TESIS DOCTORAL

CARACTERIZACIÓN DE COMPUESTOS SOLUBLES EN PIENSOS PARA LOS ANIMALES DOMÉSTICOS

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presentada por **Cécile Soltane**

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Que la memoria titulada "**CARACTERIZACIÓN DE COMPUESTOS SOLUBLES EN PIENSOS PARA LOS ANIMALES DOMÉSTICOS**" presentada por Dña. Cécile Soltane para optar al grado de Doctor por la Universidad de Valencia, ha sido realizada bajo su dirección y supervisión, reuniendo las condiciones necesarias para ser defendida por su autora.

Valencia, Mayo 2016

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SUMMARY

Meat-based palatability enhancers are commonly used by pet food industry to increase the acceptability of cat kibbles. The manufacturing of these enhancers involves two main steps, an enzymatic digestion and a thermal treatment, leading to many volatiles and non-volatiles compounds. It is well established that the performance of palatability enhancers can vary depending on raw materials and manufacturing processes generating different non-volatile compounds, some of which are considered as tastants. However, the reasons that explain the differences in palatability from a biochemical point of view are not known. The aim of the current project was to identify groups of taste-active compounds that correlate positively to cat palatability.

First, three raw materials (pork livers) were analysed from a biochemical point of view. Proteins, peptides, free and total amino acids, free and total fatty acids, nucleotides and minerals were analysed. Most differences between pork livers were observed for potential key tastants which concentrations depend on endogenous metabolic. Moreover, two palatability enhancers were studied and fractionated to improve their characterization. They were analysed from a biochemical point of view focusing on the proteomic study of peptides. The analysis of the peptide sequences confirmed the use of different proteolytic enzymes during the manufacturing of studied palatability enhancers. Finally, the sensory quality of each fraction was evaluated by a new technology called Microtiter Operant Gustometer (MOG) using trained rats. The sensory evaluation allowed the establishment of a range of palatability among fractions confirming the correlation between product composition and animal preferences.

RESUMEN

Los potenciadores del sabor a base de carne son usados frecuentemente para mejorar la aceptabilidad de los piensos para gatos. Los procesos utilizados para la fabricación de estos potenciadores consisten fundamentalmente en la licuefacción de las materias primas por digestión enzimática seguida de un tratamiento térmico, generándose una gran variedad de compuestos volátiles y no volátiles. Está ya bien establecido que la eficacia de los potenciadores del sabor depende de las materias primas y de los procesos de fabricación que generan distintos compuestos no volátiles, algunos de los cuales son moléculas sabrosas. Sin embargo, las razones que explican las diferencias de palatabilidad desde el punto de vista bioquímico quedan sin aclarar. Por lo tanto, la presente tesis doctoral se centró en la identificación de moléculas sabrosas que afectan de forma positiva a la palatabilidad de los piensos para gatos.

Primero, se analizaron tres materias primas (hígados de cerdo) desde un punto de vista bioquímico. Las sustancias analizadas fueron proteinas, péptidos, aminoácidos libres y totales, ácidos grasos libres y totales, nucleótidos y minerales. La mayoría de las diferencias entre los higados de cerdo se encontraron para moléculas potencialmente sabrosas cuyas concentraciones dependen del metabolismo endógeno. Además, se estudiaron dos potenciadores del sabor que se fraccionaron para facilitar su caracterización. Las fracciones se analizaron en cuanto a su composición bioquímica centrando el análisis en el estudio de los péptidos mediante técnicas de proteómica. El análisis de las secuencias peptídicas confirmó el uso de distintas enzimas proteolíticas durante el proceso de fabricación de los potenciadores de sabor estudiados. Como último paso, se evaluó la calidad sensorial de las fracciones mediante una nueva tecnología de medida del gusto en placa multipocillo empleando un panel de ratas entrenadas para dicho fin. La evaluación sensorial permitió establecer un rango de palatabilidad entre las fracciones y establecer una correlación positiva entre la composición del producto y las preferencias de los animales.

RESUM

Els potenciadors de sabor a base de carn s'utilitzen sovint per millorar l'acceptabilitat dels pinsos per a gats. Els processos utilitzats per a la fabricació d'aquestos potenciadors consistixen fonamentalment en la liqüefacció de les matèries primeres per digestió enzimàtica seguida d'un tractament tèrmic, generant compostos volàtils i no volàtils. Està ja ben establit que l'eficàcia dels potenciadors de sabor depén de les matèries primeres i dels processos de fabricació que generen distints compostos no volàtils, alguns dels quals són molècules saboroses. No obstant això, les raons que expliquen les diferències de palatabilidad des del punt de vista bioquímic queden sense aclarir. La present tesi doctoral s'ha centrat en la identificació de molècules saboroses que afecten de forma positiva a la palatabilitat dels pinsos per a gats.

Primer, s'han analitzat tres matèries primeres (fetges de porc) des d'un punt de vista bioquímic. Les substàncies analitzades han sigut proteïnes, pèptids, aminoàcids lliures i totals, àcids grassos lliures i totals, nucleòtids i minerals. La majoria de les diferències entre els fetges de porc s'han trobat per a molècules potencialment saboroses, les concentracions de les quals depenen del metabolisme endògen. A més, s'han estudiat dos potenciadors del sabor que s'han fraccionat per a facilitar la seua caracterització. Les fraccions s'han analitzat d'acord a la seua composició bioquímica centrant l'anàlisi en l'estudi dels pèptids mitjançant tècniques de proteòmica. L'anàlisi de les seqüències peptídiques ha confirmat l'ús de distints enzims proteolítics al llarg del procés de fabricació dels potenciadors de sabor estudiats. Com últim pas, s'ha avaluat la qualitat sensorial de les fraccions mitjançant una nova tecnologia de mesura del gust en placa multipou emprant un panell de rates entrenades per aquest fi. L'avaluació sensorial ha permés establir un rang de palatabilitat entre les fraccions i una correlació positiva entre la composició del producte i les preferències dels animals.

