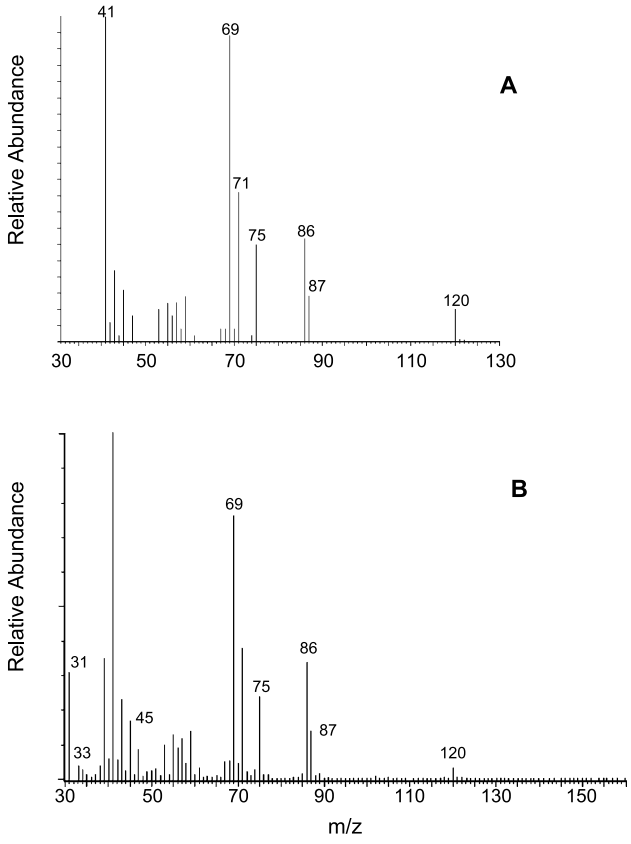

Fig. 5. Mass spectra of 3-mercapto-3-methyl-l-butanol from the Wiley MS database (Wiley Publishers, Hoboken, NJ, U.S.A.) (**A**) and the 3-mercapto-3-methyl-l-butanol standard prepared in our laboratory (**B**)

Discussion

A CE method has been developed to produce a quantitative and rapid method for determining the concentration of felinine in cat urine. The method is sensitive, accurate and requires only small amounts of urine. Felinine is present in it's free form in the urine, no urine pre-treatment is required apart from filtration. The linearity of the absorbance response was excellent between the range of 0–1 mg/ml. The reproducibility of the absorbance response and retention time for felinine was also excellent. Similarly, the recovery of synthetic felinine from spiked castrated male cat urine was greater than 95% (castrated males cat urine was used for this experiment since they contain only low levels of felinine). Given all these factors it is apparent that the newly developed CE method for determining felinine is suitable for the routine quantitative analysis of felinine in cat urine.

Westall (1953) 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 this study, synthetic felinine was shown to be stable to oxidation after 9 days exposure to oxygen while the felinine in urine was much less stable with 10% of the felinine disappearing after the same time with exposure only to air. It cannot be deduced conclusively that the disappearance of felinine from the urine in the oxidation study was as a result of oxidation of felinine. Indeed, given the apparent stability of synthetic felinine under oxidative conditions, it would appear that either, some mechanism other than oxidation is responsible for the losses of felinine or, there are oxidising agents are present in urine that cause the oxidation of felinine in urine.

The storage conditions used to store synthetic felinine caused no significant loss of felinine for the 90 day period studied. Furthermore, it is quite likely that negligible degradation of felinine would occur even with much longer storage times. It is interesting to note that felinine levels actually increased when stored at 5°C over the first 24 hours. It has been reported that felinine is also present in urine as acetyl-felinine (Hendriks et al., 2004) indeed as much as 20% of the felinine is present in this form. It is therefore, possible that the increasing felinine levels observed in this experiment could be due to the deacetylation of acetyl-felinine during storage. Storage at −20°C would have severely reduced deacetylation of felinine hence the recoveries which are close to 100%.

Felinine degradation did occur at higher temperatures although again, the stability of synthetic felinine is much higher than felinine in cat urine. In the case of the felinine in urine, the higher the temperatures, the faster the degradation that occurred. Boiling in water resulted in almost complete loss of felinine in only 2 hours.

Overall the studies show that felinine in cat urine is stable other than in the presence of very high temperatures. Exposure of the urine to the air at room temperature did not have much influence of felinine stability suggesting that felinine may not be as easily oxidised and is more stable than previously thought (Hendriks et al., 1995). It is possible that felinine integrity is affected by urine pH. For normal cat urine the pH ranges from 6.0–6.4 but can range from 5.5–7.5. It is possible that gradual felinine degradation is pH mediated. Quantitative studies on the effect of mild pH on felinine integrity may help to elucidate the behaviour of felinine in cat urine.

It is interesting to note that a strong catty odour was present in the synthetic felinine solution and urine after treatment in all experiments conducted, including the ones where very little loss of felinine was recorded (less than 2%). Since the compound responsible for the odour is thought to be a degradative product of felinine (Hendriks et al., 1995b) and since the odour existed in the synthetic felinine solution after treatment but not in the original solution, it would appear that the volatile compound responsible for the odour is derived from felinine itself. Moreover, since the odour was present even when very little felinine was lost, such as was the case with the long term storage studies which ran for several months, it would appear that the compound responsible for the odour was very potent indeed.

The identification of the compound responsible for the Tomcat odour is not certain. Joulain and Laurent (1989), suggested that two volatile compounds, 3-mercapto-3-methyl-l-butanol and 3-methyl-3-methylthio-l-butanol, can be found in cat urine and might be responsible for the cat urine odour. However, 3-mercapto-3-methyl-l-butanol could not be found in entire male cat urine in studies conducted in our laboratory. It may be that 3-mercapto-3-methyl-l-butanol is responsible for the odour but that it is so potent that the amounts required to give the odour are below the limits of detection. Furthermore, it must be pointed out that only 3-mercapto-3-methyl-l-butanol was the sole target of investigation and it is possible that other mercaptans are present in the urine and responsible for the typical cat urine odour. Perhaps further studies should focus on other reported mercaptans (Mattina et al., 1991).

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Authors' address: S. M. Rutherfurd, Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand, Fax: 64 6 350 5657, E-mail: S.M.Rutherfurd@massey.ac.nz