Developing palatants for pet food from leather industry wastes

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Philosophy

in

Animal Science

at Massey University, Palmerston North,

New Zealand

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2024

Abstract

The leather industry generates substantial waste streams, which are presenting a significant challenge in terms of both environmental sustainability and economic efficiency. Adding value to leather waste streams is a popular research idea. Most previous research has focused on recycling and reusing collagen from leather waste, or turning leather waste into bioenergy. This project concentrated on turning one leather waste stream into ingredients for pet food because the commercial value of the pet food industry is increasing. Cats are strict carnivores, and they find ingredients from animal sources highly palatable. Therefore, the aim of this project is to produce and develop edible and palatable products which are attractive to cats, and analyse these novel products in order to understand more about increasing palatability in cat food.

Chapter 2 is the literature review. The first part of the review summarises and compares research into the utilisation of leather waste streams, especially in the food industry. The second part contains basic information on palatability, and how improvements in cat food manufacture and methods for testing palatability have been made. The final part of the review considers methods for food flavour instrumental analysis especially to characterise key factors associated with palatability.

Chapter 3 details attempts to explore collagen hydrolysis. Collagen hydrolysed by water mixtures were produced for *in vivo* testing by cats using two-bowl palatability tests. However, the hydrolysed collagen solutions only had the same levels of palatability as water for cats.

Chapter 4 describes further palatability sample development and production processes. A series of samples were prepared and tested by cats using the same method in Chapter 3. The best performing of these was a raw sheep skin sample hydrolysed by phosphoric acid at 105°C and then adjust its pH to 5.5, which showed that a novel palatable product for cats can be produced from leather waste.

This sample was further analysed in Chapter 5. Proximate analysis, volatile compounds characterisation, total amino acid, free amino acid and fatty acid analyses were carried out. Those tests allowed full nutritional mapping of the sample.

The future work chapter indicates the remaining gaps in the finished lab work and the suggests further work that needs to be carried out.

Key words:

Leather waste, palatability, cat food, hydrolysis, instrumental analysis

Acknowledgements

First, I sincerely thank all my current and previous supervisors: Associate Professor David Thomas, Professor Mark Waterland, Associate Professor Don Otter, Dr Rafea Naffa, and Dr Sue Cooper. You are all professional and talented. I have learnt so much from all your guidance and had a great time worked with all of you.

My main supervisor Dave, thank you for all your supports and understanding. You are the best supervisor in the world! You usually give me positive feedback of my work and my stress was always reduced a lot after our meeting. There are too many specific stories about you I want to appreciate from the day we met online for the interview. Thank you for giving me this chance to walk into the area of animal science. By the way, I am specifically grateful for your on-site support of all my presentations.

I really want to appreciate Mark for arranging a lab space for me at campus when I needed. I also remembered your encouragement when I was looking for the cutting-edge technology even only few of the machines in the whole world and NZ does not have one. Don, many thanks for your valuable suggestions on presenting my research poster and entering the 3MT competition at the NZIFST Annual Conference. I am also really grateful for the separation devices you borrowed for me from Auckland.

Rafea, I truly appreciate your helps throughout the first three years and all your massages after you left LASRA. I cherish the discussions we had for investigating the experimental methods, even they turned into arguments sometimes. I need these opportunities to absorb your expertise and establish my ideas. Although Sue only was my supervisor for about two months, I still appreciate the chance she provided for me to experience whole tanning processes. I enjoyed a lot and had a new hobby of make leather crafts.

Many thanks to LASRA for their collaboration and fundings. Catherine and Wenkai, thank you for helping me a lot deal with collagen and SDS-PAGE at LASRA. Goeff, thank you for helping me shave the sheep skin every time.

I would also like to express my gratitude to some other persons who helped me in this project. Karin, Rachael, and Claire, thank you for teaching me all the things I needed for cat tests and thank you for your help with my cat tests. I love cats! Trevor, thank

you for helping me operate the huge centrifuge and discuss the sample separation. Martin, thank you for carrying out volatile compounds analysis. Saeedeh, thank you for helping me carry out fatty acids analysis. Pat, thank you for the sample NMR results. Fliss, thank you for the AAs tests. Xiong, thank you for the chats about data processing by using SAS.

A big thank you to my friends Huixin, Jie, and Boning for their encouragement. The time we spent together for chatting, exercise, road trip, and sharing hotpot was the energy supplement.

I would like to express my heartfelt appreciation for my family. My parents, thank you always support my life decisions and always love me. Thank you for the financial support after my scholarship expired. My parents-in-law, thank you for your financial support for living costs. My husband, Bo'xin, I am glad that we met each other. Thank you for your love and accompany especially when I was depressed or anxious. Thank you for help me find my confidence back. Thank you for cooking and cleaning when I was writing my thesis.

Finally, I want to hug myself. I did well in the past six and half years.

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Abbreviations

AAFCO	Association of American Feed Control Officials
AAs	Amino Acids
CRD	Central Retinal Degeneration
CSW	Chromium-containing Solid Waste
DHA	Docosahexaenoic Acid
DHS	Dynamic Headspace
DM	Dry Matter
EDTA	Ethylenediaminetetraacetic Acid
EPA	Eicosapentaenoic Acid
FD	Freeze-dried
FNE	Feline Nutrition Expert
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography-Mass Spectrometry
GC–O	Gas Chromatography–olfactometry
GF	Gel Filtration
GLC	Gas Liquid Chromatography
GPC	Gel Permeation Chromatography
HPLC	High-Performance Liquid Chromatography
HPSEC	High-Performance Size Exclusion Chromatography
HS	Static Headspace
ITEX	In-tube Extraction
LASRA	NZ Leather & Shoe Research Association

LC	Liquid Chromatograph
MAE	Microwave-assisted Extraction
MPI	Ministry for Primary Industries
NMR	Nuclear Magnetic Resonance
NRC	National Research Council
OPA	O-phthalaldehyde
PCA	Principal Component Analysis
PDMS	Polydimethylsiloxane
PLSR	Partial Least-Squares Regression
PVA	Polyvinyl (alcohol)
RP HPLC	Reversed Phase High-Performance Liquid Chromatography
SAFE	Solvent-Assisted Flavour Evaporation
SBSE	Stir Bar Sorptive Extraction
SCFA	Short-chain Fatty Acids
SDE	Simultaneous Distillation/Solvent Extraction
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy
SFE	Supercritical Fluid Extraction
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
TGA	Thermogravimetric Analysis
TNBS	2,4,6-Trinitrobenzenesulfonic Acid
UAE	Ultrasonic-assisted Extraction

1. Introduction

1.1 Domestic cat (*Felis silvestris catus*) food

It is believed that cat domestication started about 4,000 BC in ancient Egypt (Bradshaw *et al.* 1996), while more recent genetic and archaeological research shows that it could be much earlier, around 10,000 years ago. Agricultural practices were just starting, when human begin to domesticate cats in the Fertile Crescent (Driscoll *et al.* 2009). The earliest direct evidence for this was a sub-circular grave (see Figure 1.1) from the Mediterranean island of Cyprus where an eight-month-old cat and a human were discovered buried together, which was discovered by Jean-Denis Vigne and colleagues of the National Museum of Natural History in Paris (Vigne *et al*. 2004). It is believed that cats became associated with humans and stayed in their houses to capitalise on mice and food residues (Driscoll *et al*. 2009). The domestic cat has become the most dominant species of felid around the world through geographical expansion (Bradshaw *et al*. 1996).

Figure 1.1 The ancient grave (9500 years ago) of a cat (inside circle) and a human (above) (Vigne *et al.* 2004)

It could be argued that cats are not totally domesticated as they revert to a feral state very easily, and also still have an extremely well-developed hunting reflex (Bradshaw *et al*. 1996). The food intake behaviour of domestic cats is also affected by their hunting instincts. Domestic cats consume multiple small meals randomly throughout the day rather than eating their daily intake in one sitting when they are given free access to enough food. Similarly, wild cats consume small prey throughout day and night (Thorne 1992).

Unlike other companion animals, but similar to other large felids such as lion, tiger and panther, the domestic cat is an obligate carnivore (Randi and Bernardino 1991), which means it needs to subsist on a meat-based diet (Bradshaw *et al*. 1996), This also means that cats require much more protein of animal origin in their diets than other pets (Hewson-Hughes *et al*. 2016). In addition to this macronutrient intake difference, cats also have some unique nutrient requirements due to evolutionary mutations picked up in their metabolic pathways which have led to some nutritional peculiarities. Taurine is an important amino acid for cats which is mainly obtained from food. It is necessary to maintain the cat's reproductive, visual and cardiac health, however because only minimal taurine is produced endogenously (MacDonald *et al*. 1984), it needs to be provided by the cat's diet. Vitamin A and fatty acids such as arachidonic acid are also essential for cats and need to be provided in the diet (MacDonald *et al*. 1984). More detailed discussion of the essential nutrients required by cats is included in the nutritional peculiarities section 1.1 below.

Cats prefer food with high levels of protein and lipids. Other additives which can stimulate the taste sense receptors (taste buds) in cats also drive this palatability (Oliveira *et al*. 2016). This is vital when considering materials to produce or develop cat food because cats might reject nutritious food which is not palatable. Therefore, cat food palatability will also be thoroughly discussed below in the diet palatability section 1.2.

Nutrition and palatability are two essential factors of cat food, and they are always both considered by pet food companies when developing new products and cat owners when choosing commercial food for their cats. Commercial cat food is commonly divided into three formats according to different water content: dry food (6%–10% moisture), semi-moist food (35% moisture), and canned food (>75% moisture). Commercial cat

food should be nutritionally complete and provide all of the essential nutrients that cats have been shown to need (NRC, 2005; AAFCO, 2025), and also be attractive to cats. Most commercial cat foods follow the criterion established by the Feline Nutrition Expert (FNE) Subcommittee of the Association of American Feed Control Officials (AAFCO) to meet the nutrition requirements and ensure palatability. A similar organisation, the Fédération Européenne de l'Industrie des Aliments pour Animaux Familiers (FEDIAF, 2025), exists in Europe and setsets similar standards. The minimum levels of protein in all types of cat food have been determined to be 26% on a dry matter basis (AAFCO, 2025). Table 1.1 indicates all detailed established nutrition standards of cat food from the AAFCO Cat Food Nutrient Profiles for growth and reproduction and for adult maintenance (AAFCO, 2025).

Table 1.1 Cat food nutrient profiles (AAFCO, 2025)

Nutrient	Units DM Basis	Growth & Reproduction Minimum	Adult Maintenance	
			Min	Max
Protein	%	30	26	-
Arginine	%	1.24	1.04	-
Histidine	%	0.33	0.31	-
Isoleucine	%	0.56	0.52	-
Leucine	%	1.28	1.24	-
Lysine	%	1.2	0.83	-
Methionine-cystine	%	1.1	0.4	-
Methionine	%	0.62	0.2	1.5
Phenylalanine-tyrosine	%	1.92	1.53	-
Phenylalanine	%	0.52	0.42	-
Threonine	%	0.73	0.73	-
Tryptophan	%	0.25	0.16	-
Valine	%	0.64	0.62	-
Taurine (extruded)	%	0.1	0.1	-
Taurine (canned)	%	0.2	0.2	-
Fat	%	9	9	-
Linoleic acid	%	0.6	0.6	-
Arachidonic acid	%	0.02	0.02	-
Calcium	%	1	0.6	-
Phosphorous	%	0.8	0.5	-
Potassium	%	0.6	0.6	-
Sodium	%	0.2	0.2	-
Chloride	%	0.3	0.3	-

Nutrient	Units DM Basis	Growth & Reproduction Minimum	Adult Maintenance	
			Min	Max
Magnesium	%	0.08	0.04	-
Iron	mg/kg	80	80	-
Copper (extruded)	mg/kg	15	5	-
Copper (canned)	mg/kg	8.4	5	-
Manganese	mg/kg	7.6	7.6	-
Zinc	mg/kg	75	75	-
Iodine	mg/kg	1.8	0.6	9
Selenium	mg/kg	0.3	0.3	-
Vitamin A	IU/kg	6,668	3,332	333,300
Vitamin D	IU/kg	280	280	30,080
Vitamin E	IU/kg	40	40	-
Vitamin K	mg/kg	0.1	0.1	-
Vitamin B1 (thiamine)	mg/kg	5.6	5.6	-
Vitamin B2 (riboflavin)	mg/kg	4	4	-
Vitamin B5 (pantothenic acid)	mg/kg	5.75	5.75	-
Vitamin B3 (niacin)	mg/kg	60	60	-
Vitamin B6 (pyridoxine)	mg/kg	4	4	-
Folic Acid	mg/kg	0.8	0.8	-
Biotin	mg/kg	0.07	0.07	-
Vitamin B12 (cyanocobalamin)	mg/kg	0.02	0.02	-
Choline	mg/kg	2400	2400	-

1.1.1 Nutritional peculiarities

Cats have different nutritional requirements from other mammals especially with unique requirements for some amino acids, vitamins and fatty acids. In contrast, no unique requirements for minerals have been determined (NRC, 2005).

1.1.1.1 Protein and amino acids

Cats are strict carnivores, so they have a higher requirement for animal sources of protein than other domestic animals. The protein requirement of an animal generally refers to the minimum nitrogen requirement and specific amino acid requirement

(MacDonald *et al*. 1984). In addition to the essential amino acids needed by most animals, cats also require some unique amino acids.

Taurine (see Figure 1.2) is a non-protein β-amino sulfonic acid. It is normally generated from methionine and cysteine in the liver or other tissues (such as brain) by most mammals (except cats) and it is not generally considered an essential free amino acid. In addition, most mammals are able to bind either glycine or taurine to bile acids which are required for fat digestion, while cats can only use taurine for bile acid conjugation.

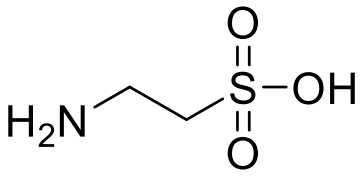
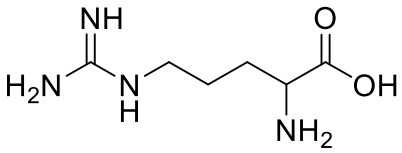


Figure 1.2 Molecular structure of taurine

In cats, however, the low biological activity of the two enzymes (cysteine sulfinate decarboxylase and cysteine dioxygenase) which are essential for the synthesis of taurine, can lead to a serious taurine deficiency. This deficiency can cause central retinal degeneration (CRD), and hence cats need to obtain sufficient taurine from their daily food (Park *et al*. 1991). In addition to CRD, taurine deficiency can also lead to reproductive failure and dilated cardiomyopathy (Zaghini and Biagi 2005).

Arginine (see Figure 1.3a), one of the most essential intermediates in the urea cycle, helps detoxify and excrete ammonia. Ammonia is the main metabolite of high protein diets and is noxious for cats if it accumulates in the body rather than being converted to urea for excretion in urine. The high concentration of arginase which breaks down arginine in the liver of cats discourages arginine releasing into the general circulation, so the kidney become the place for arginine synthesis from citrulline the precursor of arginine (see Figure 1.3b). High enough concentrations of citrulline can act as a substitute for arginine, although the utilization of citrulline is more inefficient than arginine (Morris 1985). However, unlike most mammals, cats also have very low levels of the enzymes pyrroline-5-carboxylate synthase and ornithine aminotransferase which convert glutamine and proline to citrulline (Morris 1985). Therefore, an arginine-free high protein diet for cats causes acute hyperammonaemia within three hours, with symptoms such as vocalization (moaning), emesis, ptyalism, hyperactivity, hyperesthesia, ataxia, tetanic spasms, emprosthotonus, extended limbs with exposed

claws, apnoea, and cyanosis, which in some cases even results in death (NRC, 2005). Luckily, animal tissue proteins contain sufficient arginine, so cats rarely suffer hyperammonaemia caused by arginine deficiency.



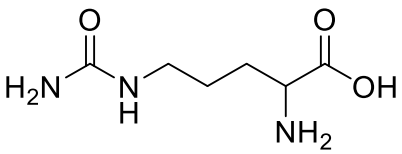


Figure 1.3a (left) and 1.3b (right) The molecular structures of arginine and citrulline

There are other reports of specific amino acid deficiencies in isolated cases. Kittens which lack threonine exhibit cerebellar dysfunction, but this can be reversed by offering extra threonine (MacDonald *et al*. 1984). Lysine deficiency has been reported after feeding diets containing high levels of cereal protein (MacDonald *et al*. 1984), while both proline and tyrosine are both dispensable (non-essential) amino acids for cats.

1.1.1.2 Vitamins

Most mammals have the ability to convert β-carotene to vitamin A in the intestinal mucosa or tissues such as liver, but cats are different. Neither feeding, nor parenterally offering, supplemental β-carotene can solve vitamin A deficiency. Cats can absorb β-carotene, but they lack the enzyme which is essential for transforming β-carotene to vitamin A, so they must obtain vitamin A from their food (Schweigert *et al*. 2002). Vitamin A deficiency results in poor night vision and skin health. However, diets containing high amounts of raw animal liver, which is extremely high in vitamin A, can be toxic for cats. Excess vitamin A can lead to lesions such as deforming cervical spondylosis (Seawright *et al*. 1967) and hepatic fibrosis (Guerra *et al*. 2014).

Most animals can synthesise vitamin D by exposing their skin to ultraviolet (UV) rays in sunlight. Morris (1999) showed that vitamin D is not able to be generated in both hairy and shaved kittens. The concentration of a precursor for pre-vitamin D, 7-dehydrocholesterol, is very low in the skin of cats probably because of the high activity of reductase which catalyses the synthesis of cholesterol by using 7-dehydrocholesterol (Morris 1999). The vitamin D requirement for cats is very low and although endogenous synthesis is unable to meet their requirements, vitamin D is present in prey species, so they rarely suffer from a deficiency,

Endogenous niacin synthesis is limited by a different mechanism to vitamin D in cats. In this case the precursor is removed very fast due to high activity of the enzyme picolinic carboxylase, so endogenous niacin levels produced down an alternative metabolic pathway are very low (NRC, 2005). However, cats can obtain enough niacin from their normal diet too.

Vitamin E deficiency has also been observed in kittens fed a high unsaturated fatty acid (canned red tuna) food (MacDonald *et al*. 1984), which caused steatitis, also known as yellow fat disease.