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Abbreviations

A amiloride
AAFCO Association of American Feed Control Officials
AAP alanyl aminopeptidase
ACN acetonitrile
AMC alanine-amido-4-methylcoumarin
ANOVA analysis of variance
AQC 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
AC radio frequency
ALA α-linolenic acid
AMP adenosine 5'-monophosphate
ArA arachidonic acid
BHMT betaine-homocysteine methyltransferase
BSA bovine serum albumin
CDD charge coupled device
CE capillary electrophoresis
Cis cystine
DC direct current
DHA docosahexaenoic acid
DTT dithiothreitol
ECD electron capture detector
EDTA ethylenediaminetetraacetic acid
EGTA ethylene glycol tetraacetic acid
EPA eicosapentaenoic acid
ESI electrospray ionization
FAA free amino acids
FAME fatty acid methyl ester
FFA free fatty acids
FID flame ionisation detector
FLD fluorescence detection
FMOC 9-fluorenylmethyl chloroformate
GC gas chromatography
GFC gel filtration chromatography
GMP guanosine 5'-monophosphate
GSH glutathione
GSSG oxidised form glutathione
HILIC hydrophilic interaction chromatography
HPLC high performance liquid chromatography
ICP-OES inductively coupled plasma optical emission spectrometry
IEC ion-exchange chromatography
IMP inosine 5'-monophosphate

IP-RP-HPLC ion-pairing reversed-phase high performance liquid chromatography
L 100% Landrace
LA linoleic acid
LC liquid chromatography
LC-MS/MS liquid chromatography coupled to tandem mass spectrometry
MALDI matrix-assisted laser desorption/ionisation
MAP methionyl aminopeptidase
MOG microtiter operant gustometer
MPA 3-mercaptopropionic acid
MRP Maillard reaction product
MSG monosodium glutamate
MS/MS tandem mass spectrometry
MUFA monounsaturated fatty acid
NPM N-(1-pyrenyl)maleimide
NEM N-ethylmaleimide
NEp New enzyme product
OEp Old enzyme product
OPA o-phthaldialdehyde
PAGE polyacrilamide gel electrophoresis
PBS phosphate-buffered saline
PCA perchloric acid
PITC phenylisothiocyanate
PLE pressurized liquid extraction
PLWL 50% Pietrain/ 25% Large White/ 25% Landrace
PLW 50% Pietrain/ 25% Large White
PMF peptide mass fingerprinting
PUFA polyunsaturated fatty acid
Q quadrupole
RAP arginyl aminopeptidase
RP-HPLC reverse-phase high performance liquid chromatography
SBB serine borate buffer
SEC size-exclusion chromatography
SDS sodium dodecyl sulfate
tCys total cysteine
TAA total amino acids
TFA total fatty acids
tGSH total glutathione
ToF time-of-flight analyser

Nomenclature for amino acids

Full name	Three-letter abbreviation	One-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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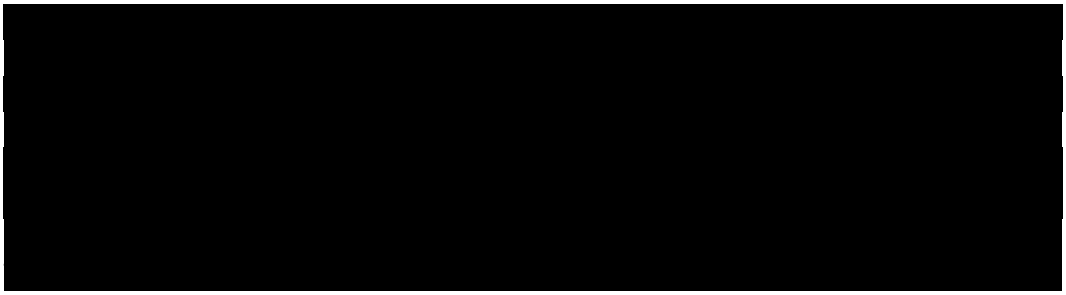

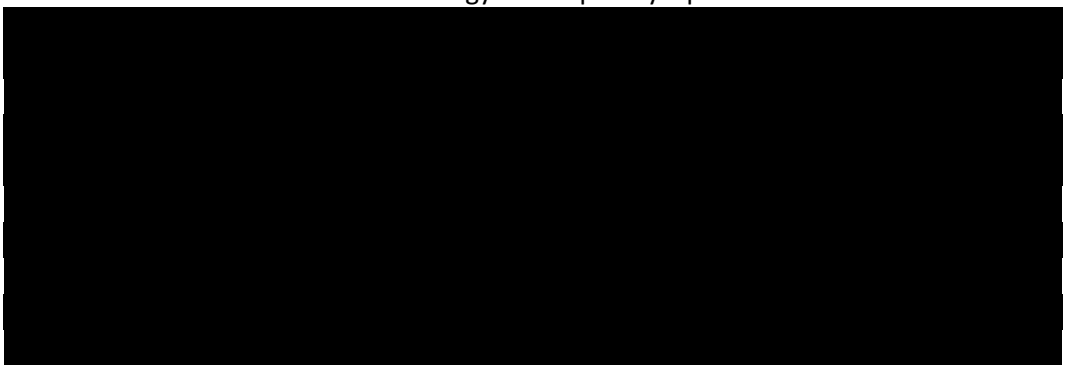

INTRODUCTION

This PhD project was financed by DIANA *Petfood* (Division of Symrise Group). The company develops innovative solutions for pet food manufacturers, intended to improve the palatability of food and the well-being of dogs and cats (http://www.spf-diana.com/).

The pet food industry is constantly offering new products to satisfy specific desires and needs for animals and pet owners. Among this offer, palatability enhancers, i.e. products that contribute to improve the stimulation of taste and/or olfactory system are the main products. To stimulate the nose and tongue sensorial sensors of cats and dogs, volatile and soluble compounds must be present in palatability enhancers. Hence, many processes used to prepare these enhancers are based on transformation of raw materials like animal by-products for instance. Processes that are involved are mainly product liquefaction via enzymatic hydrolysis, followed or not by a thermal treatment that is used initially to ensure the safety of the product.

The enzymatic hydrolysis releases “simple” products directly coming from the raw material like proteins which are not volatile and/or sapid. In a second step, these “simple” products can react to each other if a thermal treatment is applied. The Maillard reaction is an example of this thermal reaction between an amino acid and a reducing sugar, usually requiring high temperature (> 80°C). During this reaction, many flavour compounds are generated. Their nature, origin and chemical formulas bring to the final enhancers specificities in term of aroma. Consequently, a modification of the process and the raw material used to produce the palatability enhancers may directly impact on the generation of tastants and flavours. Then, the obtained palatants (in liquid or powder form) are dusted at the surface of cat or dog kibbles. The palatability of the final product is evaluated directly by animal. In fact, pets preferences for one or another product is determined by a trained panel of pets, i.e. cats or dogs, using different methodologies of food presentation as it could be done with humans.

This project is part of the DIANA *Petfood* research projects which aim to better understand the drivers for palatability in cats. More precisely, it is part of the “gustative” chapter dedicated to the identification of the tasty molecules of interest for cats.

To achieve a molecular understanding of palatant taste perception, several products of known and different palatability from DIANA *Petfood* will be studied trying to isolate chemical compounds involved in these differences. The choice was deliberately done not to focus on generated aromas, but to study more in depth the tasty fractions and their contribution to cat preferences.

1.**STATE OF ART**

The domestic cat (*Felis silvestris catus*) is a carnivorous mammal. Compared to dog, the domestication of cat arrived late [1]. The earliest archaeological finds indicate that cats were first domesticated in Egypt around 2000 B.C. More recent discoveries suggest that the cats' taming began at least 3500 B.C. in Egypt [2] but, in Cyprus, remains of a cat were found in a human grave dated as coming from 9000 to 7500 B.C. [3]. This discovery supports the hypothesis that cats' domestication begun with grains farming in the Fertile Crescent when humans used cats to control the rodent population who ate the harvest. In 2014, the genome of domestic cat was sequenced and compared with the wild cat (*Felis silvestris*) genome. Significant differences were found between the two species. These differences concerned genes involved in memory, fear and search of reward [4].

In 2014, there were approximately 400 millions of domestic cats in the world, of which more than 90 millions in the U.S.A. and 65 millions in E.U., and around $22 billion were spent by U.S. owners for cat food and treats [5]. Cats are now considered as part of the family and most owners are searching for the best products. In order to satisfy clients' requests, pet food industries are constantly improving their recipes to offer the better tasting and nutritive foods. This improvement is closely correlated with the understanding of cat behaviour and taste preferences.

1.1 Feeding behaviour and taste perception

1.1.1 Feeding behaviour of cats

Most studies of cats' eating behaviour are performed by pet food companies and data are mostly empirical. Indeed, it is easier to satisfy specific nutritional requirements when ideal alimentary environment and social habits are well known.

The domestic cat is considered as obligate carnivore because its survival depends of nutrients only found in animal flesh. Thus, it requires a diet of primarily flesh and organs. This eating behaviour suggests that cats have a number of special dietary requirements that do not apply to many other animals like humans or dogs [6]. Cats' eating behaviour includes several phases from the food research, its recognition and its acceptance to its ingestion and digestion.

Some laboratory tests have shown that if food is provided *ad libitum*, the domestic cat eats many small meals (7 – 20) per day evenly distributed between night and day. The quantity of food eaten and the frequency of meals vary among each cat [7–9]. Cats drink water as many times as they eat during nights and days [10]. This behaviour is probably a heritage of wild cat eating behaviour. In fact, wild cats eat various small preys per day to obtain their nutritional requirements [7]. In addition, they are usually considered as lonely hunters and this wild behaviour is reflected in domestic cats by an absence of strict social rules during meals. They usually eat alone but the presence of another cat does not affect their intake. Nevertheless, some studies have shown that a hierarchy exists when several cats live in the same house. Females during oestrous cycle and higher-ranking cats eat first [11]. When groups of cats are fed *ad libitum*, only 20% of meals involved two cats at the same time. In this case, two meals are considered as two only if there is more than one minute between one and another [8]. The behaviour of domestic cats before and after feeding by their owners cannot be related to cats' characteristic (sex, age...) since it appears to be highly cat-specific. However, no relation has been found between the owner characteristics and the behaviour of cats. It suggests that pet cats' behaviour at feeding is not related to owner attitudes and may be a consequence of developmental factors [12]. Even if owner attitudes are not involved, the behaviour of cats during feeding is strongly influenced by their environment. They need to feel safe and not stressed. A recent study has shown that various parameters can influence the behaviour of confined cats [13]. Indeed, attention must be paid to

both macroenvironment (room, light, noise) and microenvironment (cage dimensions, food and litter) to maintain cat welfare. All these factors have to be monitored when constituting a panel of cats for food evaluation.

During feeding sequence, taste is strongly linked with olfaction. This sequence can be resumed in four major steps which involve different senses: food selection (smell), grip (sense of touch), chewing (taste) and digestion. These steps must be well understood by pet food companies to use the most appropriate raw materials and adapt their processes. Post-meal behavioural sequences depend on the food appetence [12]. Most frequent behaviour are *stand*, *walk tail up* and *miaow* before meal, and *lick lips*, *groom face* and *groom body* after meal. To be more specific, if the cat is highly attracted by the food, it comes quickly to the bowl, licks the bowl, then its whiskers and finally, after eating, cleans its face after eating. On the contrary, if the cat is not attracted by the food, it starts by sniffing food and then licks its nose [14].

Cats have a kind of "calorie regulator" and, as other mammals, regulate their macronutrient intake. For cats, the target composition is estimated at 52% of energy brought by protein, 36% by fat and 12% by carbohydrate [15]. Nevertheless, early studies showed that dilution of cats' diet by non-nutritive substances results in a maintained bulk intake leading to a decrease in caloric intake. The bad palatability of these non-nutritive substances was a possible interpretation for the lack of caloric adjustment observed in these studies [16,17]. By contrast, they respond to dilution of their diets with water by increasing diet consumption to maintain dry matter intake [18] and can regulate energy intake regulation after food dilution when feed by commercial food [9,19]. If fed with a low-fat vs. a high-fat diet, cats regulate their daily food intake on the basis of energy density [19] .

1.1.2 Cat's taste perception

Taste is the sensory system devoted primarily to a quality check of food to be ingested [20]. The sense of taste in cats appears to be similar to that of other mammals except for sweet taste because lacking the sweet taste receptor. Actually, domestic cats are not attracted by sweet taste nor rejected it. The degree of sensitivity of cats for the five basic flavours can be classified as follows: sourness > bitterness > saltiness/umami > sweetness. The sense of taste is present five days before birth and evolves then [21].

Cats have around 475 taste buds much less than dogs (1700) and humans (9000). In vertebrates, taste buds are mainly located on the upper face of the tongue but, are also present in the mucosa of the palate, the epiglottis and the pharynx [22]. Taste buds located on cat tongue are included in the gustatory papillae and each taste bud contains polarized neuroepithelial taste cells (Figure 1). Four types of papilla are distributed on the cat tongue [23–26]:

- The filiform papillae are the most abundant and line the whole dorsal surface of the tongue. They are highly keratinized and permit the food retention on the barbed tongue surface. There are no taste buds in the filiform papillae but.