1.1.1.3 Fatty acids

Linoleic acid and arachidonic acid are essential omega-6 fatty acids for cats. Linoleic acid is linked to liver, hair and skin health, and arachidonic acid is unable to be converted from linoleic acid in cat liver due to the low activity of a vital enzyme (Δ6 desaturase) which catalyses this reaction, so cats also need to receive it from their diet (MacDonald *et al*. 1984). According to the criterion of AAFCO, minimum arachidonic acid levels should be 0.2% on a dry matter basis in food (AAFCO 2025). Arachidonic acid is important for wound healing, growth, liver and skin health.

Omega-3 fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are also essential fatty acids for cats. DHA is important for neurological and retinal development and EPA is indispensable for supporting the body's natural anti-inflammatory response (Watson *et al*. 2023). However, Lenox and Bauer (2013) reviewed literature which showed the potential adverse effects of excess supplementation of omega-3 fatty acids in dogs and cats such as altered platelet function (negative effects on wound healing), lipid peroxidation and altered immune function.

1.1.2 Diet palatability

Palatability is a term which describes the preference of an animal for a diet. Cats have a distinct predilection for some flavours. However, in addition to flavour, other factors such as food texture or feeding behaviour also influence palatability.

1.1.2.1 Flavour preference

Cats select food via both smell and taste, and taste is more reliable because smell can be disrupted by aging, adverse weather conditions, and drugs (Hullár *et al*. 2001). The vomeronasal organ, which is regarded as a third chemosensory system, only reacts to social odours (Hart and Leedy 1987). Most research has concentrated on only one of the four cranial nerves related to taste, which is the facial nerve. The chorda tympani fibre and the geniculate ganglion cell bodies are the most popular study targets (Bradshaw 1991).

Quantitatively, the major receptor group in cats detects amino acids. Amino acid receptors are very sensitive to those detected as 'sweet' in humans, such as proline, cysteine, ornithine, lysine, histidine, and alanine, while 'bitter' amino acids, such as arginine, isoleucine, phenylalanine and tryptophan, are rejected by those receptors (Bradshaw 1991). Monophosphate nucleotides, which can accumulate in killed prey animals, are inhibitors of amino acid receptors, which indicates why cats have no preference for carrion (Bradshaw 1991).

Although cats prefer sweet amino acids, in fact, cats lack the sweet taste receptor (Li *et al*. 2006). No preference was observed when cats were offered water and sucrose dissolved in water (Bartoshuk *et al*. 1971). However, cats prefer milk with added sucrose or lactose, although the reason is believed to be because of textural differences rather than taste (Gary *et al*. 1977).

The second dominant feline taste receptor group is the acid system, which is sensitive to carboxylic acids, phosphoric acids, and other Brønsted acids such as nucleotide triphosphates and protonated imidazoles. Acid receptors also respond to some amino acids such as histidine, cysteine and taurine. Cats also prefer short-chain fatty acids (SCFA) when compared to medium-chain fatty acids (Bradshaw 1991).

The remaining feline receptor units are regarded as the X group, which respond to bitter substances such as quinine, alkaloids, tannic, and phytic acids (Bradshaw 1991).

Cats can taste water itself, so tests of potential palatants in pure water might be obscured by the water taste (Bradshaw 1991).

As for olfaction, less research has been carried out in cats compared to dogs. However, studies have shown even the smell of meat cannot overcome neophobia (the avoidance

of unfamiliar food) in cats (Bradshaw 1986). This shows the link between odour and food is highly complex.

Research investigating the palatability of by-products has shown that liver, red meat and blood are the most attractive to cats (Crane *et al*. 2000), while some cats prefer fish, but others reject it (Watson *et al*. 2023).

1.1.2.2 Factors that Influence Palatability

Water content is one of the factors that can influence palatability, because cats have been shown to prefer moist canned food with a water content analogous to that of meat (70–85%) compared to semi-moist or dry food (Zaghini and Biagi 2005). As for dry food itself, size, shape and texture of the kibble are also significant aspects to food palatability. Cats do not accept sharp edged kibbles, which might injure the mouth and stomach (Trivedi and Benning, 1999). Adding fat to the surface of kibble can improve palatability due to changes in food texture, but some studies have shown increasing the fat level from 15% to 45% had no further effect on palatability (Kane *et al*. 1987). However, further studies indicates that there may be a threshold level of fat with higher content above this 15–45% level being less palatable. The addition of 25% yellow grease to a diet made it more attractive than either 10% or 50% in the diet (Kane *et al*. 1981). The temperature of food is also an important determinant of palatability, with a temperature range between 15 ‑ 50°C optimal (Sohail, 1983).

The nutritional requirements and eating behaviour of cats also influence food choice. Some researchers have identified a primacy effect, which means the food preferences of cats could be affected by the diet of their mother during gestation and lactation, or become established in early life from four weeks to six months of age (Stasiak 2001 and 2002). In addition, cats may reject unfamiliar food due to neophobia or accept it due to neophilia. Feeding behaviour of the owner also influence food preference of domestic cats. Owners can use puzzle toys to release dry food or put wet food in hollow toys to encourage interaction and activity (Herron and Buffington 2010).

1.1.3 Market scale and variety

The numbers of cat owners are increasing globally. In New Zealand, 41.6% of families keep at least one cat, which is one of the highest cat ownership rates in the world

(Euromonitor International, 2022). In 2022, more than 1.22 million pet cats were kept in New Zealand (Euromonitor International 2022). The number of households owning a cat and the population of cats are both growing steady in New Zealand (see Table 1.2; Euromonitor International, 2022). An overview of the actual and predicted growth in the retail value of cat food in New Zealand from 2008–2027 is shown in Figure 1.4. The total cat food market is currently estimated to be valued at 435.3 NZD million. What stands out in this figure is the steady growth of cat food sales through periods of global economic recession, and the growth rate was 1.2% in current terms in 2022.

Moreover, cat mixers and treats show stronger growth than prepared pet food and more companies are now concentrating on raw or fresh natural ingredients (Euromonitor International, 2022). These data provide strong evidence that the cat food market is continuing to grow in value and has potential to grow further and there are huge opportunities to develop novel cat food products.

Table 1.2 Cat owning households ('000 Households) and Cat population ('000s of animals) (Euromonitor International, 2022).

Year	2017	2018	2019	2020	2021	2022
Households owning a cat	727.8	734.8	736.3	744.8	751.9	756.4
Cat Population	1,147.0	1,165.0	1,189.9	1,219.0	1,221.0	1,218.8

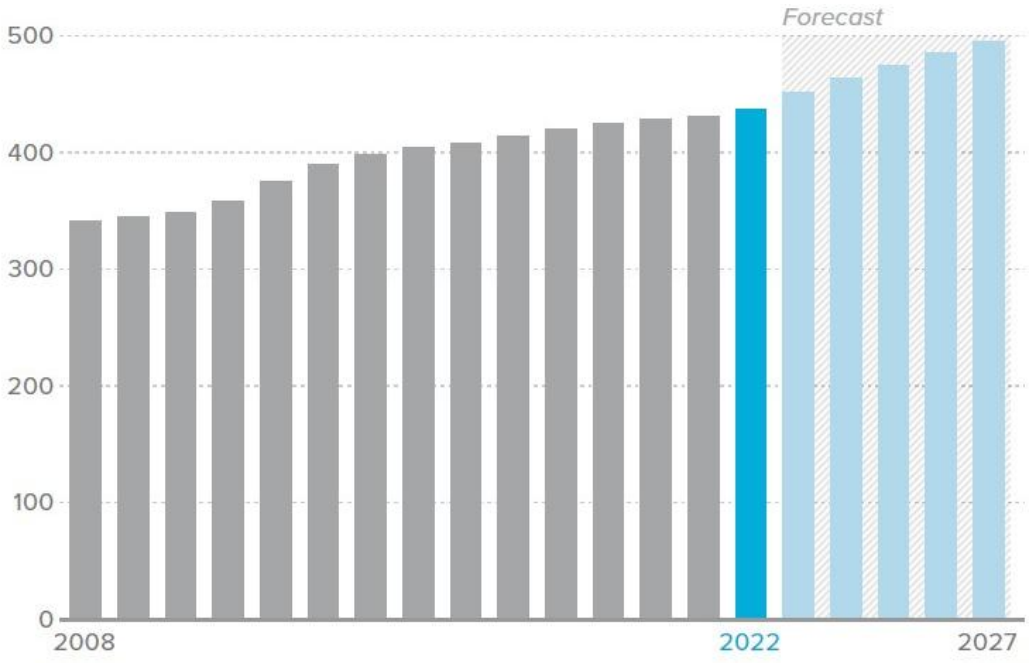


Figure 1.4 Sales of cat Food (Retail Value NZD Million) from 2008 – 2027 (Euromonitor International, 2022)

Most raw materials used for commercial pet food or pet food additives (especially protein) are from chicken, fish, and even protein-rich plants (see Table 1.3). Relatively few products use beef or lamb, so there is an opportunity to develop bovine- or ovine-sourced material for novel cat food products (Plantinga *et al*. 2011).

Table 1.3 Top protein types contained in pet food products and number of launches by region between March 2017 and March 2018 (Mintel, 2018).

Protein type	Europe	Asia Pacific	North America	Latin America	Middle East & Africa
Vegetable protein	718	88	0	8	71
Pea protein	52	28	101	1	3
Soybean proteins	48	96	3	24	4
Potato protein	39	27	29	3	2
Soy protein concentrates	2	8	17	47	6
Beans	8	64	0	5	0
Plant protein	29	30	0	1	5
Textured soy protein	0	0	4	38	0
Isolated soy protein	2	20	6	7	1
Vegetable protein isolate	15	9	0	0	7
Vegetable protein concentrate	4	2	0	12	3

Protein type	Europe	Asia Pacific	North America	Latin America	Middle East & Africa
Mange-tout protein	0	12	0	0	0
Poultry protein	174	7	0	0	9
Chicken protein	66	10	0	5	1
Meat protein	26	17	0	9	3
Fish collagen	0	51	0	0	0
Pork protein	30	8	0	4	0
Salmon protein	18	2	0	0	1
Sheep protein	**20**	**0**	**0**	**0**	**0**
Hydrolysed chicken protein	6	4	0	8	0
Pork protein isolate	0	0	0	17	0
Turkey protein	12	2	0	0	1
Beef protein	**11**	**0**	**0**	**1**	**1**
Pig collagen	0	10	2	1	0
Fish proteins	9	2	0	0	0
Duck protein	8	1	0	0	1

1.2 Leather wastes

1.2.1 Leather industry

The raw materials for producing leather are hides and skins. The external tissue covering of larger animals such as cows are called hides while that of smaller animals such as sheep are called skins (Covington 2009). Fresh animal hides and skins are very moist and begin to degrade 5–6 h after the animal is slaughtered (Kanagaraj *et al*. 2005). Although a primitive intervention of eliminating the subcutaneous fatty layer and moisture, slows down the effects of noxious bacteria and delays the decay process (Harris and Veldmeijer 2014), a more complicated tanning process is the only choice for producing a stable product – leather, which is able to be preserved for a long time (Hodges 1995; Reed 1972). Traditionally, the word “leather” refers to a group of materials obtained from raw hides and skins, which includes tanned leather, parchment, vellum, and oil or fat cured skins or hides (Hodges 1995). However, it has been suggested that leather should only refer to products from hides or skins which are kept non-putrescible under warm moist conditions (Marion and Thomson 2006), so technically, according to this definition, only hides and skins that have experienced a tanning process can be called leather.

Tanning is considered to be the first manufacturing process developed by humans (Marion and Thomson 2006), with leather one of the oldest materials used by humans (Harris and Veldmeijer 2014). Ubiquitous stone scrapers and cut marks on animal bones from the Palaeolithic period associated with skinning have been discovered, and support a hypothesis that humans migrated to the northern hemisphere and wore skins in order to keep themselves warm in the temperate climate (Charles 1997). Leather production has therefore existed from the prehistoric period, over 5000 years ago (Covington 2009). The first written evidence of leather production is found in Law 274 in the famous Hammurabi Code (1795–1750 BC) which recorded the salaries for curriers and tanners (Reed 1972; Harris and Veldmeijer 2014). The oldest leather shoe, which is 5500 years old, was discovered in a cave in Armenia (see Figure 1.5; Pinhasi *et al*. 2010). Archaeological excavations show that leather became more widespread from the Roman period onwards in Europe and the Mediterranean, and the development of vegetable tanning methods could be the reason (Driel-Murray 2000; Groenman-van Waateringe *et al*. 1999).

Figure 1.5 A photo of oldest leather shoe found to date which is 5,500 years old (Gasparian, 2010)

There are many beneficial properties of leather such as resistance to tearing, puncturing, and abrasion. Good heat insulation and water vapour transmission are also included (Kite and Thomson 2007). Currently, leather plays diverse roles in many commercial products from the more traditional clothes and footwear, to toys, artwares and car accessories.

Hides and skins are important by-products of the meat industry in New Zealand (NZ). Although export revenues decreased visibly in 2020 and 2021, it brings consistent and significant export revenue for the NZ government (see Table 1.4).

Table 1.4 New Zealand export revenue of hides and skins 2018–2024, NZ$ million (Stats NZ and MPI, 2022).

	Actual				Forecast		
	2018	2019	2020	2021	2022	2023	2024
Hides and Skins	396	354	241	201	295	300	310

The number of leather enterprises in New Zealand have declined in the last 20 years, although numbers have stabilised since 2012 (see Figure 1.6).

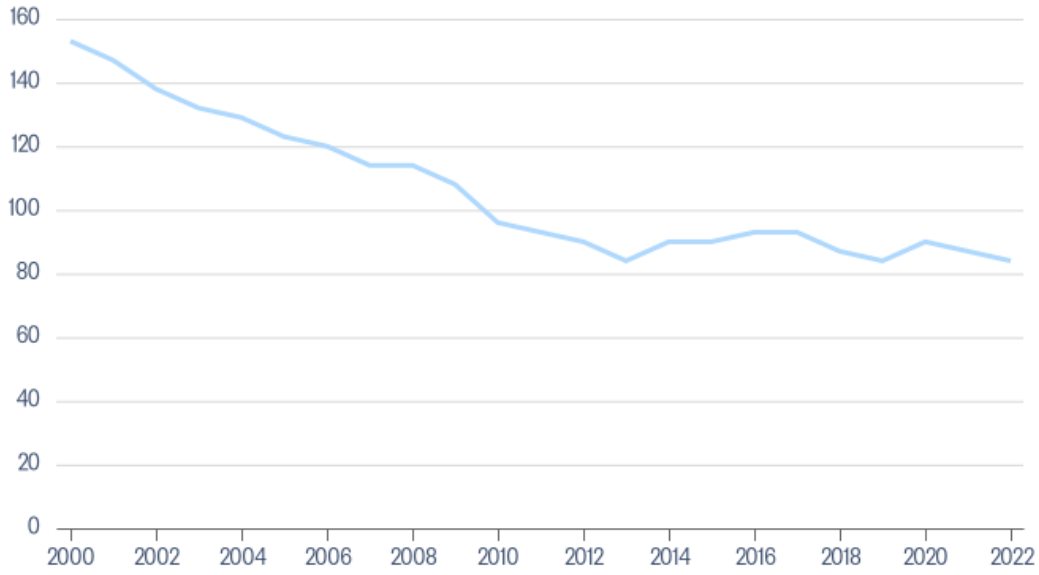


Figure 1.6 The numbers of enterprises in the leather tanning, fur dressing and leather product manufacturing industry in New Zealand (Stats NZ 2022)

The data presented do not fully capture the economic potential of the hide, skin and ultimately leather industry and the waste streams they produce. There are significant opportunities to add value to the hide, skin and leather industry. Understanding tanning process is the first step in order to achieve this goal.

1.2.2 Tanning process

Modern industrialised leather manufacturing consists of three steps: pre-treating, tanning and post-tanning (Kanagaraj *et al*. 2015). There are a series of steps that make up the pre-treatment stage: curing, soaking, fleshing, trimming, liming, deliming, bating and finally pickling (see Figure 1.7).

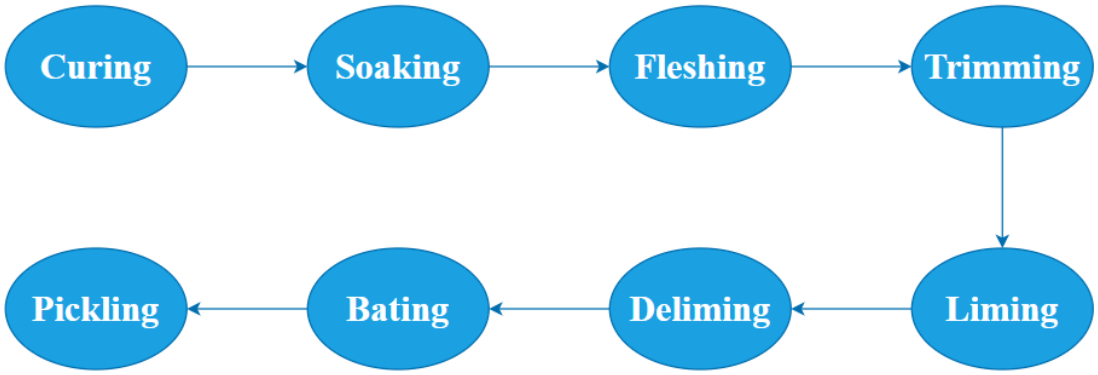


Figure 1.7 Progress of leather pre-treating

Curing preserves the raw animal skins or hides using salt in order to prevent microbial attack (Fela *et al*. 2011). Soaking is then a simple rehydration process which also removes the preserving salts, dirt, blood, and unstructured proteins or proteoglycans (Leafe 1999). Fleshing is the chipping-off of the rest of the meat layer of the hides or skins, and trimming is the cutting of the edges of the hides and skins to obtain the shape required. The addition of lime (liming) then opens up the fibre structure which is mainly for the removal of hair and epidermis. Calcium hydroxide suspension is commonly used for liming. After liming, the pH of hides or skins is high, so deliming neutralises the alkali, lime and caustic soda. Ammonium salts such as ammonium chloride (NH_4Cl) and ammonium sulphate ($(NH_4)_2SO_4$) are commonly used for deliming. Bating is the process that removes non-structural proteins, hair root and pigment mainly using proteases such as pancreatic enzymes and bacterial proteases, which makes the hides and skins soft. Finally, the hides and skins are stored at a low pH to maintain stability before the tanning process takes place. This final pre-treatment stage is called pickling, which allows hides and skins to be transported across the world for extended periods of time (Covington 2009).