- The fungiform papillae usually contain only one or few taste buds at their top. They are mixed with the filiform papillae over the tongue.

- Only 1 to 6 circumvallate papillae are present on cat's tongue but their role in taste perception is very important since they contain up to 1000 taste buds in their side walls. They are the biggest papillae and are located on the posterior third of the tongue.

- The foliate papillae (6 to 8 on cat's tongue) are located on the lateral parts of the tongue and are almost deprived of taste buds.

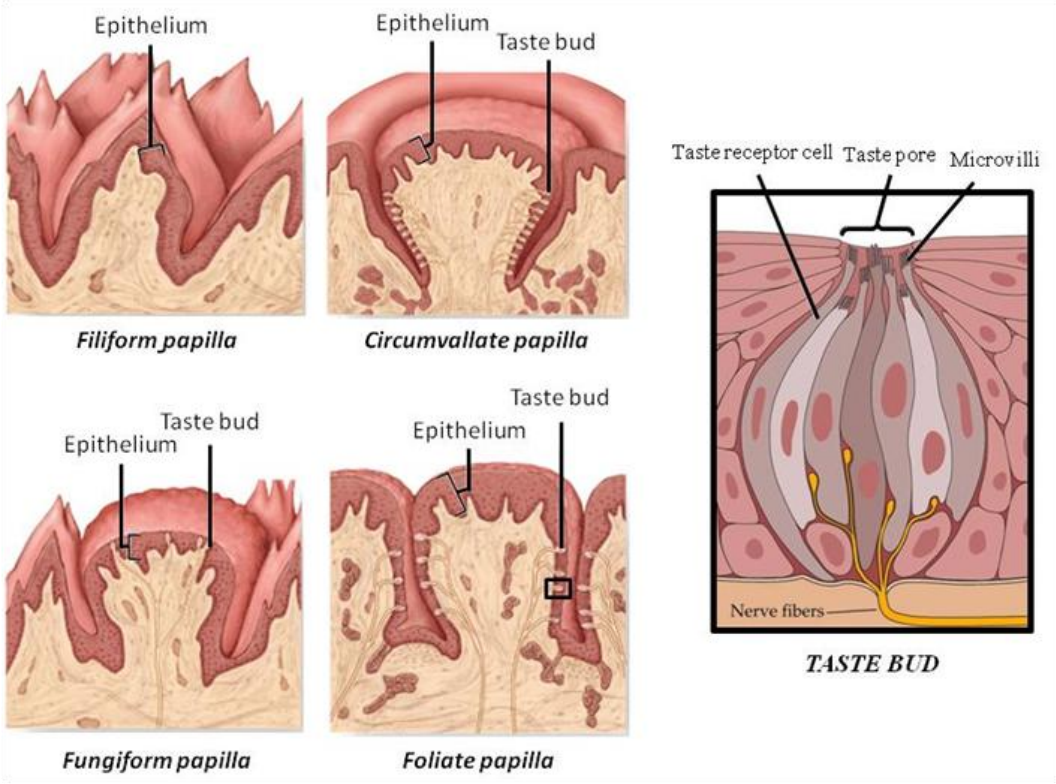

Figure 1. Papillae and taste bud (adapted from [27,28])

As mentioned before, each papilla contains several taste buds. To understand the role of each bud, the taste sensitivity of cat was first studied by stimulation of taste bud, connected to cranial nerves, by various substances. After bud stimulation, a neurologic signal was registered. Earlier studies attempted to classified taste receptors by measuring the electro-physiological responses of neurons innervating taste buds on cat's tongue. Boudreau [29,30] proposed to divide these chemoresponsive tongue units in three functional groups: Group I responded to acids; Group II responded to amino acids among others; Group III was discharged by nucleotides. Neurophysiological studies

were also done on goat and rat geniculate ganglion [31,32]. Chemoresponsive units of these species can also be classified in groups and present similarities with those found for cats. Thus, a basic model of four model groups was established for mammals: acid units, salt units, amino acid units and X units where X can be an alkaloid in the case of cats [33].

More recently, in mammals, the classification of taste buds was reconsidered based on ultrastructural features of taste cells. Taste buds were described as containing four types of cells [34]. Type I cells have voltage-dependent outward currents implicated in salt taste transduction [35]. Type II cells exhibit G-protein coupled receptors binding sweet, bitter or umami compounds [34]. Apparently, type II cells are not stimulated by salty or sour stimuli. Type III cells respond directly to carbonated solutions and sour taste [36,37]. Type IV cells are largely described as undifferentiated cells and their exact importance as cell population remains unexplained.

In mammals, the transduction of chemical stimuli provided by food into a neural signal involves taste receptors located in the taste cell microvilli [22]. These receptors are ionic (for salty or sour stimuli) or metabotropic (for sweet, bitter or umami stimuli). In case of salty flavour, sodium ions are transported through the membrane by sodium channels inducing the depolarization and the liberation of neurotransmitters into the cranial nerves. The acid compounds release protons H^+ responsible of acid flavour. In this case, two types of transduction exist: the H^+ acts like a sodium ion as described before, or it blocks potassium channels causing an increase of potassium ions concentration into the cell, a depolarization and the liberation of neurotransmitters into the cranial nerves. Bitter, sweet and umami flavours use the same transduction pathway. This transduction is more complex than the others since G protein-coupled receptors, T1Rs and T2Rs, are involved. T2Rs are specific of bitter taste. Umami flavour transduction use two receptors (dimmers), T1R1 and T1R3, and sweet flavour transduction, T1R2 and T1R3 [38]. In the

case of cats, the sweet taste receptor is missing. They are unable to taste sweet compounds due to defects in a gene that controls the structure of the sweet taste receptor [39]. The mammalian sweet taste receptor is actually made up of two coupled proteins generated by two separate genes: known as *Tas1r2* and *Tas1r3* [40]. In the case of cats, *Tas1r2* is a pseudogene and the heteromer T1R2/T1R3 cannot be formed [41]. Comparison of the domestic cat receptors with their human ortholog has also been done to understand cat taste perception, in comparison to human perception, showing that umami and bitter receptor functional characteristics were distinct between the two species [42,43].

1.2 Cat's food preferences and nutritional requirements

1.2.1 Food preferences

Cats are very sensitive to the taste, odour and texture of foods and their preferences are highly related to its obligate carnivore character. Thus, increasing the protein content of a defined food is likely to improve its attractiveness to cats. Taste is an important component for cat food preferences and it is very unified to their well developed sense of smell [44]. Generally, cats first smell food and if they find it attractive, they taste it. Texture and size of kibbles also influence food preferences. Adult cats have 30 teeth including 4 sharp and pointy teeth designed to capture prey, 12 small incisors and 14 no-rounded molars (Figure 2). Consequently, cats are incapable of chewing efficiently so they reduce the size of food by cutting it into smaller pieces before swallowed. Moreover, they frequently reject kibbles pieces with sharp edges. The temperature of food is also an important factor. Cats prefer food at blood temperature (around 35°C) certainly due to their original diet composed of fresh preys. They prefer moist food with

moisture content similar to that of meat (70-85%) but semi-moist and dry food are also accepted. The appetite also influences food choice. In fact, cat will eat a low attractive food only if it is very hungry but a high palatable food will be generally tasted in any case [14].

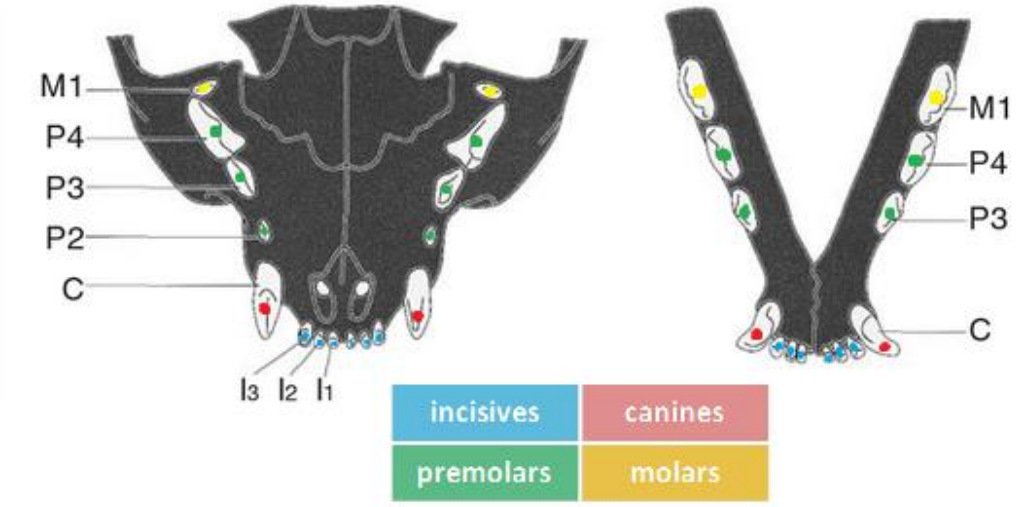

Figure 2. Feline dentition (adapted from [45])

Beauchamp et al. also examined flavour preferences in cats [46]. They observed that domestic cats do not show any preference concerning sweeteners diluted in water or saline solution. Solutions of hydrolyzed protein and amino acids or emulsified fat mixtures are preferred to the diluents. This avidity for proteins and fats and not for carbohydrates is related to the strict carnivore feature of cats. Eisert [47] proposed that the higher protein requirement of domestic cats is a consequence of a high amino acids conversion into glucose to supply the needs of brain and tissues. Cats are particularly attracted to amino acid stimuli especially proline, cysteine or alanine described as "sweet" by human. These amino acids activate amino acids units in cat. However, they reject stimuli that taste bitter or very sour to humans such as arginine, phenylalanine or

tryptophane which inhibit amino acids units [46]. It has been suggested that cats may evaluate the quality of meats by tasting adenosine triphosphate. Moreover, monophosphate nucleotides, when accumulated at high level in prey tissues after death, inhibit the amino acids units in cats and by this way, may avoid them to feed on carrion [6]. Even if cats do not show a large synergism between monosodium glutamate (MSG) and/or amino acids and inosine monophosphate (IMP) (unlike mice [48], dogs [49] and humans [50]), they are attracted by this "umami combination" which enhances meat flavour and food acceptance. In fact, the combination of MSG and IMP activate the same response pathway as NaCl in humans [51] and mixes of MSG and/or amino acids and IMP are widely used to enhance palatability of cat foodstuffs [52]. Animal protein hydrolysates, animal proteins and fat, emulsified meats and acids are flavours generally highly preferred by cats. They also are particularly attracted by fish, liver, meat, yeasts extract and acidic flavours [53]. Others flavours such as vegetable oils, fibres, vegetable proteins have negative effects on acceptance, or at least less positive acceptance [26]. Cats also reject medium chain fatty acid and caprylic acid [54]. Cats are indifferent to sucrose diluted in water but when NaCl is added, they drink the solution enthusiastically [55]. Paradoxically, they prefer milk if sucrose or lactose is added [46]. The optimum pH range for increasing salivation is 4.5 - 5.5, and taste response is increased when food temperature is about 30°C [26].

Feeding past and, especially, early feeding experiences guide individual food preferences of adult cats [56]. Two opposite effects of early experiences have been described in the literature [9,57–59]. The first effect, also called neophobia, is a propensity to reject unfamiliar food and to accept early experienced food in contrast to the second effect, called neophilia. Nevertheless, cats are largely described as neophobes more than neophiles. Kittens tend to eat and like the same foods that their mother ate during pregnancy and lactation and may develop a strong preference for this [60]. This neophobia can explain why some owners have difficulties to change their cat's diet. It

can also explain differences of food preferences between domestic and free-ranging cats. Farm cats prefer raw beef to canned meat while domestic cats reject raw beef probably due to neophobia. In the same way, kibbles are accepted by domestic cats but mostly rejected by farm cats [61].

1.2.2 Nutritional requirements

Cats have specific needs of nutrients from animal origin due to their specific metabolism. Cat's nutritional requirements are highly related to his strict carnivore behaviour. For example, cats' metabolism is unable to synthesize some essential nutrients such as retinol, taurine and arachidonic acid (ArA) out of vegetable matter and rely on animal protein in their diet to supply these elements. The typical energy intake repartition of a feral cat is estimated to 52% crude protein, 46% crude fat and only 2% N-free extract [62].

1.2.2.1 Protein and amino acids

Cats get most of their protein from animal products such as meat or fish. Animal-based proteins are usually easier to digest than plant-based protein and are better suited by cat's digestive system [63].