The tanning process is the conversion of putrescible organic compounds, especially proteins, of raw hides and skins into more stable compounds which resist decay and are appropriate for multiple usages. Tanning technology has a long history which can be traced back to the Palaeolithic period (Ian, 2010). The ancient Greeks were able to tan leather using tree barks and leaves, and this was the first record of vegetable tanned leather which is still manufactured today (Covington 2009). The initial advantage of vegetable tanning was the use of plant polyphenols to stabilise the collagen in the skins or hides to prevent decomposition (Covington 2009). Vegetable tanning was almost the only leather processing method used up until the end of nineteenth century (Covington 2009), when problems began to occur due to changing fashions and the wearing of corsets. The iron bracing strips in corsets reacted with the plant polyphenols in vegetable tanned leather when worn close to the skin, and dark stains appeared on the garments. Mineral tanning utilising chromium salts was developed to solve this problem. Chrome tanning is currently the most common mainstream industrialised leather tanning process, not only because it prevented garments from developing dark stains, but also because it was faster and more reliable than vegetable tanning and many leather industries converted to chrome tanning at the beginning of twentieth century (Covington 2009). It is estimated that approximately 90% of the leather in the world is currently processed by chrome tanning (Covington 2009), and basic chromium(III) sulphate is the most common tanning agent. In 1858, Friedrich Knapp used chrome alum and a series of processing steps which occurred consecutively in the same container. This ‘single bath’ process is generally regarded as the start of the establishment of chrome tanning (Knapp 1858). The technique was subsequently improved by Augustus Schultz who was granted the first commercial chrome tanning patent in 1884 with a novel process which is referred to as the ‘two-bath’ process (Schultz 1884). Researchers are attempting to find alternative metals which have similar properties to chrome for tanning due to the negative environmental impact of chrome tanning. Cations such as aluminium (mainly potash alum), titanium, zirconium, and iron have all been investigated, but only at small-scale so far and chrome tanning still holds a dominant position. Other novel tanning agents (such as some unsaturated oil and synthetic polymers) are also used for particular leather production (such as oil tanning for chamois leather), or as attempts to improve the tanning process (Covington 2009).

Post-tanning processes are the methods that occur after the main tanning reaction, and include re-tanning, dyeing and fat-liquoring. Re-tanning uses a wide variety of agents to modify the properties and performance of the leather. Dyeing is the colouring process that changes the appearance of leather, while fat-liquoring prevents fibre sticking and controls the level of softness and water resistance using different functional oils (Covington 2009).

1.2.3 Leather waste stream

A great deal of waste is generated from the processing steps described in the previous section, so the leather industry is regarded as a highly polluting industry (Fela *et al*. 2011). A detailed description of the tanning process and the waste generated from it, is shown below (see Figure 1.8). The leather waste consists of three parts: solid waste, water waste, and gaseous waste. Four million tons of solid waste is generated by the global leather industry each year (Fela *et al*. 2011). It is calculated that only 27% of the raw hides or skins become leather products (Fela *et al*. 2011).

The solid waste generated during the production of leather consists of trimmings, fleshings, shavings, buffing dusts and hair, which are normally disposed of on the land surface (land treatment), buried in landfills or incinerated (Hughes 1988). Land treatment relies on the capacity of soil to degrade the solid waste, and it can reduce the harmfulness of potential pollutants by adsorption or precipitation. Land treatment is much cheaper than the use of landfill or incineration and can also utilise the waste as both a fertilizer and soil ameliorant (Hughes 1988). However, because the population of the world has exploded since the 20th century (from 1.6 billion in 1900 to over 8 billion by 2023), available land for the degradation of the waste has decreased significantly. The recovery or reuse of tannery waste has become an important research target, and some examples of types of research being undertaken will be discussed in the literature review Chapter 2.1.

Solid waste from the leather industry is classified into two types, hide and skin waste and tanned waste. Hide and skin waste, which are also regarded as untanned waste are generated during the pre-treating process. This type of waste mainly contains raw trimmings, fleshing, and shaved hair. Tanned waste, which should be strictly termed leather waste, is generated during the tanning process. Tanned waste contains leather

trimmings and shavings. Different strategies of recycling and re-use are applied to each category. It is known that approximately 80% of waste is untanned waste (Puntener 1995), and this project only deals with this kind of waste. However, research into applications for recycling tanned waste may still provide some relevant ideas, so both waste streams will be discussed.

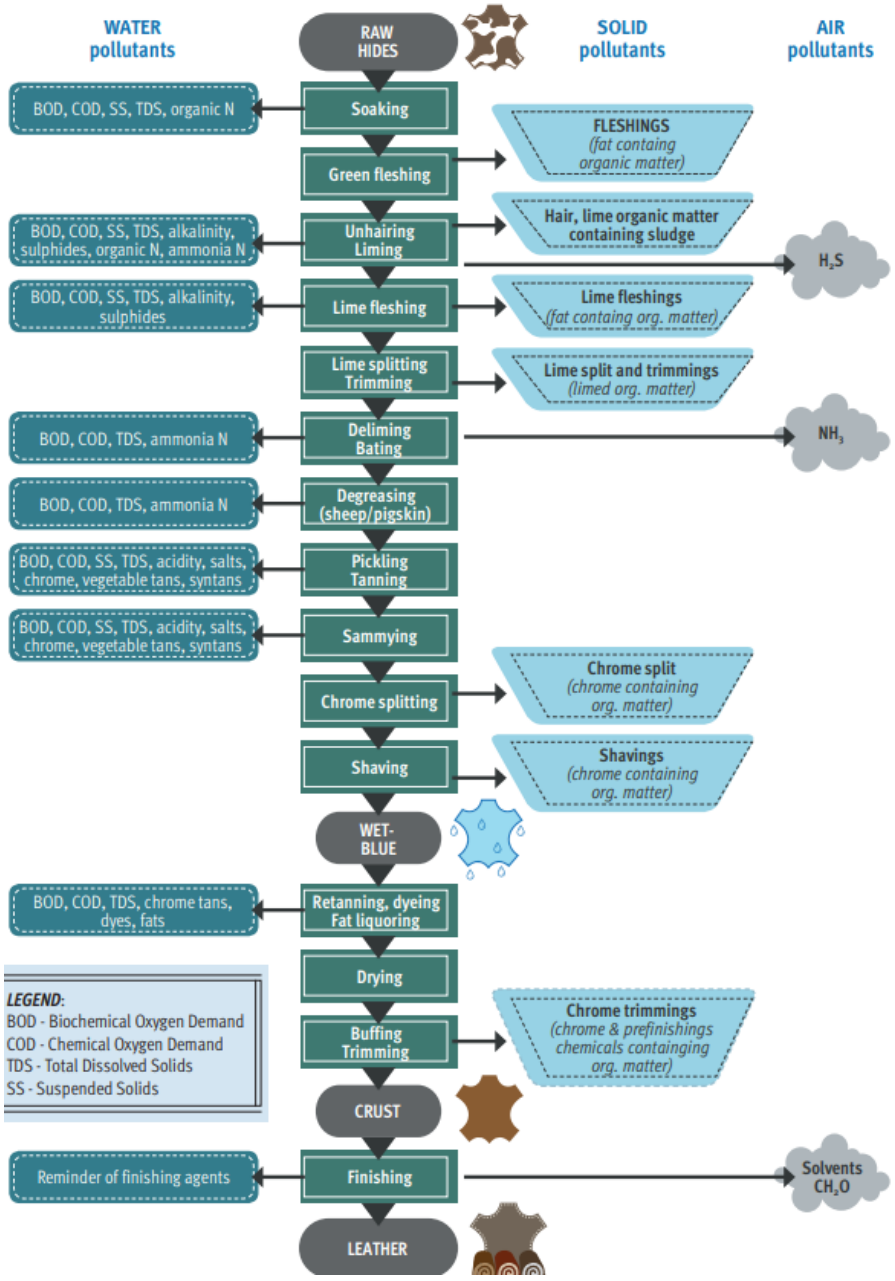


Figure 1.8 Description of the tanning process and the waste generation (Buljan and Král 2019)

1.2.3.1 Raw trimmings

Raw trimmings are limed raw hides and skins which contain little harmful mineral chemicals. They are composed of approximately 60–70% water, 20–30% protein, 2–5% lipids, other carbohydrates and mineral salts (Naffa 2017). The dominant protein in raw trimmings is collagen, the name for a large triple helical protein group, with at least 28 different types of collagens identified (Kadler *et al*. 2007). Some vertebrate tissues such as tendon, cartilage, bone and skin rely on collagen to provide distinct and vital tensile functions (Kadler *et al*. 2007). There are nine types of collagens in hides and skins; types I, III, IV, V, VI, VII, XII, XIV, and XVI (Naffa 2017). Type I collagen is the most abundant and important in animal skin, so the term ‘collagen’ usually refers to type I collagen. Similarly, in this review unless specifically mentioned, collagen refers to type I collagen.

Collagen is a nutritionally incomplete protein, with two major differences in amino acid content compared to other proteins. Firstly, the amino acid tryptophan (see Figure 1.9a) is absent (Tarannum *et al*. 2016), and secondly, the amino acid hydroxyproline (see Figure 1.9b) is a unique amino acid which exists in very high levels in collagen and is normally used to calculate the purity of collagen. Hydroxyproline is a metabolite of proline (Wu *et al*. 2011). In 1902, hydroxyproline, also formerly known as "oxyproline" was first isolated from acidic hydrolysates of gelatin by Emil Fischer at the University of Berlin (Wieland and Bodanszky 1991). Fischer was also the second winner of the Nobel Prize for Chemistry in the same year (Wieland and Bodanszky 1991). Therefore, through testing the levels of hydroxyproline, the collagen content of hides and skins can be measured.

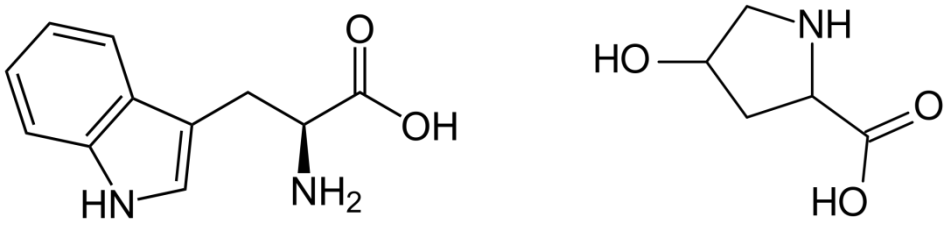


Figure 1.9a (left) and 1.9b (right) Structure of tryptophan and hydroxyproline

The structure of collagen has a repeating domain of triplet amino acids: -(Gly-X-Y)$_n$-. As a result, about 33% of the amino acids in collagen are glycine (Gly). Glycine was

first isolated from gelatin by Henri Braconnot in 1820 in France (Wieland and Bodanszky 1991). Additionally, the X in the triplet is usually proline (Pro) and Y in the triplet is usually hydroxyproline (Hypro). It has further been shown that 12% of triplets are –Gly-Pro-Hypro-, 44% are –Gly-Pro-Y- or –Gly-X-Hypro- and 44% are -Gly- X-Y-, where X and Y are other amino acids (Covington 2009). These triplets build two αI(1) chains and one αI(2) chain, which together form the triple helix structure of collagen (see Figure 1.10), which was first described in 1968 (Ramachandran *et al*. 1968). In Figure 1.10, a single polypeptide chain of $(Gly\text{-}Pro\text{-}Pro)_8$ folds into a triple helix. The sequence along the chain is A, B, C and the chain B runs in the opposite direction to chain A and C. The dash lines are NH···O hydrogen bonds and the arrows are the direction from the donor to the acceptor.

Figure 1.10 Triple helix structure of collagen (Ramachandran *et al.* 1968)

1.2.3.2 Fleshing

Fleshing is mainly subcutaneous tissue or hypodermis which mostly contains lipids (Pappas 2014), although the nutrient content of this component is not the same across different species. The lipid content of sheep skin is higher than that of cow hide, while the protein content of sheep skin is lower than cow hide (Ozgunay *et al*. 2007). There is little historical research on the utilisation of fleshing waste, with most of the literature published in the last twenty years. This section will be introduced in Chapter 2.1.2.

1.2.3.3 Tanned wastes

Most tanneries around the world use the chrome tanning method, so tanned waste usually consists of chromium-containing solid waste (CSW). Compared with untanned wastes, CSW contains harmful chromium, and collagen crosslinking makes it more stable. More uses for CSW have been published compared to untanned waste, and these can be divided into two types: direct utility and chrome eliminated utility. More detailed information will be presented in Chapter 2.1.3.

1.3 Aims and objectives

The ultimate aim of this project is to produce an edible and palatable product which attracts cats from the waste stream associated with leather production and characterise the palatants in this product.

This will be achieved by first reviewing literature of both usage and application of leather wastes, and the nutrient requirements and palatability enhancement of cat food. Secondly, choosing one type of suitable leather waste. Developing and refining a hydrolysis methodology for the raw material is the next step. The product will be provided to cat palatability panels at the Centre for Feline Nutrition and palatable sample will be selected. Finally, characterising the palatants and explaining the mechanism of palatability enhancement by multiple analytical chemistry methods are necessary.

In the future, the established methodology of product preparation and analysis can be applied on other type of leather wastes or quality control for palatable products from leather wastes.

2. Literature review

This review will cover the different aspects of the project. The first section describes the utilisation of different kinds of leather wastes, especially their possible use in the animal food industry. The second section describes methods of palatability testing and the development of palatants for cat food. Finally, food flavour analysis will be summarised in the context of palatability.

2.1 Utilisation of leather wastes

Leather waste consists of solid waste, water waste, and gaseous waste. However, only solid waste, which is the bulk of the waste produced during leather production, is the focus of this project. This includes untanned wastes such as trimmings and fleshings and tanned wastes.

2.1.1 Trimmings

Most research which discusses the recycling and reusing of trimmings usually concentrates on collagen extraction. To give a more comprehensive perspective, this review will also discuss some of collagen extraction techniques from other animal source such as fish and chicken. In addition to food additives, other utilisations of collagen such as clinical applications will also be covered.

2.1.1.1 Collagen extraction

Collagen extraction should be carried out without any alteration of its structure which will then affect its physical and chemical properties. However, if the temperature is higher than 37°C, gelatin is generated, which is large protein molecule like collagen but without some of its bioactivities. Many classical techniques are used for collagen and gelatin extraction such as acidic, neutral-salted and enzymatic methods. Alkaline extraction methods (pH 12) always break down the large collagen molecules (>100 kDa) into smaller molecules (<100 kDa) (Morimura *et al*. 2002), so it is rarely used for native collagen extraction. In addition to raw trimmings, extracting collagen from other sources such as fish skin and scales are also worthy of exploration in this review.

Some inorganic substances which exist in limed raw trimmings affect the yield of collagen during the extraction process, so researchers have investigated a variety of pre-treatment methods to increase the extraction yield. Li *et al.* (2008) compared the pre-treatment effects of ethylenediaminetetraacetic acid (EDTA) and hydrochloric acid (HCl) and the results indicated the HCl method (calf trimmings soaked in two volumes of 15% NaCl solution, then adjusted to pH 2 with HCl) produced higher collagen yields. Morimura *et al.* (2002) also used 0.6M HCl to remove inorganic compounds, and hexane to remove fats from pig skin before collagen extraction. Butyl alcohol (10%) has also been used to remove fats (Kittiphattanabawon *et al*. 2005). In addition to the removal of inorganic substances and fats, some non-collagenous proteins can also be removed by endogenous proteases, before collagen extraction (Muralidharan *et al*. 2013). Non-collagenous proteins can also be removed by optimized alkaline and then acidic pre-treatment, and this pre-treatment method can also provide a suitable pH environment for gelatin extraction from Alaska Pollock skin (Zhou and Regenstein 2005).

Acetic acid (0.5M) or citric acid (0.5M) are the most common acids used for native collagen extraction at low temperature (e.g., 0–4 °C for 24–48 h). Many researchers use the two acid extraction techniques with slight modifications. Sadowska *et al.* (2003) successfully extracted collagen from Baltic cod skin using the two acids, while others have shown phosphoric acid, in addition to acetic acid and citric acid, also has potential for use in collagen extraction (Chakarska *et al*. 2006).

Neutral salt extraction methods are used by some research groups. Heinrich *et al.* (1971) used collagen cyanogen bromide (CNBr) digestion under two different extraction regimes, one using 0.05 M pH 7 tris-HCl buffer with 1M NaCl at 4 °C and the other using acetic acid. A water–salt extraction method for gelatin was developed by Neklyudov *et al.* (2003), where the optimum conditions for pig skin gelatin extraction were at the ratio of 1:5 (w/v) with 5% of sodium chloride at 50 °C for 4 h.

Many enzymes have been used in collagen extraction. Muralidharan *et al*. (2013) chose to add 1% papain to chicken feet at 30 °C for 28 h to extract collagen, and achieved a yield of 32.16 %. Suphatharaprateep *et al.* (2011) isolated two strains of bacteria, a gram positive *Bacillus cereus* CNA1 and a gram negative *Klebsiella pneumoniae* CNL3

to produce collagenases which were used together with 0.5 M acetic acid for collagen extraction from salmon skin. The yields were 54.6 % and 53.0 % respectively.

Some novel or assorted methods have also been established. A combination of acidic and enzymatic treatment methods has been developed. Li *et al.* (2008) added 0.5 M acetic acid and 2 % pepsin to extract collagen from limed raw trimmings at 4 °C for 48 h and achieved a 41.31 % yield.

Huang *et al.* (2016) established an extrusion–hydro-extraction method to obtain gelatin from tilapia fish scales. They smashed the scales and added double-distilled water (ddH_2O) to scale powder, and then 1.26 % citric acid (pH 2), or 9.37 % acetic acid (pH 2) at a ratio of 4.7:1 (w/v). This method increased the gelatin yield 2-3-fold.

2.1.1.2 Utilisation

Collagen is widely used for clinical applications such as tissue adhesion, vascular grafts, aortic heart valves, drug delivery matrices, wound dressing, and tissue engineering scaffolds (Stenzel *et al*. 1974). These uses are due to its unique properties such as low biodegradability and weak antigenicity (Lee *et al*. 2001). Li *et al.* (2003) showed that collagen extracted from limed calf trimmings, using pepsin at low temperatures, had similar qualities to commercial collagen. An alkali-treated collagen gel was produced by Saito *et al.* (2008) which showed enhanced collagen stability against enzymatic degradation and improved mechanical strength *in vivo*. Collagen products are required in surgery as prostheses, and to provide mechanical protection of surgically repaired nerves (Sundar *et al*. 2011). Collagen can also be used as a carrier or shield for drug delivery (Lee *et al*. 2001), as a covering for wounds, and as a membrane for haemodialysis (Sundar *et al*. 2011). Collagen biopolymers are biomaterials which are regenerative, biocompatible, nontoxic and cost effective, and can also be used for skin burn treatments (Sundar *et al*. 2021).