1.2.2.1.1 Protein requirements

First studies determining the protein requirements of cats were performed using foodstuffs or purified compounds and without knowing the amino acid requirements [64,65]. Therefore, the protein requirements were about 30% of the diet for kittens and about 20% for adult cats. Further studies were done ensuring that all amino acid requirements were met [66], thus, the protein requirements were lower, around 18% for kittens and between 10 and 16% for adult cats [63,67]. Currently, recommended protein concentration in cat food for adult maintenance of body weight is evaluated by

the Association of American Feed Control Officials (AAFCO) as 26% based on dry matter [68]. As others strict carnivores, cats have higher protein requirements for maintenance than omnivores do [10,69,70]. This specificity is not well understood. The most plausible reason is the high activity of enzymes involved in the nitrogen metabolism (protein-degrading) that cannot be shut off. In contrast to most omnivorous, cats have a limited ability to reduce nitrogen metabolism enzyme activities when fed diets low in protein [71]. Consequently, their rate of nitrogen loss is higher than in omnivorous species. Nevertheless, this hypothesis is probably not the only explanation for cats' high protein requirements. More recently, the hypothesis for a relation between protein intake and metabolic reactions such as protein oxidation or ureagenesis has been tested but unfortunately the reason of the high protein requirement remains unclear and requires further research [72,73]. Eisert proposed that glucogenesis from amino acids represents a significant metabolic sink for amino acids that increases the minimum protein requirements [47].

1.2.2.1.2 Essential amino acids

Proteins include 21 amino acids and only 10 are essential for cats meaning that they must be provided in the diet [63]. The deficiency of one of these essential amino acids can seriously compromise the health of both kittens and adult cats. Meat including organ meats, fish proteins and cereal glutens are the most common source of amino acids. Rogers and Morris [70] tested the essentiality of ten amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) on growing kittens. Deficiency in any of these amino acids resulted in a decrease in food intake, a loss of body weight and a low concentration of the deleted amino acid in the plasma. Others health problems due to a deficiency or an excess of some of these amino acids are described below.

Lysine is important for the synthesis of all proteins. Lack of lysine can result in weight loss. Anderson et al. [74] has established requirement estimates for arginine and lysine. First, they suggested a requirement of 0.80% for lysine and then, established a requirement of 0.83% for arginine at this level of lysine. AAFCO [68] recommends a lysine concentration in adult cat food of 0.83% in dry basis.

Arginine is essential for growth and urea production. However, a diet without arginine can provoke hyperammonemia in near-adult cats [75] which causes vomiting, hypersalivation and nerve problems. Recommended arginine concentration in adult cat food is 1.04% in dry basis [68].

Histidine has a structural function in proteins and is also a precursor for some neurological compounds such as histamine. In cats, a marginal deficiency during a long period of time can result in cataracts [76]. Recommended histidine concentration in adult cat food is 0.30% in dry basis [68].

Methionine and cysteine are particularly important for the keratin synthesis. Nevertheless, kittens do not grow normally when the diet contains 2% or more methionine [77]. Their body gain weight is smaller than in control conditions (0.5% Met diet). Recommended methionine concentration in adult cat food is 0.2% in dry basis and should not exceed 1.5% [68]. Cysteine can be synthetised from methionine but if supplied by diet, methionine can be used for other functions. Cysteine is also involved in the synthesis of felinine, a putative pheromone precursor excreted in the urine [78,79].

Threonine is a precursor of active molecules of metabolism such as pyruvate. Threonine deficiency can result in weight loss and nervous system issues affecting the mobility [80]. Recommended threonine concentration in adult cat food is 0.73% in dry basis [68].

Phenylalanine and tyrosine are aromatic amino acids involved in hair pigmentation [81]. Only phenylalanine is considered essential since tyrosine can be synthetised from

phenylalanine but if tyrosine is supplied by diet, phenylalanine can be used for other functions. Phenylalanine and tyrosine are essential for thyroid and adrenal gland functions. They ensure an appropriate functioning of the brain and are required for reproduction. Signs of deficiency include neurological dysfunction, uncoordinated gait and hyperactivity in cats [82]. Recommended phenylalanine-plus-tyrosine concentration in kitten food and in adult cat food were, respectively, 1.92% and 1.53% in dry basis [68]. Approximately 73% of that concentration can be provided with tyrosine.

Tryptophan is required for hormone production such as serotonin and melatonin. Deficiency can lead to refusal to eat and weight loss. Tryptophan minimal requirement of the kitten was evaluated at 1.1 g of tryptophan per kg of diet for a maximal growth and nitrogen retention [83]. More recently, AAFCO evaluated the optimal concentration of tryptophan in cat food at 0.25% (dry basis) for kitten and at 0.16% for adult cat but should not exceed 1.7%.

Unlike other essential amino acids, taurine does not have any role in protein synthesis but deficiency of taurine is associated to serious clinical problems [84]. Retinal degeneration is associated with a decrease in taurine in cats [85]. Dietary taurine deprivation has an effect on reproduction. Cats feed taurine deficient diets have poor reproductive performance [86] associated to congenital birth defects. The maturation of the cerebellum is delayed in taurine deprived kittens [87]. Moreover, Pion et al. [88] proposed a direct link between decreased taurine concentrations in the myocardium and decreased myocardial mechanical function. Taurine myocardial concentrations depend on plasma concentration which is modulated by taurine concentrations in diets. Recommended taurine concentration in dry cat food is 0.10% in dry basis, and 0.20% for canned food. The same concentration is recommended for kitten food [68].

Others amino acids are not essential but can have repercussions for cats' health. For instance, glutamic acid at or above 9% of the diet inhibits normal growth in kittens [89].

1.2.2.2 Lipids and fatty acids

In many commercial pet foods, 50% or more of the energy comes from fat even if the recommendation for crude fat in cat foods is around 9% in dry basis [68]. Dietary fats supply essential fatty acids that cannot be synthesised in the body and provide the necessary environment for absorption of fat-soluble vitamins. Essential fatty acids are part of two families: omega-3 and omega-6. In all animals, linoleic acid (omega-6; LA) and α-linolenic acid (omega-3; ALA) are essential but contrary to most animals, cats were shown to be incapable to convert them into ArA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively [90,91]. This conversion involves the action of elongase and desaturase enzymes. Pawlowsky [92] demonstrated that Δ6 desaturase activity does exist in the feline but only at low level and, as a consequence, cats need foods from animal origin as source of ArA, EPA and DHA. This low activity may be another inheritance of their strict carnivorous nature. Essential fatty acids play an important role in cell structure and function. Fatty acids are required for maintenance of skin and coat, strong immune system and reproductive system [10]. Omega-3 deficiencies can result in abnormalities in the nervous system like visions problems or learning difficulties. Omega-6 deficiencies are associated to physiological issues [93]. Minimum requirements of EPA and DHA for cats are not well established. AAFCO recommends an ArA concentration in adult cat food of 0.02% in dry basis [68].