Approximately 6.5 % of the total gelatin gel production is used for the covering of capsules in the pharmaceutical industry. Gelatin can also be treated as a binding or compounding agent for medicated pills and pastilles (Liu 2002).

Native collagen is of limited use in the food industry, because of its insufficient water holding capacity and ability to form emulsions (Neklyudov *et al*. 2003). It is also chemically inert under physiological conditions, so collagen is hard to digest by animals

directly (Harkness *et al*. 1978). Hydrolysed collagen fractions, however, can be used for food-related applications, and can contribute much more than just acting as nitrogen sources. Collagen hydrolysates have the ability to facilitate digestion and remove heavy metals, toxins, and carcinogens (Neklyudov *et al*. 2003). In addition, collagen hydrolysates can also be used as protein supplements for animal feeds and can even be functional after hydrolysis.

In general, most acid protein hydrolysates are used to enhance food flavour. One advantage of acid hydrolysis of protein is the low cost of the process. However, disadvantages include the total destruction of tryptophan, a limited loss of methionine, and the conversion of glutamine into glutamate and asparagine into aspartate (Pasupuleti 2008). In 1820, the first successful acid hydrolysis of gelatin at a high temperature was carried out by the French chemist Henri Braconnot (Wieland and Bodanszky 1991). Complete collagen hydrolysis using 6 M HCl at 110 °C for 2–6 h produces peptides (Pasupuleti 2008), and for 24 h produces amino acids (Naffa 2017). Alkaline hydrolysis can produce tryptophan, but most other amino acids are completely destroyed, so this method is not popular in the food industry (Pasupuleti 2008).

Collagen hydrolysates also have some functional properties and can be vital for some functional foods. Limed raw trimming collagen is a desirable material for producing edible meat sausages casings (fibrous collagen) (Gennadios 2002), fillers or thickening agents (Sundar *et al.* 2011). Soluble collagen fragments can be used as binders, while insoluble fragments can be used as texturizers (Henrickson *et al*. 1984).

Collagen digested products are also used in cosmetic materials. Morimura *et al.* (2002) extracted gelatin at pH 3 and 60 °C for 1 h, and produced a product that had a high water retention capacity and could be used for repairing rough skin.

Other more uncommon uses for collagen digested products also exist. Vasileva-Tonkova *et al.* (2007) hydrolysed collagen from calf skin waste using an alkaline proteinase (subtilisin DY), which is produced by *Bacillus subtilis* strain DY. This commercial subtilisin DY is inexpensive and has high specific activity. The hydrolysates were then used as peptones for bacterial culture.

2.1.2 Fleshings

Some researchers have used fleshing as an energy substitute for replacing fossil fuels. Colak *et al.* (2005) converted sheep fleshing into biodiesel due to its considerable lipid content. Biodiesel is a cleaner and more renewable fuel which is obtained from biological sources such as vegetable and animal oils and lipids (Shemelis and Jorge 2017). Data showed fleshing biodiesel had acceptable quality, so fleshing is definitely a potential material for producing biodiesel. Devaraj *et al.* (2018) developed fat extraction methods requiring less energy consumption and characterized their production by Fourier Transform Infrared (FTIR) spectroscopy, Scanning Electron Microscopy (SEM), Thermal Gravimetric Analysis (TGA), Gas Chromatography - Mass spectrometry (GC-MS) and Proton Nuclear Magnetic Resonance spectroscopy (H^1 NMR) spectroscopy. In addition to biodiesel, biogas is another renewable energy that can be produced by using leather fleshing. However, the yield of biogas from fleshing waste is much lower than that from carbohydrate rich solid wastes due to its high pH and low C:N (3:2). However, a co-digestion with organic municipal solid waste in a pH 6.5 environment can increase the biogas yield markedly (Shanmugam and Horan 2009).

In addition to using fleshing as the raw material to produce fuel directly, an indirect utilisation of fleshing has been established and hydrogen has been generated. Hydrogen is one of the most efficient and cleanest energy sources and most research attention into hydrogen production has been aimed at artificial photosynthesis. Anaerobic fermentation using organic carbon compounds, however, results in comparatively higher productivity than photosynthesis. In addition, the technology of anaerobic fermentation is much simpler with lower energy and money costs (Kubendran *et al*. 2017). Aathika *et al.* (2018) co-digested fleshing waste with secondary tannery treatment plant sludge under anaerobic condition and produced biohydrogen.

There has also been some food-related research using fleshing. Early work utilised partially rendered fleshing which was added into the diet of rainbow trout (Cowey *et al*. 1979). After treatment, the hide fleshing mainly contained collagenous protein and saturated fat and was regarded as a source of non-essential amino acids and energy. Before being added into fish feed, HCl was used to hydrolyse proteins in the fleshing.

The results indicated that the weight gains and protein utilisation of both test and control groups of rainbow trout were similar and no adverse effects on the fish were observed.

Bhaskar *et al.* also published a series of studies looking at fleshing hydrolysis and its application for feed supplements. In 2007, they delimed sheep and goat skin fleshing by using H_2O_2 and HCl producing very little or no H_2S successfully. They also tried to ferment delimed fleshing by culturing with *Pediococcus acidolactici* and hydrolysis by an enzyme named Protex-6L with the optimized condition calculated by response surface methodology (RSM). This method was suggested for the production of an arginine, phenylalanine and tyrosine supplement (Bhaskar *et al*. 2007). In 2009, they used formic acid and propionic acid (1:1) to hydrolyse steam cooked (80°C, 15min) fleshing. Response surface methodology was also used for better antioxidant properties design. This method was suggested for production of an arginine, leucine and lysine supplement (Amit 2009). In 2010, they concentrated on fermentation again and this time *Enterococcus faecium* HAB01 (GenBank #FJ418568) was used (Kumar Rai *et al*. 2010). In 2016, they reported that a fleshing fermentation or hydrolysis product has some inherent biofunctionalities such as antioxidant and antibacterial properties and tested its acute and sub-acute toxicity. The results showed both delimed fleshing fermentation and hydrolysis products were safe for feeding to animals (Jini *et al*. 2016).

Other uses of fleshing waste are significant, such as, as biological fertilizers and nanofibrous materials. Ravindran *et al.* (2019) compared the effects of solid state and submerged state hydrolysed fleshing for compost and vermicompost production to improve growth and yield of tomato plants. Selvaraj *et al*. (2019) investigated developing nanofibrous materials from acid fleshing hydrolysates mixed with polyvinyl (alcohol) (PVA) through an electrospinning process. This novel material had better thermal stability than neat PVA material, and also covering traditional coir material gave the material enhanced sound absorbing ability, especially at lower frequencies.

2.1.3 Tanned wastes

Direct applications of CSW are chromium recovery as chrome cake for the tanning or re-tanning processes (Dettmer *et al*. 2010, Khan *et al*. 2018, Tahiri *et al*. 2007), incineration for energy (Pati *et al*. 2014), biogas (Pati 2013, Yılmaz *et al*. 2007), and as

an adsorbent for water decontamination (Oliveira *et al*. 2011, Kantarli and Yanik 2010, Kong *et al*. 2013, Fathima *et al*. 2012).

Only the application of waste eliminated chrome is discussed in this part of the literature review. CSW contains shavings, which are tiny and thin leather fractions generated during the shaving operation which creates the desired thickness of leather, and trimming. The shavings are rich in chromium cross-linked protein collagen, so chrome-removed CSW has similar treatment as untanned wastes such as hydrolysis. Paul *et al*. (2013) used an oxidation method for de-chroming CSW and then thermal and enzymatic treatments were used to produce gelatin solutions and collagen concentrates for poultry feed. Quadery *et al.* (2016) used 0.1 % of a trypsin enzyme to also obtain hydrolysates from chrome shavings for poultry feed. Pati and Chaudhary (2015) hydrolysed and purified CSW at a chemical engineering scale for fertilizer production.

Finally, Ocak *et al.* (2018) investigated green composite films using Alcalase-treated collagen hydrolysate from leather solid wastes and chitosan to developing polymeric materials to replace plastic packaging and wrapping products.

2.1.4 Conclusion

Many solid leather wastes utilisation processes concentrate on reusing proteins and lipids. The extraction and hydrolysis of proteins, especially collagen and gelatin, are the most important processes used for animal feeds, food additives, medical and cosmetic materials, bioenergy, bio-materials, and fertiliser by acidic, alkali, and enzymatic methods. Lipids are mainly used for producing bioenergy. As for animal food utilisation, no research group has investigated using leather waste for cat food.

2.2 Developing palatants for cat food

This review part will discuss some research about possible palatability enhancers of cat food and the methods to test whether cats like a particular food.

2.2.1 Palatability improvements

Little research has been published on palatability enhancement, however one study showed that cats prefer hydrolysed/digested protein and emulsified fat mixtures diluted

in water to water alone, and samples with higher concentrations of proteins and fats were more palatable (Gary *et al*. 1977).

Many cat food palatant patents refer to phosphorus-based compounds. It is known that using phosphoric acid in preparation of cat food has three main advantages: it avoids the creation of struvite kidney and bladder stones, improves oral health and develops palatability. Brand and Bryant (2012) suggested that phosphorus-based compounds are palatable because they interact the TIR1 and T1R3 acid receptors in cats. Shao *et al.* (2005) used potassium pyrophosphate as a palatability enhancer, while Lee *et al.* (2007) used tripolyphosphate as a palatability enhancer.

Further work has shown that a combination both sodium pyrophosphate and yeast extract can improve palatability (Oliveira *et al*. 2016). Yeast extract contains proteins, peptides, nucleotides, and some free amino acids such as alanine, lysine, and glutamic acid, which trigger amino acid receptors, and 2 % yeast extract can enhance palatability of dog food (Teshima *et al*. 2007). However, the addition of 1.5 % yeast extract to a cat dry diet mixed with moist diet reduced palatability (Ogoshi *et al*. 2014).

Sodium glutamate and sodium inosinate, which are flavouring agents, also work as palatable additives for cat food (Trivedi and Benning, 1999).

Besides additives, some process methods for food are able to improve palatability, such as the Maillard reaction. The Maillard reaction is a complex chemical reaction between amino acids and reducing sugars which occurs when they are heated together and is a non-enzymatic browning reaction which produces distinct flavour compounds (Maillard 1912). Extrusion can also enhance palatability, particularly for diets containing vegetable ingredients such as soybean and corn meal (Hullár *et al*. 1998).

2.2.2 Methods for palatability testing

There are two types of animal panels used for palatability assessment: expert panels which are kept in pet colonies (laboratory), and normal pets which stay in homes with their owners. The expert panels are exposed to intensive testing, so they are used to a variety of foods. Therefore, expert panels are more accurate and dependable than in-home panels (Larose 2003). It is also significant to compare the results from both panel types, with some studies suggesting that more consistency is observed between the two when feeding wet food rather than dry food (Griffin *et al*. 1984).

The primary challenge for palatability testing of pet food is that animals lack the linguistic abilities to express themselves, and pet preferences are not always obvious and easy to interpret. Establishing and developing indirect objective methodologies are vital to obtain preference information from eating behaviour and the reaction of cats to different diets (Koppel 2014).

Palatability is related to both preference and acceptance, so different trials are carried out for each. For preference testing, the most popular method used is the two-bowl test (or paired stimulus or *versus* test). In this test, two different diets (usually one test sample and one control sample) are offered simultaneously to the cats for a period of time to obtain the total intake and mean intake for each diet, and then a comparison is made using statistical analysis. Griffin (1996) summarized the data analysis strategy for a two-bowl test. The two-bowl test is arranged in individual enclosures to prevent any social disturbance or competition between cats. A positional switch of the bowls is also important between each day of testing because some cats have a left or a right bias (Waterhouse 1967). In contrast, acceptance testing only uses one bowl and the test can be run for a few minutes, over a longer period, or even *ad libitum* to simulate natural situations (Christelle *et al*. 2015). The one bowl test is more suitable for in-home panels because it is similar to the situation when new food is introduced by the cat owner (Smith *et al*. 1984).

More novel complementary techniques and criteria have been applied to palatability trials based on those basic methods. The operant conditioning or concurrent schedule paradigm concentrates on the animal's motivation for consuming a diet (Rashotte and Smith 1984). A liking test offers more meaningful factors than a simple one bowl test such as the percentage of finished bowls, the eating speed, and a comparison with the reference consumption rate of each animal (Becques and Niceron 2014). Consumption kinetics can also provide quantitative information about the average time before the food starts to be consumed, average consumption per meal and number of gaps between meals (Roguès 2014). Some research also focusses on cat body language and behaviour while they are selecting food. Two taste reactivity patterns were found by van den Bos and co-workers (2000), cats which lick or sniff the food bowl with a lip lick and then groom their face, are showing hedonic taste reactivity patterns; while cats that lick or sniff the food with a nose lick, are showing aversive taste reactivity patterns. Some cat behaviours such as 'flick ears backwards', 'flick tail' and 'groom body' have been

related to aversive taste reactivity patterns (Savolainen *et al*. 2016). Real-time monitor systems for capturing palatability testing which have a video recording camera and automatic weigh scales to measure food intake and feeding behaviour have been developed (Al-Souti 2012). Similar systems have revealed that cats spend significantly longer sniffing at a diet they find less attractive (Becques *et al*. 2014).

2.2.3 Conclusion

There are limited published papers or accessible patents on palatability enhancers for cat food. As for typical palatability test methods, they are well-established and developed for *in vivo* novel food testing. However, the palatability tests only show preference or food choice. To understand the drivers of palatability, more tests are needed such as food flavour analysis.

2.3 Food flavour analysis

Classic measurements of palatability for pet food concentrates on the differences in food consumption which can only provide limited information of food preference (Watson *et al*. 2023). Understanding why palatable pet food attracts pets is not only important for further research, but also essential for the pet food industry. Although many factors could influence palatability, flavour is an important aspect just like human food. Flavour is a sensory impression with many factors (Reineccius and Peterson 2013). Two of the major factors, aroma and taste will be discussed here.

The stimulation of chemoreceptors (taste buds) located in the oral cavity by food can provide the taste, while stimulation of the olfactory receptors (olfaction) located in the nasal cavity by volatile components can provide the smell (Aldrich and Koppel 2015). Aroma and taste analysis can be included in sensory analysis.

Sensory analysis can be carried out by a variety of instruments, which not only allows identification of components of palatability, but also is beginning to explain the reason in a fundamental way. However, even for human food, identifying aroma or taste compounds is a difficult task because laboratory instrumentation is not as sensitive to many compounds as the human sensory system (Reineccius and Peterson 2013). Moreover, some compounds in food contribute much more flavour in extremely small

quantities than abundant compounds in the same food (Reineccius and Peterson 2013), which makes analysis more difficult. Therefore, pet food flavour research presents a challenge, as like infants, pets cannot communicate with us directly by talking (Koppel 2014). Very few studies have focussed on pet food flavour analysis, so instead human food such as meat and broth flavour analysis will be discussed.

2.3.1 Volatile compounds analysis

Aroma compounds contribute the chemical stimuli responsible for the odour of foods, so they must be volatile to be sensed (Reineccius and Peterson 2013). It is not rare for the browning flavours in meats to have nearly 1000 volatile substances, and over 8000 volatile constituents have been found in foods (Reineccius and Peterson 2013).

To be analysed, aroma must first be extracted and gathered from the food. Food is normally crushed, homogenized, or blended using a variety of methods. Flavours can alter quickly in most fresh plant and animal tissues, because they contain active enzyme systems (Dirinck *et al*. 1981; Drawert *et al*. 1965). The common methods for aroma extraction can be divided into few specific types: headspace methods, distillation methods, solvent extraction, sorptive extraction, and others.

The static headspace (HS) method is direct and very simple, but its low sensitivity is an obvious defect (Reineccius and Peterson 2013). For more complete research of aroma compounds in a food, static headspace is usually incorporated with other aroma isolation techniques such as purge and trap methods, and solvent-assisted flavour evaporation (SAFE) (Qian 2000). The purge and trap method is also known as dynamic headspace (DHS), which is a headspace concentration method (Reineccius and Peterson 2013). The modified DHS can work with small samples in order to be automated which is called in-tube extraction (ITEX) (Reineccius and Peterson 2013). According to research using coffee powder, DHS is the most sensitive method while HS is not as sensitive (Gil *et al*. 2007).

The most traditional steam distillation method is simultaneous distillation/solvent extraction (SDE), which is also known as the Likens-Nickerson method. This is also one of the oldest methods used for aroma extractions (Reineccius and Peterson 2013). This method allows good sampling of the volatile isolates in a food, but the accurate measurement of their proportions is limited (Pollien and Chaintreau 1997).

Aroma extraction by direct solvent extraction can only be carried out on samples which don't contain any lipids. This is one of the simplest and most efficient methods (Reineccius and Peterson 2013), although not much relevant research has incorporated this method recently.

Solid phase extraction (SPE), stir bar Sorptive extraction (SBSE), polydimethylsiloxane (PDMS) foam and solid phase microextraction (SPME) are all sorptive extraction approaches which are relatively novel methods for aroma extractions. SPME is the most broadly used method for isolation of volatiles in food (Reineccius and Peterson 2013). The SPME device is shown in Figure 2.1. An inert fibre which is coated with one kind of adsorbent, is located in the headspace of tube with samples or alternatively put into the sample and adsorbs aroma compounds (Reineccius and Peterson 2013). After allowing enough time for the volatiles to be adsorbed, the fibre is then injected into a GC and thermally desorbed volatiles are released into the carrier flow for analysis (Reineccius and Peterson 2013).