1.2.2.3 Minerals

Twelve minerals are essential for cats [63].

Calcium is necessary for bones and teeth formation and also involved in nerve impulse transmission. Deficiency in calcium may compromise growth while excess of this mineral results in bones abnormalities and increased bone mineral density. Recommended concentration in adult cat food is 6 g/kg in dry basis [68].

Potassium is essential to maintain the acid-base balance in cells and to ensure nerve pulse transmission. It also plays an important role in the production of energy at cellular level. Deficiency in potassium can cause significant retarded growth and neurological disorders. Recommended concentration in adult cat food is 6 g/kg in dry basis [68].

Phosphorus is necessary for strong bones and teeth, energy production and is a structural component of DNA and RNA. Deficiency in phosphorus can result in slow growth, loss of appetite and locomotion disturbances. Recommended concentration in adult cat food is 5 g/kg in dry basis [68].

Chlorine is important for maintaining the acid-base balance in cells and the osmolarity of extracellular fluids. Deficiency in chlorine increases sodium concentration in renal fluid and can result in an excess of potassium excretion causing symptoms of potassium deficiency. Recommended concentration in adult cat food is 3 g/kg in dry basis [68].

Sodium is involved in regulation of osmotic pressure and nerve impulse generation and transmission. Deficiency in sodium is rare in cats but can be detected by increased heart rate and increased urine output. Excess of sodium consumption can cause dry mucous membranes. Recommended concentration in adult cat food is 2 g/kg in dry basis [68].

Magnesium is essential for healthy bone structure and nervous system. Deficiency in magnesium can lead to hyperextention of carpal joints and loss of appetite while excess in magnesium can result in urinary tract stone formation. Recommended concentration in adult cat food is 0.4 g/kg in dry basis [68].

Iron is essential for oxygen transport as is a vital component of hemoglobin and myoglobin. Deficiency in iron can lead to poor growth, pale mucous membranes and diarrhea. Very high level of iron can result in vomiting and diarrhea. Iron is also involved in many enzymatic reactions. Recommended concentration in adult cat food is 80 mg/kg of food in dry basis [68].

Zinc is essential for skin and coat health, and for reproductive function. Zinc is also a co-factor for several enzymes involved in cell replication. Deficiency in zinc can cause skin lesions, poor growth and testicular atrophy. Excess zinc can cause seizures in cats. Recommended concentration in adult cat food is 75 mg/kg of food in dry basis [68].

Manganese is involved in the formation of bones and cartilages. It also plays a structural role in many enzymes. Manganese deficiency in cats was not studied but it was reported that in dogs, deficiency can result in shortening of the front legs during growth. Extended manganese excess can cause iron deficiency. Recommended concentration in adult cat food is 7.6 mg/kg of food in dry basis [68].

Copper is necessary for melanin pigment (hair) and myelin (nervous system) formations. It also plays a role in iron metabolism and in defending cells against oxidation damage. Deficiency in copper can result in anæmia and hair depigmentation. Toxicity is rare although copper is stored in liver. Recommended concentration in adult cat food is 5 mg/kg of food in dry basis [68].

Iodine is involved in thyroid hormone synthesis impacting growth and metabolic rate regulation. Symptoms of deficiency are enlargement of the thyroid glands, hair loss and weight gain. Excess iodine leads to same signs to those observed in deficiency. Recommended concentration in adult cat food is 0.6 mg/kg of food in dry basis [68].

Selenium is essential to reduce cellular damage caused by free radicals and as support for immune response. Deficiency can result in depression and coma. Excess can appear if fed with high amount of fish. Recommended concentration in adult cat food is 0.3 mg/kg of food in dry basis [68].

1.2.2.4 Vitamins

Vitamins are organic compounds which can be divided in two families: fat-soluble vitamins, including vitamins A, D, E and K and water-soluble vitamins mainly vitamins B and C. Some vitamins, such as vitamin A and vitamin B3, cannot be synthesised by cats from precursors which must be directly provided by diet. Therefore, vitamin mixes are sometimes added to pet food during the process to complete the naturally-present vitamins of pet food raw materials. Vitamins have many functions such as vision (vitamin A), skin health (B2, B3, B5, B7), formation of blood cells and blood clotting (B9, B12 and K), protection of cell membranes from free radicals (vitamin E) and regulation of calcium metabolism (vitamin D). As a consequence, deficiencies can result in eyes problems, muscle weakness, blood and nerve disorders. Some vitamins are also toxic if consumed in high doses such as vitamin A which excess can cause skeletal lesions especially in growing kittens [94]. Vitamin C is not essential for cats since they are able to synthesise enough by their own.

Choline is generally classified as a B vitamin even if all animals can synthesise it to some degree. In cats, the production does not always cover requirements and choline must be provided by diet. Choline may prevent fatty liver disease (in diabetics) and cognitive disorder in cats and humans [95,96].

Recommended concentrations for vitamins in adult cat food are presented in Table 1.

Table 1. Recommended cat food vitamin profile based on dry matter [68]

Vitamin	Recommended concentration
A	3332 IU/kg
D	280 IU/kg
E	40 IU/kg
K	0.1 mg/kg
B1	5.6 mg/kg
B2	4.0 mg/kg
B3	60 mg/kg
B5	5.75 mg/kg
B7	4.0 mg/kg
B9	0.8 mg/kg
B12	0.020 mg/kg
choline	2400 mg/kg

1.2.2.5 Others nutrients

1.2.2.5.1 Carbohydrates

Carbohydrates are not essential in the cat's diet since cats can synthesise their glucose from amino acids but they are an abundant source of energy. In pet food, cereals, vegetables and legumes are the main source of carbohydrates and fibres. There is neither recommended concentration of carbohydrates/fibres in cat food nor evidence of deficiency symptoms. Nevertheless, too much or too little fibre may reduce faeces quality and excess carbohydrates can cause diarrhoea or contributes to feline diabetes and obesity [97].

1.2.2.5.2 Nucleotides

Nucleotides are not essential in cat's diet. Nevertheless, they are considered as immunomodulatory nutrients [98]. The appropriate quality and quantity of nucleotides in cat food are not established.

All these nutritional recommandations do not consider the palatability of the product and the economic reality. Moreover, the nutritional contribution of palatability enhancers is very low since they only represent from 0.5 to 3% of the final product while their sensorial values are essential.