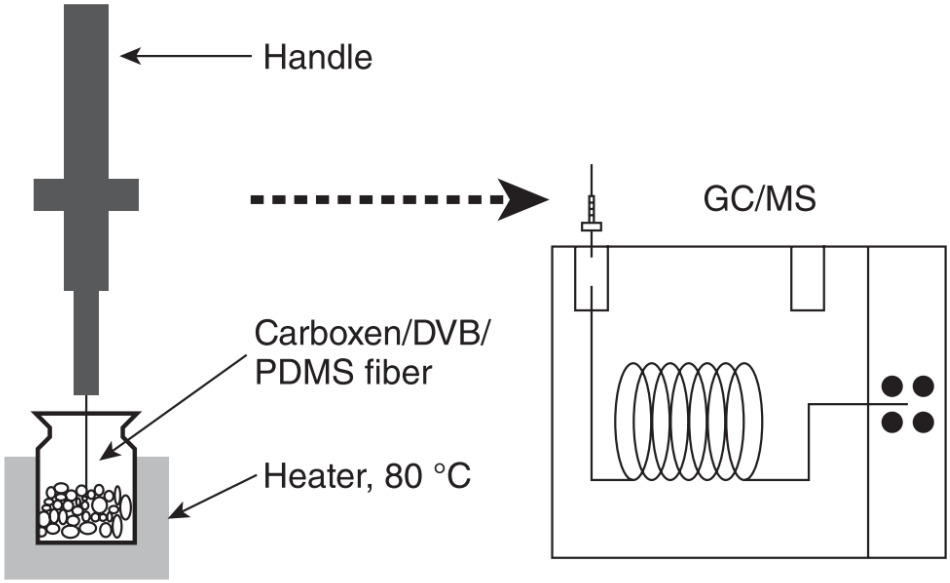


Figure 2.1 Schematic of a SPME device (Marsili 2002)

Gas chromatography (GC) is often used for aroma analysis after extraction. The most common method used is gas chromatography-mass spectrometry (GC–MS). Yin *et al*. (2020) extracted volatiles from six dog foods using HS-SPME, and then 55 aroma compounds were identified by GC-MS and followed by partial least-squares regression (PLSR) analysis. Nine compounds were selected for preference testing and three key

aroma compounds were determined (Yin *et al*. 2020). In other work aroma components of three different extruded dry dog food were isolated by SPME and then analysed by GC-MS (Di Donfrancesco and Koppel 2017).

Recently, gas chromatography–olfactometry (GC–O) has become more popular and uses the human nose as the detector (Sohail *et al*. 2022). This approach has solved the problem of matching GC peaks with odour descriptions (Reineccius and Peterson 2013). A review was published in 2022 which summarised in total 332 odorants characterised in thermally cooked meat food by GC–O (Sohail *et al*. 2022). The group have also discussed the formation mechanisms of aromas which are mainly lipid degradation, Maillard reaction and others (Sohail *et al*. 2022).

The limitations of GC-MS and GC-O, which are mainly the differences between observations of a single aroma compound and a combination of volatiles, is discussed in a review by Chambers and Koppel (2013). Aroma analysis is still a formidable task, and methods are still developing. For instance, studies have concentrated on a single food aroma, such as soy sauce aroma type baijiu, but its key aroma compounds are still unknown (Duan *et al*. 2022).

2.3.2 Non-volatile compounds analysis

In contrast to aroma compounds, taste compounds are commonly non-volatile. They contain a wide range of compounds with molecular weights ranging from 100 – 20,000 Da such as small peptides, nucleotides and salts (Laing and Jinks 1996). There are five basic tastes: sweet, salty, sour, bitter, and umami (Delwiche 1996).

Different techniques have been used to attempt the quantify taste compounds. Flavour amino acids and free amino acids in beef broth were tested using an amino acid analyser after being separated by gel filtration (GF) (Cambero *et al*. 1992). Other research has used an Agilent 1100 liquid chromatograph (LC) with a UV detector to analyse amino acids (Zhan *et al*. 2020).

Peptide distribution is normally tested by using LC with a GF column and UV detector (Lan *et al*. 2010). Zhang *et al*. (2021) characterised flavour peptides in bone meal hydrolysate by using ultrafiltration and GF for separation, RP-HPLC for purification and LC-MS/MS for analysis. Nucleotide flavour compounds were also identified in a study of mutton soup (Zhao *et al*. 2021).

Degree of protein hydrolysis is the vital factor to monitoring the whole hydrolysis reaction (Nielsen *et al*. 2001). Many methods for the measurement of the degree of hydrolysis of proteins have been used, such as pH-stat, trinitrobenzenesulfonic acid (TNBS), o-phthalaldehyde (OPA), trichloroacetic acid soluble nitrogen (SN-TCA), and formol titration, and these have been summarised and compared by Rutherfurd (2010).

As for lipids extraction and analysis, Hewavitharana *et al*. (2020) reviewed fat extraction methods from food and derivatization of fatty acid methyl esters for GC-MS analysis. In addition to traditional isolation approaches, microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), and supercritical fluid extraction (SFE) methods followed by acid, alkali, and improved derivatization were discussed (Hewavitharana *et al*. 2020). Caponio *et al*. (2003) studied degradation of the lipid fraction from bouillon cubes by using high-performance size exclusion chromatography (HPSEC), and Wood *et al*. (2007) described fatty acids in meat products, especially in pig, cattle and sheep.

The ultrafiltration, GF, gel permeation chromatography (GPC) and size exclusion chromatography (SEC) are all typical separation (pre-treatment) methods used in taste compounds analysis. Ottinger and Hofmann (2003) used ultrafiltration with a molecular weight cut off of 1000 Da, followed by GPC, and then the selected low molecular weight fraction was analysis by High-Performance Liquid Chromatography (HPLC) in order to identify a taste enhancer (Alapyridaine) in beef broth.

2.3.3 Other analysis

The electronic nose (E-nose) is an analysis system with a series of electronic chemical sensors which can recognise specific aroma compounds and link them to an existing odour, while the electronic tongue (E-tongue) has the capability to identify some taste compounds through liquid media (Cheli *et al.* 2017). A study analysed twelve wet cat foods by using the combination of sensory analysis, GC-MS, texture measurements and gas sensors (E-nose) (Denis *et al*. 1999). The researchers believed that although E-nose presented less information on sample features than the other types of analysis methods, it was still reliable and fast. A combination of E-nose and E-tongue technology has been used for developing flavour and even predicted pet food preference (Oladipupo *et al*. 2011). Commercial canned pet food has be classified by E-nose and E-tongue (Éles

2013). However, E-nose and E-tongue can only provide the fingerprint characteristics of flavour compounds of pet food, so it could have challenges to understand the actual sensory properties (Koppel 2014).

A review has summarised both traditional sensory analysis techniques and the latest methods with consumer research for developing new meat foods (Ruiz-Capillas *et al*. 2021). As for pet food, consumer research can provide information from both owners and pets (Koppel 2014). These studies commonly use questionnaires with the questions about income, age, level of education, living area, number of pets, and relationships about pets (Forrest *et al.* 2023).

Some researchers use human panels to test pet food. Lin *et al*. (1998) studied the effects of lipids (beef tallow and poultry fat) in extruded dry pet food by using human sensory judges. Four commercial dried cat food products were profiled using 20 flavour attributes and 4 texture terms by a trained six-person human panel (Pickering 2009). There are disadvantages in using humans, because human taste and aroma perception are different to that of a pet (Koppel 2014). Olfactory perception has developed further in cats and dogs, while the sense of taste in humans is more advanced than that of cats and dogs (Neufeld 2012).

2.3.4 Conclusion

The methodology for volatile (aroma) compounds analysis is well established and systemic. The study of non-volatile (taste) compounds is much less developed without standard procedures, and methods used to investigate these vary depending on the target compound. Using humans for pet food analysis has its limitation.

3. Collagen hydrolysis and *in vivo* testing

3.1 Introduction

Leather wastes originate from a natural material, which usually has a complex chemical composition. The chemical composition of the leather waste also varies widely according to both leather processing stages and the different selection criteria of hides and skins (species, age, sex, nutrition, group, etc.). The morphological structure of animal skin is complex (Simeonova and Dalev 1996). The structure of skin consists of three clearly defined layers which are the epidermis, dermis and flesh layer (hypodermis) (see Figure 3.1). These three layers are characterised by their distinct organisation and distribution of macromolecular components that influence their biological functions (Montagna 1974). The outer epidermal layer mainly contains keratinocytes and barrier lipids while the inner hypodermis is mainly fat (Montagna 1974). The middle dermal layer makes up 90% of the weight of skin which is subdivided into the grain and the corium layers (Naffa 2017). The grain layer consists of a precise and loose collagen fibre network which contributes to the unique appearance of leather with a higher proportion of collagen III, while the corium layer shows a thicker and packed collagen fibrous structure lying parallel to the surface of the skin which contributes to skin strength (Covington 2009). Fibroblasts, which are the sites of biosynthesis of collagen, elastin and proteoglycans can be found in both the grain and corium layers (Montagna 1974). Additionally, these layers also contain immune-competent mast cells and macrophages (Driskell and Watt 2015).

Untanned solid leather wastes have a similar chemical composition to raw hides and skins and do not contain chromium compounds (Kanagaraj *et al*. 2006). The most obvious differences in the chemical constituents in raw hides and skins are proteins, fats and water. Raw hides of cattle are usually composed of 60–65% water, 30–35% protein, 1–10% fat, 1% carbohydrates and 0.5–1% mucopolysaccharide (Mocanu *et al*. 2015). The fat content can reach up to 15–20% in the sheep skins. Collagen comprises 90–95% of the protein component in skins and hides (Mocanu *et al*. 2015).

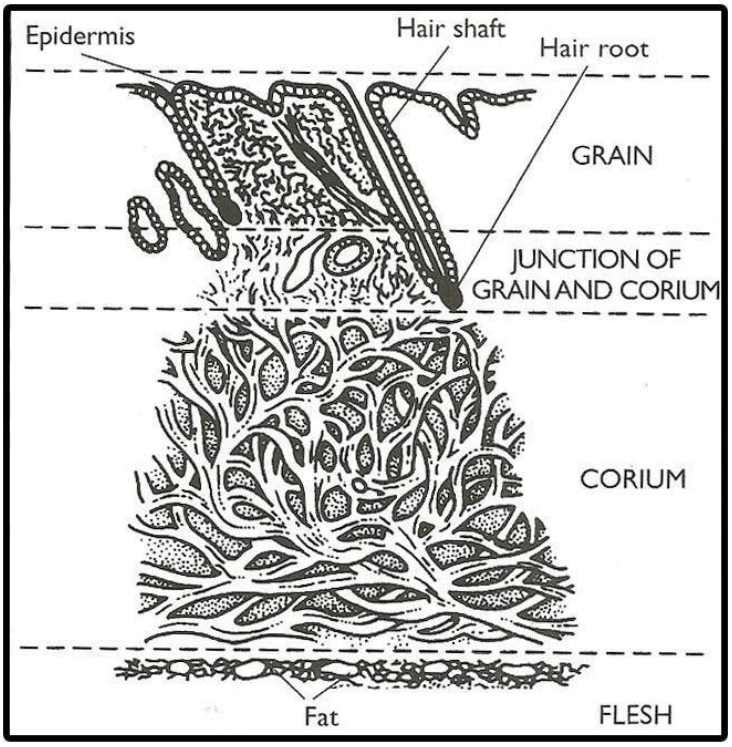


Figure 3.1 Anatomical features of skin showing the grain and corium layers (from Sharphouse 1983)

As previously stated above, except for water the highest chemical constituent of hides and skins is protein and the highest component of protein is collagen. Therefore, collagen was chosen as the raw material to investigate at the beginning of this project. Native collagen has limited application in the food industry due to its inadequate water-holding capacity and incomplete emulsifying properties (Neklyudov *et al*. 2003). Collagen is chemically stable under physiological conditions, which means it difficult for animals to digest. (Harkness *et al*. 1978). Hydrolysed collagen fractions, however, can be used for food-related applications, can contribute more than simply a source of nitrogen, and have the ability to facilitate digestion (Neklyudov *et al*. 2003). All sample preparation in this chapter was conducted in the Research lab at NZ Leather and Shoe Research Association (LASRA).

Protein hydrolysis produces a mixture of peptides and amino acids. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a commonly used laboratory method to understand hydrolysates molecular weight distribution. Traditional glycine SDS-PAGE is better for high molecular weight proteins (>20 kDa) and novel tricine SDS-PAGE is better for low molecular weight proteins (<20 kDa) (Schägger 2006).

A trained panel of eight domestic short-haired cats were used to test the preference for the samples produced. All testing was conducted at the Centre for Feline Nutrition, Massey University, Palmerston North.

3.2 Materials and methods

3.2.1 Materials

Raw materials

All animal skins and hides used in this work were from New Zealand farm-raised animals commonly used to produce leather. Hides from 5 year old cows were locally sourced, slaughtered at AFFCO Land Meats, Whanganui and then transported to LASRA for further treatment or temporary preservation at -20°C. Sheep skins were from 11–12 months old Romney crosses reared on local farms in the Manawatu and processed at Ovation Ltd, Feilding. Some cow hides and sheep skins were freeze dried and pulverised into powder and collagen were extracted by LASRA and tested before use.

Chemicals and regents

Hydrochloric acid (HCl), 37 % (w/w) was sourced from Carlo Erba Reagents S.r.l., Italy; Deionized water was obtained from a MilliQ Ultra- pure ThermoFischer water system (Dubudue, IA, USA), and 12 % and 16 % SDS-PAGE gel were produced and provided by LASRA.

3.2.2 Methods

3.2.2.1 Collagen hydrolysis

Three different solutions were prepared; deionized water, 1 M HCl and 6 M HCl. Collagen (extracted from cow hide) were dispersed into these solutions (w/v: 10:1), and were kept in an oven set at different temperatures for 4 h. The oven temperatures were 25 °C, 50 °C and 105 °C. The summarised treatments comparisons were shown below (see Table 3.1). The samples were centrifuged at 4500 x g for 10 min. The upper layers

were removed and run through an SDS-PAGE 12 % gel in the Research lab at LASRA. Three replicates of each sample were prepared.

Table 3.1 Collagen hydrolysis comparisons

Sample No.	Solution	Temperature (°C)
1	Deionized water	25
2		50
3		105
4	1 M HCl	25
5		50
6		105
7	6 M HCl	25
8		50
9		105

3.2.2.2 Additional material hydrolysis

Collagen (extracted from sheep skin), cow hide powder and sheep skin powder were hydrolysed by water at 50°C for 4 h. The samples were also centrifuged at 4500 x g for 10min. The upper layers were removed and run through an SDS-PAGE 16% tricine gel in the Research lab at LASRA. The process was repeated 3 times.

3.2.2.3 *In vivo* Tests

Collagen (extract from cow hide) was hydrolysed by water at 50 °C and 105 °C for 4 h. The mixtures were dialysed to remove salt and centrifuged at 4500 x g to obtain supernatant samples for feeding cats. A two-bowl test was used: one bowl contained a test sample and the other bowl contained a control sample. A 10 g test sample and 10 g control water or sample were offered to each cat in the panel for 2 h in a single cage. Three rounds of testing were carried out; a 50 °C hydrolysed sample compared with water, a 105 °C hydrolysed sample compared with water, and a 50 °C hydrolysed sample compared with a 105 °C hydrolysed sample. All three tests were run over 5 days with the position of each bowl (left or right) changed daily.

3.3 Results

3.3.1 Collagen hydrolysis

A brief description of the samples and photos of their appearance are given below (see Table 3.2; Figure3.2):

Table 3.2 Description of the sample appearance after collagen hydrolysis

Sample No.	Solution	Temperature (°C)	Solubility	Colour	Undissolved solid shape
1	Deionized water	25	-	Jellylike	Sticky Solution like glue
2	Deionized water	50	Almost all	Transparent	A trace of small-suspended solids
3	Deionized water	105	Almost all	Little yellow/brown	A trace of small-suspended solids
4	1M HCl	25	Rarely	White	Big floccule
5	1M HCl	50	Almost all	Little yellow/brown	A trace of small-suspended solids
6	1M HCl	105	Almost all	Little yellow/brown	A trace of small-suspended solids
7	6M HCl	25	Almost all	Transparent	A trace of small-suspended solids
8	6M HCl	50	Almost all	Transparent	A trace of small-suspended solids
9	6M HCl	105	Good	Brown	A trace of small-suspended solids with a trace of black spots

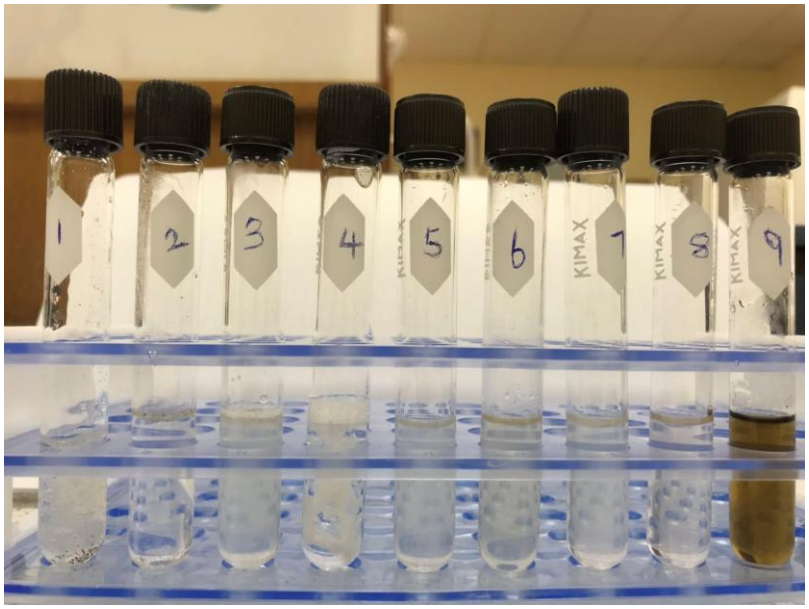


Figure 3.2 Sample appearance after collagen hydrolysis

The results of hydrolysis were influenced by solutions and temperatures. The higher concentration of acid was used, the better solubility was observed. At the same time, the higher the temperature used, the better solubility was observed. Sample 1 showed that collagen could be partly dissolved in water even at room temperature (25 °C), but it was a very viscous and sticky like glue. At the higher 50 °C and 105 °C temperature water hydrolysis the samples dissolved better. Generally, acid hydrolysis demonstrated significantly better solubility at all temperatures. The brown colour of sample 9 demonstrated that the Maillard reaction occurred with the highest acid concentration and highest temperature.

The gel results (see Figure 3.3) showed the peptide molecular weight distribution in the collagen hydrolysis samples. These results generally supported the observational results. Samples 1 and 4 were too viscous to be pipetted properly and injected in the sample loading place on the gel, so their bands on the gel were almost empty. This confirmed their low hydrolysis level among all samples. Samples 2, 3, 5 and 7 had a clear staircase distribution of bands, which indicated significant amounts of peptides in the samples. Samples 6, 8, and 9 also had almost empty bands, however the interpretation for this is they contained peptides with lower molecular weight than the markers on the gel. For sample 9, the peptides broken down into amino acids because the hydrolysis conditions of sample 9 are usually used for amino acids analysis (Naffa 2017).

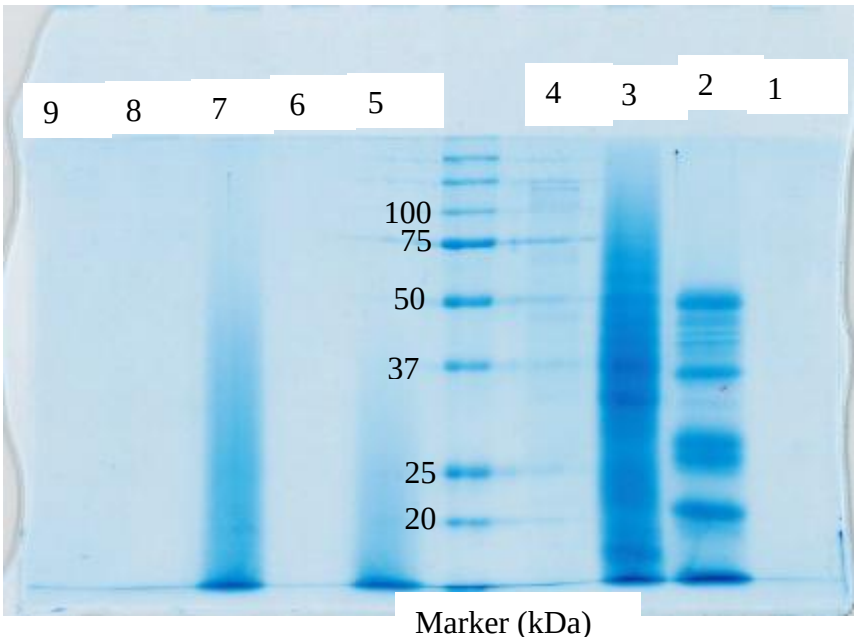


Figure 3.3 SDS-PAGE results on 12 % gel for the collagen hydrolysis trial.

3.3.2 Additional collagen hydrolysis

The hydrolysis conditions selected for three additional materials were water and 50 °C based on the previous collagen hydrolysis results (Section 3.3.1). These conditions gave clearer bands on the gel results and showed more information for analysis. The appearance for each sample is shown below (see Figure 3.4) with sample 9- a sheep skin powder hydrolysate, sample 10- a cow hides powder hydrolysate and sample 11- a collagen (extracted from sheep skin) hydrolysate. Sample 2 is the collagen (extracted from cow hide) hydrolysed also by water and 50 °C.

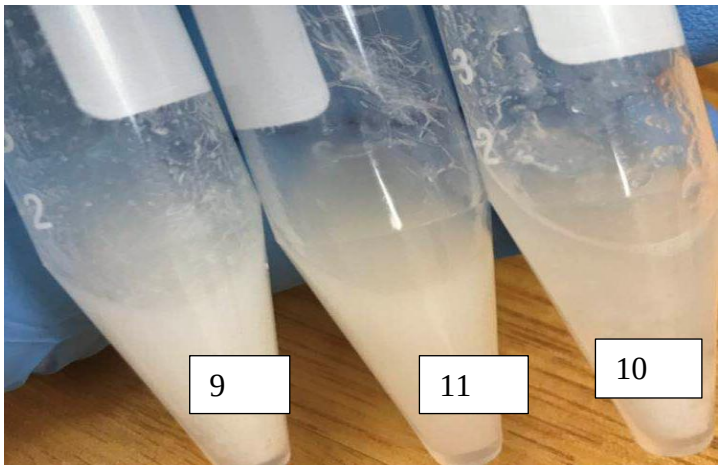


Figure 3.4 Appearance for hydrolysed samples by water at 50°C:

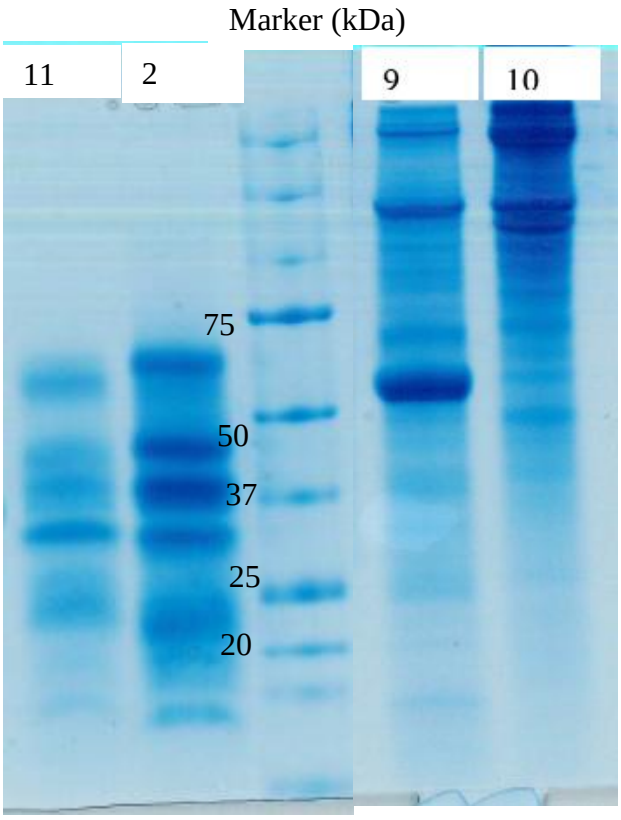


Figure 3.5 SDS-PAGE results on 16 % tricine gel for hydrolysis

Collagen extracted from sheep skin and collagen extracted from cow hide had similar solubility in water and were partly dissolved. Sample 2 and sample 11 also showed similar staircase distribution bands on the gel (see Figure 3.5). In contrast, the sheep skin powder and cow hide powder were barely dissolved. The gel bands of sample 9 and 10 showed their molecular weight distribution were similar to pure collagen. Although the hydrolysis levels of samples 9 and 10 were lower than the pure collagen sample, the sheep skin powder was hydrolysed more than the cow hide powder in the same solutions at the same temperatures.

3.3.3 *In vivo* test

In addition to measuring the total intake of the test and control samples, the sample first chosen by the cats in each test and whether they didn't drink any sample at all were also recorded. The first choice indicates the sample that was chosen first by a cat while the none-drinking times means a cat did not drink anything during the 2 h test period. The

number of timepoints was 40 (8 cats tested for 5 days), and the summarised results are shown below (see Table 3.3).

Table 3.3 Summary of all cat tests

A	B	Mean daily intake of A (g)	Mean daily intake of B (g)	First choice of A (times)	First choice of B (times)	Not drinking at all (times)
50 °C hydrolysed sample	Water	3.6	3.8	11	17	12
105 °C hydrolysed sample	Water	4.7	3.8	19	15	6
50 °C hydrolysed sample	105 °C hydrolysed sample	1.8	3.2	5	10	22

Test 1: 50 °C hydrolysed sample (A) compared with water (B)

The mean intake of A was 3.6 g while the mean intake of B was 3.8 g. Cats drunk A first 11 times while cats drunk B first 17 times. Cats did not drink at all 12 times. No statistical difference (P>0.05) was shown between A and B using t-test and chi-squared analysis.

Test 2: 105 °C hydrolysed sample (A) compared with water (B)

The mean intake of A was 4.7 g while the mean intake of B was 3.8 g. Cats drunk A first 19 times while cats drunk B first 15 times. Cats did not drink at all 6 times. No statistical difference (P>0.05) was shown between A and B using t-test and chi-squared analysis.

Test 3: 50 °C hydrolysed sample (A) compared with 105 °C hydrolysed sample (B)

The mean intake of A was 1.8 g while the mean intake of B was 3.2 g. Cats drunk A first 5 times while cats drunk B first 10 times. Cats did not drink at all 22 times. No statistical difference (P>0.05) was shown between A and B using t-test and chi-squared analysis.

In summary, the mean intake of 105 °C hydrolysed sample was numerically higher than both 50 °C hydrolysed sample and water. The number of times the 105 °C hydrolysed

samples were chosen first was also more than other two samples. However, all three group tests showed no statistical difference in intake between A and B.

3.4 Discussion and conclusion

At the beginning, the selection of collagen hydrolysis conditions for this research had to consider the following *in vivo* test. Water was the first choice. As the obligate carnivores with a naturally low thirst drive, cats are predisposed to low water intake (van der Meer 2021). This could cause feline lower urinary tract disease, chronic kidney disease, and even impacts on body weight and overall metabolic balance (van der Meer 2021). Therefore, increasing palatability of water for cats is significant and water can be used as control in two-blow tests.

When comparing acid hydrolysis with alkaline hydrolysis, acid hydrolysis for collagen is more popularly used in food industry because alkaline hydrolysis can produce tryptophan, but most other amino acids are completely destroyed (Pasupuleti 2008). Therefore, acid hydrolysis is another choice.

The results of the two-bowl test showed there was no statistical difference between the collagen water hydrolysate samples and water, which means cats equally like to drink water and samples. On the one hand, the collagen hydrolysate samples are not palatable for cats, which means these samples are not the target sample for this project. On the other hand, however, cats also did not refuse to drink the collagen hydrolysate samples, which means this can be researched in the future when looking at collagen functional food for cats.

Although cats accepted collagen hydrolysate samples as the same level as they accepted water, the 105 °C hydrolysed sample still performed better. Cats drunk this sample more than the others and more cats wanted to drink this sample first. A hypothesis can be established from this result, which is that a higher temperature hydrolysis could increase palatability. Higher temperature hydrolysis samples contain smaller peptides from SDS-PAGE gel results. This could also mean that the higher degree of hydrolysis of collagen could increase palatability.

Pure collagen perhaps was not a suitable raw material for developing palatable food. Luckily, raw hides or skins can be partly hydrolysed directly. The SDS-PAGE gel

results showed that the sheep skin had a higher degree of hydrolysis than cow hide under the same hydrolysis conditions. A hypothesis can be also established from this result, which is sheep skin is more suitable for enhancing sample palatability than cow hide.

Collagen water hydrolysate solution samples are equally attracted to cats as water due the *in vivo* test results. The raw material is still needed to be selected and the hydrolysis conditions still needed to be improved. The detailed raw material and hydrolysis conditions selection progress are shown in Chapter 4.

4. Further palatable sample development

4.1 Introduction

The ultimate aim of this project was to find an extraction process that could be used to convert leather waste into edible and palatable products which could be consumed by cats. Collagen was initially tried as a raw material (Chapter 3) and the samples produced had a neutral palatability which was equivalent to water. Therefore, in an attempt to produce improved palatant fractions, a wider range of raw materials and extraction techniques were investigated. Some further hypotheses were developed which considered the hydrolysis conditions that could produce a more palatable sample for cats based on the previous test results and other published references. The next step in the project was the selection of the raw materials and the conditions used to create the samples. The same *in vivo* testing procedure could then be used to examine whether they are palatable for cats.

The raw materials available from the leather waste could can be compounds extracted from hides and skins such as lipids. However, lipids can be highly influenced by light, heat, and oxygen during the extraction process, which can significantly alter their sensory properties such as smell and taste. Lipid oxidative degradation often leads to the formation of undesirable off-flavours and odours, commonly characterised as rancidity (Rosa *et al*. 2020). Therefore, effective management of these factors during extraction and storage is essential to preserve the sensory quality and overall stability of lipids. These approaches could increase the cost of this process, and thus the commercial production of the fraction in the future. The consideration of cost is an important aspect given this project is focussed on adding value to leather production waste streams.

The most suitable raw material for this project is untreated leather waste such as raw hides and skins. The most common animals used for leather industry are sheep and cows. The previous results in Chapter 3 showed that the sheep skin hydrolysed mixture contained smaller peptides than cow hide hydrolysed sample, and a previous PhD thesis had indicated that ovine edible products are more palatable than similar bovine edible

products (Watson 2023), which together suggested that sheep skin may be a more appropriate raw material than cow hide.

The hydrolysing agent is one of the vital components of hydrolysis, and a range of options exist such as acids, enzymes, water or different combinations of each. Phosphoric acid, hydrochloric acid and acetic acid are the best options because phosphoric acid and acetic acid are commonly used in food industry while hydrochloric acid is commonly used for hydrolysis. There are also a few papers which indicate the importance of phosphoric acid in increasing palatability of cat food (Brand and Bryant 2012, Shao *et al.* 2005, and Lee *et al.* 2007), in a similar to that discussed in Chapter 2. In order to improve hydrolysis, pepsin was chosen to be used together with phosphoric acid. The reason for choosing pepsin was because it is one of the most common

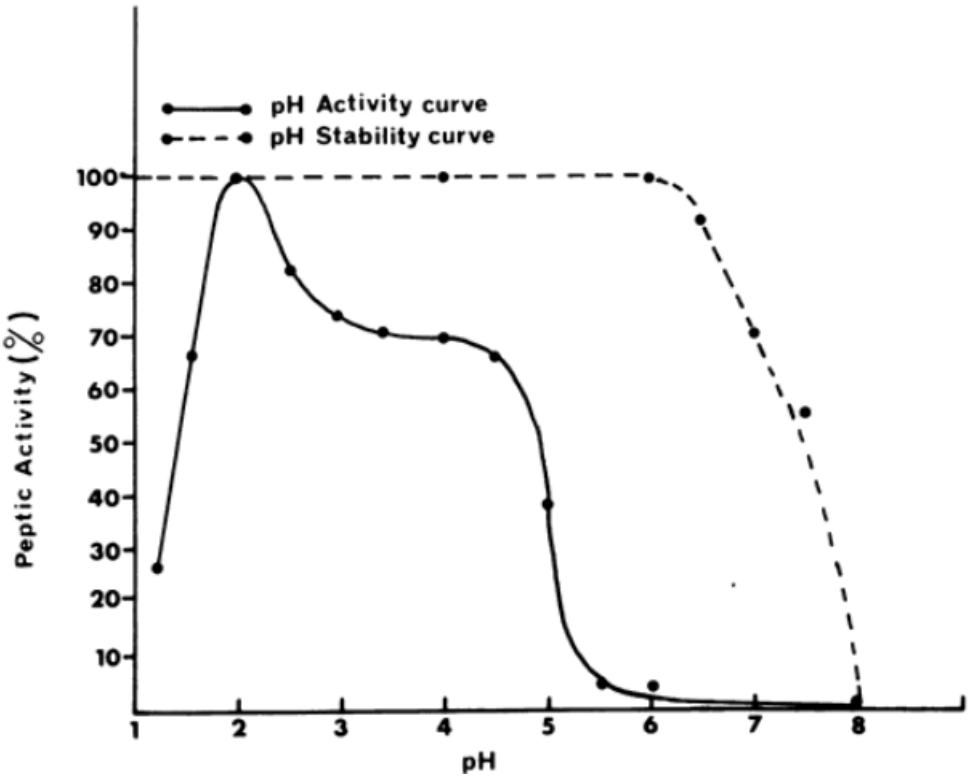


Figure 4.1 The pH stability and pH activity of pepsin (Piper *at el.* 1965)

enzymes used in the food industry and it is active in an acidic environment (see Figure 4.1) especially pH 1.5 to 2.5 (Piper *at el.* 1965). The sequence of testing was to use phosphoric acid with pepsin and then try the single acid, and finally only water for the hydrolysis.

The temperature selected for the hydrolysis was 105°C due to previous results presented in Chapter 3. This higher temperature produced smaller peptides as shown in the SDS-

PAGE results and it was hypothesised that these smaller peptides might be more palatable due to previous *in vivo* tests.

Other details in the sample preparation were also considered. For example, the pH of the hydrolysed sample was monitored and may need to be adjusted if it is too low for cats. A pH adjustment step may also produce phosphates which might influence the palatability, so this also needs to be considered.

4.2 Materials and regents

Raw materials

The sheep skins used in this work were from 11-12 months old Romney crosses taken from local farms in the Manawatu, and processed at Ovation Ltd, Feilding.

Chemicals and regents

Ortho Phosphoric acid (85% wt) and NaOH pellets were supplied by the Materials Store, at the School of Natural Sciences, Massey University. Deionized water was obtained from a Milli-Q Ultra-pure ThermoFisher water system (Dubudue, IA, USA); 16 % SDS-PAGE gels were produced and provided by LASRA.

4.3 Methods

4.3.1 Sheep skin preparation

Fresh sheep skins (see Figure 4.2) were received and the fleshing layer was removed (see Figure 4.3). After the layer was removed, the sheep skins were shaved to remove most of the wool fibres and then cut into small pieces (see Figure 4.4). The sheep skin pieces were either frozen at -20°C before continuing or the sample preparation was carried out immediately.

Figure 4.2 fresh sheep skins

Figure 4.3 Removal of the fleshing layer by the hydraulic fleshing machine

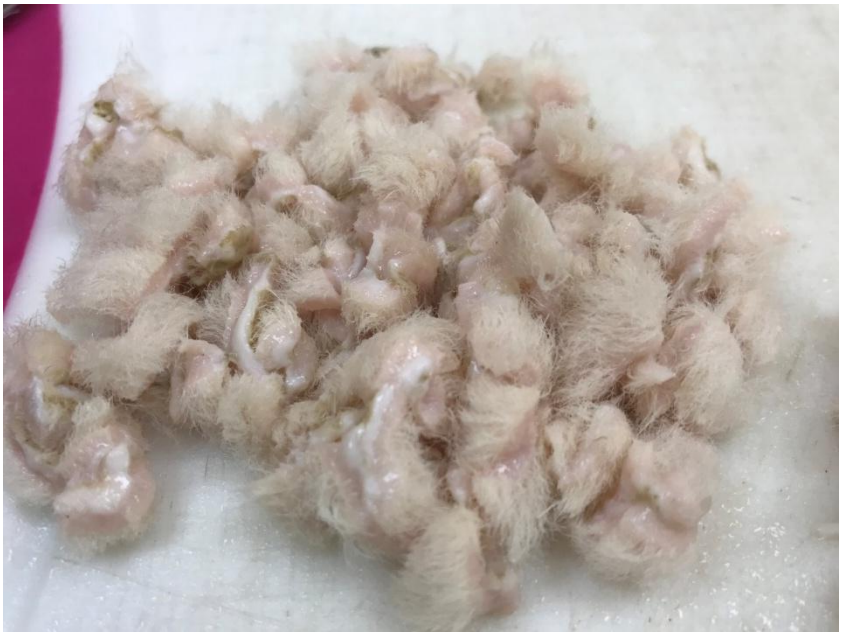


Figure 4.4 Small pieces of shaved sheep skins

4.3.2 Development of the sample preparation technique

Sample 1

Sheep skin pieces were added to 0.5 M phosphoric acid (w:v = 1:10) and 5 % pepsin and stirred for 1 h. After blending (using stick mixer) the mixture was put into an oven at 105 °C for 4 h and then cooled to room temperature. 10 M NaOH was used to adjust the pH to 5.5 then the mixture was centrifuged at 4500 x g for 10 min. The supernatant was removed and dialysed (molecular weight cutoff was 0.5–1 kDa) at 4 °C overnight. This test sample was offered to cats with a control water sample (Test 1).

Sample 2

Sheep skin pieces was added to 0.5 M phosphoric acid (w:v = 1:10) and 5 % pepsin and stirred for 1 h. After blending the mixture was put into an oven at 105 °C for 4 h and then cooled to room temperature. 10 M NaOH was used to adjust the pH to 5.5 then the mixture was centrifuged at 4500 x g for 10 min. The supernatant was removed and this test sample was offered to cats with a control sample (sample 1 above) (Test 2).

Sample 3

Sheep skin pieces was added to 0.5 M phosphoric acid (w:v = 1:10) and stirred for 1 h. After blending the mixture was put into an oven at 105 °C for 4 h and then cooled to room temperature. 10 M NaOH was used to adjust the pH to 5.5 then the mixture was centrifuged at 4500 rpm for 10 min. The supernatant was removed and this test sample was offered to cats in two tests; one with a control sample (sample 2 above) and one with a control sample (water) (Tests 3 & 6). The SDS-PAGE result for sample 3 was provided by LASRA.

Sample 4

0.5 M phosphoric acid was stirred for 1 h and then put into an oven at 105 °C for 4 h and then cooled to room temperature. 10 M NaOH was used to adjust the pH to 5.5 then the mixture was centrifuged at 4500 rpm for 10 min. The supernatant was removed and this test sample was offered to cats with a control sample (sample 3 above) (Test 4).

Sample 5

Sheep skin pieces was added to water (w:v = 1:10) and stirred for 1 h. After blending the mixture was put into an oven at 105 °C for 4 h and then cooled to room temperature. The mixture was then centrifuged at 4500 rpm for 10 min. The supernatant was removed and this test sample was offered to cats with a control sample (sample 3 above) (Test 5).

Table 4.1 and 4.2 show the summarised methods below:

Table 4.1 Development of the different palatability samples

	Sheep skins	H_3PO_4	pepsin	dialysis
Sample 1	√	√	√	√
Sample 2	√	√	√	
Sample 3	√	√		
Sample 4		√		
Sample 5	√			

Table 4.2 The *in vivo* testing schedule

	A	B
Test 1	Sample 1	Water
Test 2	Sample 2	Sample 1
Test 3	Sample 3	Sample 2
Test 4	Sample 4	Sample 3
Test 5	Sample 5	Sample 3
Test 6	Sample 3	Water

4.3.3 *In vivo* testing

A two-bowl test was used as previously described: one bowl contained a test sample and the other bowl contained a control sample. A 10 g test sample and 10 g control water or sample were offered to each cat in the panel for 2 h in a single cage. Six rounds of testing were carried out (see Table 4.2). All six tests were run over 5 days with the position of each bowl (left or right) changed daily.

4.4 Results and discussions

Samples 1, 2, 3 and 5 had a similar cloudy appearance even after centrifugation (see Figures 4.5 and 4.6).

Figure 4.5 Centrifuged sample

Figure 4.6 Collected sample ready for *in vivo* testing using the panel of cats.

Sample 1 was the sample produced using all of the hypothesised conditions which might enhance palatability of sheep skins. Test 1 compared sample 1 against water using the cat panel in order to determine if the palatability was increased. The mean intake of sample 1 was 6.6 g and the mean intake of water was 2.8 g. There was a

statistical difference ($P<0.05$) in intake between sample 1 and water, which indicated that sample 1 was more palatable than water. The conditions needed to enhance the palatability of the first sample had been successfully generated.

Once this palatable sample had been produced, the conditions were reduced one by one to confirm their necessity. Sample 2 was made by all of the procedures used to produce sample 1 except the removal of the dialysis step. Test 2 compared sample 2 against sample 1 using the same cat panel in order to confirm if the dialysis step could be removed. The mean intake of sample 2 was 5.0 g while the mean intake of sample 1 was 5.4 g. There was no statistical difference ($P>0.05$) between sample 2 and sample 1. This result indicated that the dialysis step could be removed as it had no influence on the palatability of the fraction.

After removed dialysis, the next step was investigating whether the addition of pepsin was necessary. Sample 3 was only hydrolysed by phosphoric acid. Test 3 compared sample 3 against sample 2. The mean intake of sample 3 was 3.7 g and the mean intake of sample 2 was 4.0 g. There was also no statistical difference ($P>0.05$) between sample 3 and sample 2. The result also indicated that pepsin wasn't required and could be removed.

The aim of producing sample 4 was similar to the aim of producing sample 2, and investigated the influence of phosphates on palatability. Sample 2 was used to identify the influence of phosphates produced during pH adjustment step using the skin raw material, but sample 4 was produced to confirm if the only palatable part of the sample was the phosphate from the phosphoric acid. Test 4 compared sample 4 against sample 3. The mean intake of sample 4 was 1.4 g and the mean intake of sample 3 was 3.1 g. There was a statistical difference ($P<0.05$) in intake between sample 4 and sample 3. This result suggested that it wasn't the phosphate from the phosphoric acid that was driving the palatability of the fractions for to cats.

The best extraction conditions that appeared to enhance palatability in the fraction was achieved using phosphoric acid. This was confirmed in the following piece of work. Sample 5 was produced using only water to hydrolyse the sheep skin. Test 5 compared sample 5 against sample 3. The mean intake of sample 5 was 1.9 g and the mean intake of sample 3 was 5.8 g. There was a statistical difference ($P<0.05$) in intake between

sample 5 and sample 3. The result indicated that phosphoric acid was vital in the preparation of a palatable sample from sheep skin.

Finally, in test 6 sample 3 was compared against water. The mean intake of sample 3 was 6.6 g and the mean intake of water was 1.6 g. There was a statistical difference ($P<0.05$) in intake between sample 3 and water.

This group of two-bowl tests demonstrated that sample 3 was both palatable, and relatively easy to produce. The summary table of the results is shown below (see Table 4.3):

Table 4.3 A summary of the results from the *in vivo* tests

Test	A	B	Mean intake of A (g)	Mean intake of B (g)	Statistical difference	More palatable
1	Sample 1	Water	6.6	2.8	P<0.05	Sample 1
2	Sample 2	Sample 1	5.0	5.4	-	-
3	Sample 3	Sample 2	3.7	4.0	-	-
4	Sample 4	Sample 3	1.4	3.1	P<0.05	Sample 3
5	Sample 5	Sample 3	1.9	5.8	P<0.05	Sample 3
6	Sample 3	Water	6.6	1.6	P<0.05	Sample 3

The result of SDS-PAGE for sample 3 is shown in Figure 4.7. There were no discernible protein bands but there was a large smear of stain below 37 kDa which suggested the presence of a large amount of low molecular weight material and also the there was a high salt content that disrupted the electrophoresis. The bands were merged so it was not easy to determine the specific molecular weight of the fraction. It may indicate that the cloudy liquid contained more than protein. It did appear that there were more peptides present with molecular weights lower than 37 kDa than larger peptides. However further analysis was required in order to better characterise this palatable sample.

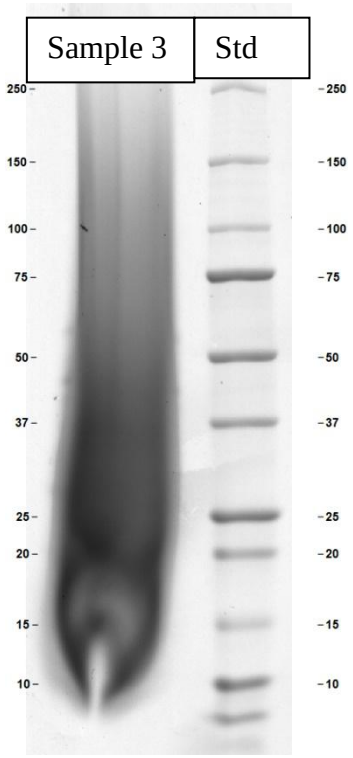


Figure 4.7 SDS-PAGE result

4.5 Conclusion

A novel palatable sample for cats was successfully produced from untreated leather waste and was ready for further analysis. The hydrolysis methods developed might be applied to other leather wastes such as fleshing to produce a highly palatable cat food.

5. Palatable sample analysis

5.1 Introduction

Sample analyses are essential to understand the reason why a productmaterial is effective for attracting cats. Proximate analysis can provide basic information for samples and describes the protein, fat, fibre, moisture and ash content. Both chemical characterisation and whole sample compounds fingerprint mapping are critical. Chemical characterisation can identify specific compounds which are important to increasing palatability in the sample. At the same time, characterised compounds also can be compared with palatable compounds which have been previously described. The amount of these palatable compounds can also predict the palatability level of the sample. Sample fingerprint results are vital for quality control. In addition to sample quality, existing fingerprint results could be used to compare or predict palatability for any new samples generated.

Flavour is an important aspect of cat food, just like human food. It is a complex sensory perception influenced by multiple factors that interact to create the overall experience. (Reineccius and Peterson 2013). The perception of taste is given by the stimulation of chemoreceptors, commonly known as taste buds, located within the oral cavity; whereas the perception of smell arises from the activation of olfactory receptors situated in the nasal cavity by volatile compounds present in food (Aldrich and Koppel 2015). Aroma and taste compound analysis can be involved in sensory analysis.

Sensory analysis can be carried out by *in vivo* tests and some instruments. Instrumental sensory analysis not only offers the characterisation of components of palatability, but also is beginning to explain the basis of palatability in a fundamental way. However, even for human food, identifying aroma or taste compounds is a hard task because laboratory instrumentation is not as sensitive to many compounds as is the human sensory system (Reineccius and Peterson 2013). Moreover, some compounds in food make a major contribution to the overall flavour even in extremely small quantities (Reineccius and Peterson 2013), which makes analysis more difficult. Therefore, research into pet food flavours present huge challenges, and like infants, pets cannot communicate with us directly by talking (Koppel 2014).

Both volatile compounds and non-volatile compounds in the hydrolysed test fraction, raw skin and precipitate produced in Chapter 4 were charactered in this project. All tests in this chapter were carried out by commercial research institutions using well-established methods due to the shortage of instruments in the research group.

5.2 Analysis methods

5.2.1 Sample preparations

The sample preparation procedures used were identical to those used to produced sample 3 in Chapter 4: Sheep skin pieces was added 0.5 M phosphoric acid (w:v = 1:10) and stirred for 1 h. After blending the mixture was put into an oven at 105 °C for 4 h and then cooled to room temperature. 10 M NaOH was used to adjust the pH to 5.5 then the mixture was centrifuged at 4500 rpm for 10 min. The supernatant was the target sample which was palatable to cats. The raw sheep skin, hydrolysed supernatant and precipitate were all freeze dried (FD) for further analysis.

5.2.2 Proximate analysis

All proximate analyses were performed by staff of the Massey University Nutrition Laboratory (Palmerston North, New Zealand) in duplicate. In all analytical analyses, a precision balance was used, and weights were rounded to four decimal places. All determinations were calculated and expressed as g per kg dry matter (DM) unless stated otherwise. All analysis was carried out according to AOAC International approved analytical methods (Horwitz & Latimer, 2005) and for gross energy content using bomb calorimetry. The dry matter and moisture contents were determined using a convection oven at 135 °C (AOAC 930.15). Crude ash content was determined using a furnace at 600 °C (AOAC 942.05). The nitrogen content was determined according to the combustion method (AOAC 992.15) using the Elementar rapid MAX N exceed (Elementar Analyesensysteme GmbH, Langenselbold, Germany). Compressed helium gas (99.99 %) was used as a carrier gas and ethylenediaminetetraacetic acid (EDTA) was used as a nitrogen standard for calibration. Nitrogen content was converted to crude protein by multiplying by 6.25. The crude fat content of the diet was determined using the gravimetric (Mojonnier) method (AOAC 954.02), while the crude fat content of the

faeces was determined using the Randal modification of the standard Soxhlet extraction (AOAC 2003.06). The crude fibre content was determined using the fritted glass crucible method (AOAC 978.10).

5.2.3 Volatile compounds analysis

The volatile compounds analysis was carried out by Food Industry Science Centre, The New Zealand Institute for Plant and Food Research Limited. The methodology they provided are shown below:

Samples were analysed using a Shimadzu TQ8050 GC-MS equipped with a GL Sciences Optic-4 cryo-focusing injector unit using a 50/30 um DVB/Car/PDMS SPME fibre, (Supleco Cat # 57298-U). The GC column was a DB-Wax column (Agilent Technologies) 20 m x 0.18 mm ID x 0.18 µm film thickness, using helium at 0.9 mL min^{-1} column flow.

Vials were equilibrated at 50 °C for 5min, before sampling by SPME for 30min at 65 °C. The sample was injected for 4min in splitless mode with an injection temperature of 240 °C.

The cryo-trap was held at -150 °C for 245 sec, then heated at 60 °C sec^{-1} to 250 °C. The GC temperature program was 35 °C for 2 min, then 8 °C min^{-1} to 80 °C, then 12 °C min^{-1} to 240 °C, with a hold at 240 °C for 18 min. Volatiles were identified from their retention indices and by comparison with commercial mass spectral databases and authentic compounds. The data were processed using Shimadzu GC-MS PostRun Analysis and GC-MS Browser software.

5.2.4 Amino acids (AAs) analysis

Both free AAs and total AAs in the samples were analysed. The AAs analysis was carried out by the Nutrition Laboratory, School of Food and Advanced Technology, Massey University. The Methodology they provided were shown below:

Total AAs (acid stable): HCl hydrolysis followed by RP HPLC separation using AccQ Tag derivatization. AOAC 994.12

Free AAs: HPLC separation, AOAC 994.12

Cysteine and Methionine: Performic acid oxidation, AOAC 985.28

Tryptophan: Alkaline Hydrolysis, sub-contracted

5.2.5 Fatty acids analysis

The fatty acids analysis was carried out by the Nutrition Laboratory, School of Food and Advanced Technology, Massey University. The Methodology they provided were shown below:

Fatty acids: GCL based on Sukhija and Palmquist (1988), in house method.

5.3 Results and discussion

5.3.1 Proximate analysis results

The proximate analysis results were shown below (see Table 5.1).

Table 5.1 Proximate analysis results

Sample name	DM % (As-is basis)	Ash % (DM basis)	Protein % (DM basis)	Fat % (DM basis)	Crude fibre % (DM basis)
Raw sheep skin	27.4	3.28	86.68	18.98	0.36
Hydrolysed supernatant	6.3	69.84	19.84	1.59	<1.59
Hydrolysed precipitate	21.4	18.69	58.41	16.36	5.61

The results of raw sheep skin are typical constituents of animal skins, which contains 72.6 % of moisture, 23.75 % of protein, 5.2 % of fat and 0.9 % of ash. High protein value confirms that raw sheep skin can be a potential source for protein-rich palatants in cat food. Moderate fat value also suggested the possibility that raw sheep skin might be the palatability enhancer for cat food. Very low ash content indicates only minimal contamination exists. The negligible fibre content is expected, as animal-derived materials typically contain slight fibre.

DM value for the hydrolysed supernatant were lower than other samples, because a large amount of water has been added during sample production and it is a liquid hydrolysates mixture. The ash remaining after incineration primarily consists of the inorganic minerals. The contents of ash in hydrolysed samples were higher than in raw sheep skin, because the hydrolysis procedure introduced more inorganic elements, especially from the pH adjustment step.

The hydrolysed supernatant contains moderate amounts of protein, likely due to the soluble peptides and amino acids released during hydrolysis. The crude fibre in hydrolysed precipitate is higher than other samples. This may be because the invisible contaminants such as plant materials, dirt, or feed residues trapped in the hair accumulated during sample preparation.

5.3.2 Volatile compounds results

Four samples were arranged for volatile compounds test: freeze-dried (FD) raw sheep skin, hydrolysed supernatant, hydrolysed precipitate, and the original liquid of hydrolysed supernatant. The total volatile compounds characterisation results are shown in Table 5.2, while the GC-MS spectrum results for four samples are shown in Figure 5.1. The original liquid of hydrolysed supernatant was the palatable sample fed to cats. Volatile compounds fingerprint might be different between FD hydrolysed supernatant and original hydrolysed supernatant due to their unstable characteristic – some of the volatile compounds might be lose during FD. Over one hundred volatile compounds were detected and charactered. The sequence number is ordered by the retention time. The most arresting odour descriptor is blue cheese.

Table 5.2 Characterised volatile compounds obtained from freeze-dried (FD) raw sheep skin, hydrolysed supernatant, hydrolysed precipitate, and the original liquid of hydrolysed supernatant by GC-MS.

	Compound	Type	Odour descriptor	Threshold	Mass (m/z)
1	Heptane				100
2	Carbon disulfide	Sulfur		very low threshold	76
3	Methylamine, N,N-dimethyl-				58
4	Acetone				58

	Compound	Type	Odour descriptor	Threshold	Mass (m/z)
5	Butanal	Aldehyde			72
6	Methacrolein				70
7	2-Butanone	Ester			72
8	Isovaleric aldehyde	Aldehyde			58
9	Ethanol	alcohol			45
10	Methyl vinyl ketone	ester			70
11	Furan, 2-ethyl-				96
12	2,3-Butanedione or pentanal				86
13	CH_2Cl_2 or Penten-3-one				84
14	2-Butenal	Aldehyde			70
15	Toluene	aromatic hydrocarbon			91
16	2,3-Pentanedione	ketone			57
17	Dimethyl disulfide	Sulfer		very low threshold	94
18	Hexanal	Aldehyde			56
19	.beta.-Pinene	terpene	pine		93
20	Benzene <ethyl->	aromatic hydrocarbon			91
21	2-Pentenal, (E)-	Aldehyde			84
22	2-butanol	alcohol			69
23	o-Xylene	aromatic hydrocarbon			91
24	1-Butanol	alcohol	unpleasant, body odour		56
25	2-penten-1-ol, (E)-	alcohol			57
26	1-Penten-3-ol	alcohol			57
27	3-Methylcyclopentyl acetate	ester			104
28	Heptanal	Aldehyde			70
29	2-Butenal, 3-methyl-	Aldehyde			84
30	1-Butanol, 3-methyl-	alcohol	unpleasant, body odour		70
31	2-Hexenal, (E)-	Aldehyde			83
32	1-Decanol, 2-hexyl-	alcohol			57
33	1-Methoxy-2-propyl acetate	ester	mild, sweet, fruity odour		72
34	Furan, 2-pentyl-				81
35	2-Heptanone, 6-methyl-	ketone			58
36	4-Heptenal, (Z)-	Aldehyde			84
37	n-Pentanethiol	sulfur	sulfurous fatty roasted	low threshold	104
38	2-Methyl-3-trans-propenylpyrazine	Pyrazine		very low threshold	119

	Compound	Type	Odour descriptor	Threshold	Mass (m/z)
39	Cymene <para->	terpene			119
40	Pseudocumene or Mesitylene				105
41	Octanal	Aldehyde			84
42	1-Octen-3-one	ketone			70
43	2,5-Octanedione	ketone			99
44	2-Heptenal, (Z)-	Aldehyde			83
45	Acetaldehyde <ethyl-, phenethyl-> acetal	Aldehyde			105
46	5-Hepten-2-one, 6-methyl-	ketone			108
47	2-Methyl-1-undecanol	alcohol			57
48	Pyrazine, 2,3-dimethyl-	Pyrazine	nutty	very low threshold	108
49	1-Hexanol	alcohol			56
50	Diacetone alcohol	alcohol			59
51	Pyridine, 2,4,6-trimethyl-	Pyrazine	nutty	very low threshold	TIC
52	Dimethyl trisulfide	Sulfer		very low threshold	126
53	1-Ethylpentyl acetate	ester			TIC
54	2-Nonanone	ketone			58
55	Nonanal	Aldehyde			98
56	Ethanol, 2-butoxy-	ether			57
57	Pyrazine, trimethyl-	Pyrazine	nutty, musty	very low threshold	122
58	2-Hexanone, 6-methoxy-				98
59	Terpinyl acetate <cis-dihydro-alpha->	terpene	floral		TIC
60	2-Octenal, (E)-	Aldehyde			83
61	2-Dodecenal, (E)-	Aldehyde			83
62	Cymene <ortho->	hydrocarbon			119
63	3-Heptenoic acid	acid	Blue cheese	low threshold	68
64	Acetic acid	acid	sharp pungent sour vinegar	low threshold	60
65	1-Octen-3-ol	alcohol			57
66	1-Heptanol	alcohol			70
67	Furfural				96
68	Hexanol <2-ethyl->	alcohol			57
69	Decanal	Aldehyde			82

	Compound	Type	Odour descriptor	Threshold	Mass (m/z)
70	1-Heptanol, 3-methyl-				84
71	Benzaldehyde	Aldehyde	almond oil		106
72	4-Ethylcyclohexanol				69
73	Linalool	terpene	floral		93
74	1-Octanol	alcohol			56
75	Butanoic acid, 2-methyl-	acid	Blue cheese	low threshold	60
76	3,5-Octadien-2-one				95
77	2,3-Butanediol				45
78	Acetaldehyde, methoxy-				109
79	Propanoic acid, 2-methyl-	acid	Blue cheese	low threshold	88
80	Propanoic acid, 2,2-dimethyl-	acid	Blue cheese	low threshold	57
81	Cyclohexanol, 2,6-dimethyl-	alcohol			95
82	Furan, tetrahydro-2,5-dimethyl-				85
83	Hexanoic acid, hexyl ester	ester	apple		117
84	2-Decen-1-ol, (E)-	alcohol			110
85	Butyrolactone	lactone	creamy oily fatty caramel		86
86	Butanoic acid, 4-hydroxy-	acid	Blue cheese	low threshold	86
87	n-Caproic acid vinyl ester	ester			99
88	Phenylacetaldehyde	Aldehyde			91
89	Benzeneacetaldehyde	Aldehyde	sweet, floral odour		91
90	Levomenthol				138
91	Dec-(2E)-enal	Aldehyde			83
92	1-Decanol, 2-hexyl-	alcohol			57
93	1-Nonen-4-ol	alcohol			83
94	Acetophenone	ketone			105
95	2-Furanmethanol				98
96	5-Undecene, 8-methyl-, (E)-				43
97	1-Nonanol	alcohol			56
98	Propanoic acid, 2,2-dimethyl-	ester			57
99	Butanoic acid	acid	Blue cheese	low threshold	60
100	Butanoic acid, 3-methyl-	acid	Blue cheese	low threshold	60

	Compound	Type	Odour descriptor	Threshold	Mass (m/z)
101	Y-Hexalactone	lactone	herbal, coconut		85
102	Benzaldehyde, 4-(1-methylethyl)-	Aldehyde			148
103	2-Decen-1-ol, (E)-	alcohol			57
104	1-Decanol	alcohol			112
105	Acetamide				59
106	2,5-Hexanediol, 2,5-dimethyl-				113
107	Formamide, N,N-dibutyl-				114
108	Ethanol, 2-(2-butoxyethoxy)-	ether			57
109	2-Dodecanone	ketone			58
110	trans-Geranylacetone	ketone	fresh green fruity		69
111	Benzyl alcohol	alcohol	floral		108
112	Hexanoic acid	acid	Blue cheese	low threshold	60
113	Dimethyl sulfone	sulfur			94
114	Butanamide, 3-methyl-				59
115	Hexanamide				59
116	3-Oxobutan-2-yl 2-methylbutanoate	ester			85
117	Phenol	phenol	Sweet and tarry, disinfectant	low threshold	94
118	Heptanoic acid	acid	Blue cheese	low threshold	60
119	Butanoic acid, 3-methyl-	acid	Blue cheese	low threshold	60
120	Pantolactone	lactone	Sweet, slightly caramel-like odour		71
121	Cyclohexanamine, N-butyl-				112
122	2,5-Dimethylfuran-3,4(2H,5H)-dione				85
123	2-Pyrrolidinone				85
124	p-Cresol	phenol	pig odour	low threshold	107
125	Hexadecanal	Aldehyde			82
126	Octanoic acid	acid	Blue cheese	low threshold	60

	Compound	Type	Odour descriptor	Threshold	Mass (m/z)
127	Phenol, 4-ethyl-	phenol	Smoke, phenolic, creosote	low threshold	107
128	Phenol, 3-ethyl-	phenol	Smoke, phenolic, creosote	low threshold	107
129	Nonanoic acid	acid	Blue cheese	low threshold	60
130	Phenol, 3-propyl-	phenol	Smoke, phenolic, creosote	low threshold	107
131	Decanoic acid	acid	Blue cheese	low threshold	60

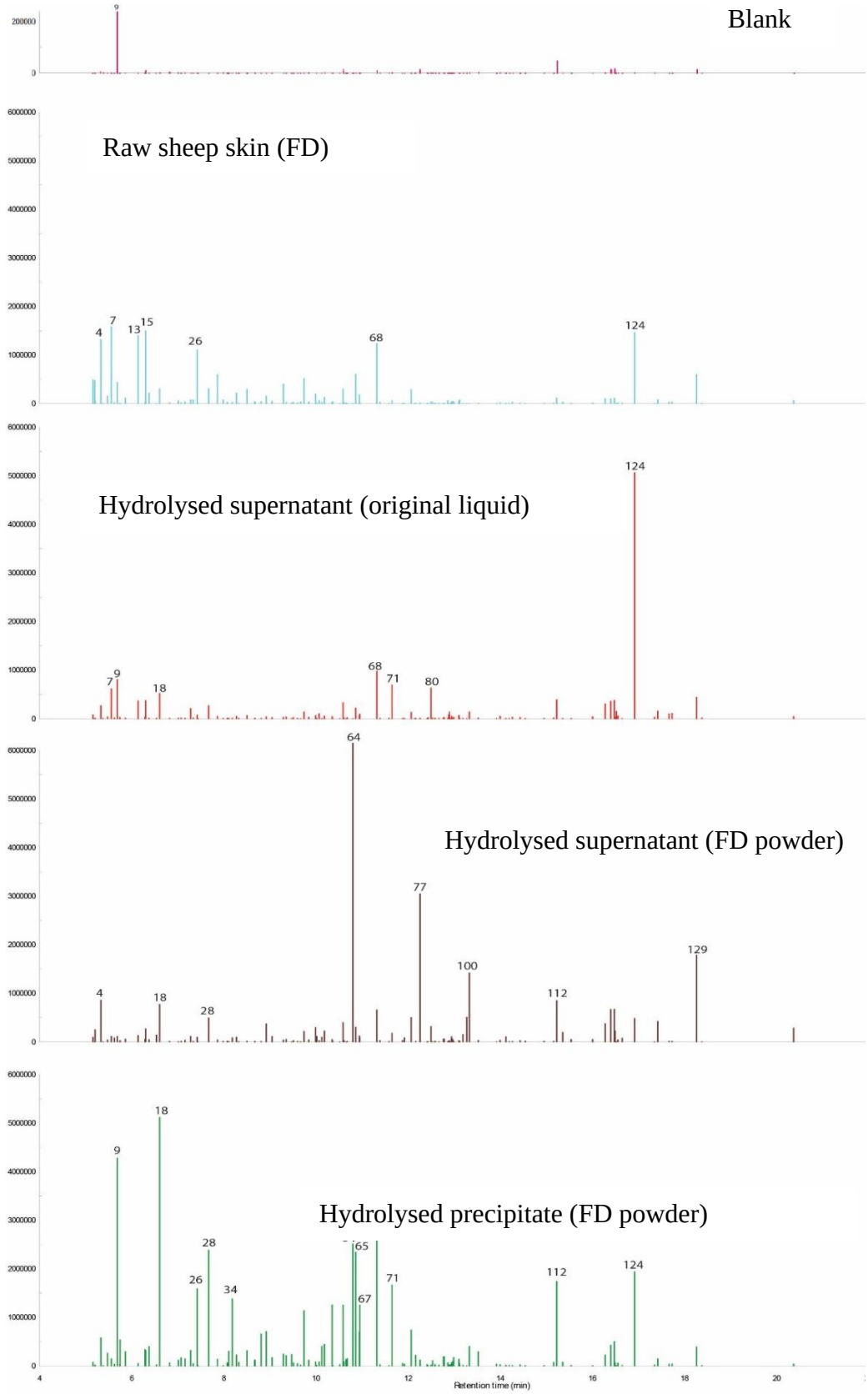


Figure 5.1 Volatile compounds obtained from freeze-dried (FD) raw sheep skin, hydrolysed supernatant, hydrolysed precipitate, and the original liquid of hydrolysed supernatant by GC-MS GC results

The GC results supported the hypothesis that the contents of volatile compounds might be different between FD hydrolysed supernatant and original hydrolysed supernatant. The highest peak on the original hydrolysed supernatant results is compound No. 124 which is p-cresol with a pig odour. In the contrast, the p-cresol content is much lower in FD hydrolysed supernatant. The p-cresol is increased during hydrolysis compared its content in raw sheep skin and the original hydrolysed supernatant.

Except for p-Cresol, the volatile compound content of the original hydrolysed supernatant is generally less than that in raw sheep skin. One reason for this may be the loss of volatile compounds during hydrolysis especially the heating step.

The highest peak on the FD hydrolysed supernatant results is compound No. 64 which is acetic acid with the smell of vinegar. However, this compound is not obvious in other samples.

Compounds No. 33, 89 and 120, which are 1-Methoxy-2-propyl acetate with a mild, sweet, fruity odour, benzeneacetaldehyde with a sweet, floral odour, and pantolactone with a sweet, slightly caramel-like odour, are absent in raw sheep skin but exist in the other samples. These three volatile compounds appeared to be generated during the sample preparation.

5.3.3 Amino acids (AAs) results

The total AAs results are shown in Table 5.3 and the free AAs results are shown in Table 5.4. Hydroxyproline is present in significant amounts as all of the samples contain collagen. The free AA content in the hydrolysed supernatant is very low, with many of them not detected. This result supports the hypothesis that the hydrolysed supernatant contains more peptides than free AAs. More analysis needs to be carried out to confirm this.

Table 5.3 Total AAs characterisation results

Total AAs (mg/100 mg)	Raw sheep skin	Hydrolysed supernatant	Hydrolysed precipitate
Aspartic Acid	1.02	0.08	0.77
Threonine	0.50	0.03	0.52
Serine	0.66	0.05	0.71
Glutamic Acid	1.51	0.12	1.29
Proline	1.50	0.14	0.61
Glycine	2.46	0.25	0.67
Alanine	1.09	0.10	0.47
Valine	0.58	0.04	0.57
Isoleucine	0.35	0.02	0.34
Leucine	0.81	0.05	0.84
Tyrosine	0.41	0.02	0.53
Phenylalanine	0.45	0.04	0.40
Histidine	0.16	0.01	0.12
Lysine	0.61	0.05	0.46
Arginine	1.26	0.10	0.90
Taurine	0.01	<0.01	ND
Hydroxyproline	1.14	0.13	0.08
Cysteine	0.43	0.01	0.69
Methionine	0.32	0.01	0.22
Tryptophan	0.09	-	0.12

Table 5.4 Free AAs characterisation results

Free AAs (mg/g)	Raw sheep skin	Hydrolysed supernatant	Hydrolysed precipitate
Aspartic Acid	-	0.01	-
Threonine	-	-	-
Serine	0.04	-	-
Glutamic Acid	0.30	0.02	0.01
Proline	0.01	<0.01	0.01
Glycine	0.19	0.03	0.07
Alanine	0.06	<0.01	-
Cystine	-	-	-
Valine	0.02	-	-
Methionine	<0.01	-	-
Isoleucine	0.01	<0.01	<0.01
Leucine	0.02	-	-
Tyrosine	0.01	0.01	0.19
Phenylalanine	0.01	<0.01	<0.01
Histidine	0.11	<0.01	0.02
Lysine	0.01	<0.01	-
Arginine	0.03	-	-
Taurine	0.13	0.01	-
Hydroxyproline	-	-	-
Tryptophan	-	-	-
Asparagine	0.01	-	-
Glutamine	0.12	-	-

5.3.4 Fatty acids

The total fatty acids results are shown in Table 5.5. A lot of the fatty acids were not detected in hydrolysed supernatant which may be due to their dilute concentration in the fraction.

Table 5.5 Fatty acids characterisation results

Fatty acids (g/100 g)	**Raw sheep skin**	**Hydrolysed supernatant**	**Hydrolysed precipitate**
C6:0 Caproic	<0.01	<0.01	-
C8:0 Caprylic	<0.01	-	<0.01
C10:0 Capric	0.01	<0.01	<0.01
C11:0 Undecanoic	-	-	-
C12:0 Lauric	<0.01	-	<0.01
C13:0 Tridecanoic	<0.01	-	<0.01
C14:0 Myristic	0.08	<0.01	0.05
C14:1n5 - cis-9-Myristoleic	<0.01	-	<0.01
C15:1n5 - cis-10-Pentadecenoic	-	-	-
C16:0 Palmitic	1.03	0.01	0.70
C16:1n7 - cis-9-Palmitoleic	0.08	<0.01	0.05
C17:0 Margaric	0.03	<0.01	0.02
C17:1n7 - cis-10-Heptadecenoic	-	-	-
C18:0 Stearic	0.55	<0.01	0.45
C18:1n9t Elaidic	0.01	-	0.01
C18:1n7t Vaccenic	0.06	<0.01	0.06
C18:1n9c Oleic	2.13	0.01	1.24
C18:1n7c Vaccenic	0.04	<0.01	0.02
C18:2n6t Linolelaidic	-	-	-
C18:2n6c Linoleic	0.05	<0.01	0.05
C20:0 Arachidic	0.01	-	0.01
C18:3n6 - cis-6,9,12-Gamma linolenic	<0.01	-	<0.01

Fatty acids (g/100 g)	**Raw sheep skin**	**Hydrolysed supernatant**	**Hydrolysed precipitate**
C20:1n9 - cis-11-Eicosenoic	<0.01	<0.01	<0.01
C18:3n3 - cis-9,12,15-Alpha linolenic	0.03	<0.01	0.02
C21:0 Heneicosanoic	-	-	-
C20:2n6 - cis-11,14-Eicosadienoic	0.04	<0.01	0.06
C22:0 Behenic	0.01	-	0.01
C20:3n6 - cis-8,11,14-Eicosatrienoic	<0.01	-	<0.01
C22:1n9 - cis-13-Erucic	<0.01	<0.01	<0.01
C20:3n3 - cis-11,14,17-Eicosatrienoic	<0.01	-	<0.01
C20:4n6 - cis-5,8,11,14-Arachidonic	0.01	<0.01	0.02
C23:0 Tricosanoic	<0.01	-	<0.01
C22:2n6 - cis-13,16-Docosadienoic	-	-	-
C24:0 Lignoceric	0.01	<0.01	0.02
C20:5n3 - cis-5,8,11,14,17-Epa	0.01	<0.01	0.01
C24:1n9 - cis-15- Nervonic	<0.01	-	<0.01
C22:5n3 - cis-7,10,13,16,19-DPA	0.01	-	0.01
C22:6n3 - cis-4,7,10,13,16,19-DHA	<0.01	-	<0.01

6. Future work

The method developed in the current work for producing palatable samples from sheep skin might be also applied on other raw materials of leather wastes such as raw hides and skins from other animals or fleshings. A small-scale test of a fraction obtained from the hydrolysis of sheep fleshing using this method was carried out and the sample appeared to be palatable to cats using a quick acceptance test. However, this test was not a standard test, so it was not included in the thesis. The classic two-bowl test and repeat testing ideally using the same panel of cats should be arranged in the future to prove the general application of this hydrolysis method.

The characterisation of aroma active compounds or taste active compounds, is still a tough challenge. One approach maybe to separate the hydrolysed supernatant into further fractions by some standard such as molecular weight, and then feed each one to cats and find out which part is most palatable. By repeating this, the range of aroma active compounds or taste active compounds would be narrowed down. However, methods for separation liquid mixture at very large scale (in order to feed cats) are limited. More separation methods need to be tested in the future.

More data processing is also needed to be run in the future. Chapter 5 only presented and discussed very basic results. For example, principal components analysis (PCA) could be used to explore this dataset further.

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