1.3 Cat food and palatability improvement

The commercial pet food industry started in England around 1860 with the invention of the first dog biscuit by James Spratt. Commercial pet food gained popularity in the 1900s with the introduction of canned cat food, dry-meat meals for dogs and then, dry expanded type pet foods. Since the 1960s, feeding table scraps was considered as "dangerous" and a great range of pet foods emerged. More recently, a new type of pet food appeared, called "prescription" foods, especially formulated to meet the needs of a certain age group, animals affected by a specific disease or animals with nutritional and physiological specific requirements.

1.3.1 Commercial cat food trends

Nowadays, most pet owners take for granted that dogs and cats need a balance diets. Consequently, commercial pet foods represent more than 90% of the calories consumed by pets in North America, Japan, Northern Europe, Australia and New Zealand [53]. Emerging countries follow the same trend. They are purchasable in three basic forms

long-standing defined by pet food industry: moist, semi-moist and dry food (kibbles). Treats and supplements are also available.

1.3.1.1 Moist food

Moist pet foods contain between 60 and 87% of water [53]. The other nutrients are found in the dry matter part. To avoid the presence of “free” water, gelling agents and gums are often used to solidify the loaf and imbibe water in high-moisture foods. A lot of moist cat foods contain high levels meat or meat by-products and as a result, the level of protein, fat, phosphorus and sodium in these products is generally higher than in dry pet foods. These nutrients act enhancing the palatability of moist foods and are considered as supplement to the dry main meal [53]. Moreover, palatability enhancers such as protein digests can be added during the process. Different packages are found in the market of moist foods such as plastic tubes, steel cans or aluminium trays (Figure 3).

Figure 3. Examples of moist foods for cats and traditional packaging

1.3.1.2 Semi-moist food

Semi-moist pet foods contain between 25 and 35% of water [53]. To avoid growth mold and to control the water activity, humectants are usually added to semi-moist foods. An alternative is to acidify the product. This second option gives an acidic note which is very appreciated by cats. A lot of semi-moist foods are designed to be attractive to the owners, by mixing forms and colours (Figure 4) but have the disadvantage that is not healthy for all cats because of its high sugar and its low fibre content [53]. Additionaly, some humectants such as propylene glycol, which has been banned as cat food additive by the U.S. Food and Drug Administration [99], can damage cat's red blood cells [100,101].

Figure 4. Examples of semi-moist foods for cats

1.3.1.3 Dry food

Dry pet foods contain between 3 and 11% of water [53]. They are usually made by extrusion cooking but flaking, baking or crumbling are other possible manufacturing methods (Figure 5). In general, protein, fat and mineral contents are lower in dry cat

food than in moist cat food (in dry basis). Indeed, some heat-sensitive vitamins can be destroyed and protein quality can decrease during the extrusion process [102]. Such vitamins and fat, as well as protein hydrolysates, can be sprayed on the kibbles after the extrusion process to increase palatability. Dry cat foods are also cheaper to manufacture than moist and semi-moist foods based on cost-per-calorie mostly due to the low water content. They are accepted by most cats but are generally less attractive than moist foods. Dry cat foods are also convenient to use by owners due to long shelflife, low level of unpleasant odour compared to moist foods.

Figure 5. Examples of dry foods for cats

1.3.2 General industrial processing of pet food palatability enhancers

As mentioned before, palatability enhancers can be added to cat foods during manufacturing especially to dry foods which are less palatable. In fact, most dry cat food kibbles are coated with them. A huge range of ingredients is considered as palatability enhancers such as fat, monosodium glutamate, yeasts extracts, cheese powder or whey for example. Palatability enhancers may be a single ingredient or a mix of several

ingredients, in raw form or processed, natural or synthetic. In this section, the main steps of palatability enhancer manufacture are described.

1.3.2.1 Source of meat by-products

As defined by AAFCO, meat by-products are the non-rendered, clean parts, other than meat, derived from slaughtered animals. It includes, but is not limited to lungs, spleen, kidneys, brain, livers, blood, bone, partially defatted low temperature fatty tissue, stomachs and intestines freed of their contents. It does not include hair, horns, teeth and hoofs. Obviously, it shall be suitable for use in animal food [103]. Meat by-products are used as protein sources.

Among meat by-products, cats are particularly attracted by liver. Beef, pork and chicken livers are the more common meat by-products used in pet foods but others species, such as lamb, kangaroo, duck or turkey, may be used depending on the regional availability. According to U.S. Department of Agriculture nutrient databases, pork liver composition (Table 2) is rather different from other pork organ compositions essentially due to its high protein content, which is a real asset for cat food palatability. This organ is also a good source of iron, zinc and phosphorous and it is relatively rich in carbohydrates, especially in glycogen, which is an important reserve of glucose for Maillard reaction. Furthermore, many flavour compounds have been identified in both raw and cooked liver which reinforce its attractiveness for cats [104–106].

As a first step of palatability enhancer manufacturing, meat by-products are grinded before being processed as described below.

Table 2. Nutrient composition of raw pork liver [107]

Nutrient	Unit	Value per 100 g
Proximates		
Water	g	71.06
Energy	kcal	134
Energy	kJ	561
Protein	g	21.39
Total lipid (fat)	g	3.65
Ash	g	1.44
Carbohydrate	g	2.47
Fiber	g	0
Amino Acids		
Tryptophan	g	0.301
Threonine	g	0.91
Isoleucine	g	1.085
Leucine	g	1.906
Lysine	g	1.649
Methionine	g	0.53
Cystine	g	0.404
Phenylalanine	g	1.047
Tyrosine	g	0.729
Valine	g	1.321
Arginine	g	1.317
Histidine	g	0.582
Alanine	g	1.276
Aspartic acid	g	1.937
Glutamic acid	g	2.782
Glycine	g	1.239
Proline	g	1.146
Serine	g	1.157

Nutrient	Unit	Value per 100 g
Lipids		
Fatty acids		
total saturated	g	1.17
C14:0	g	0.02
C16:0	g	0.44
C18:0	g	0.7
total monounsaturated	g	0.52
C16:1	g	0.03
C18:1	g	0.46
total polyunsaturated	g	0.87
C18:2	g	0.35
C18:3	g	0.03
C20:4	g	0.44
C20:5 n-3 (EPA)	g	0
C22:5 n-3 (DPA)	g	0.03
C22:6 n-3 (DHA)	g	0.02
Cholesterol	mg	301
Minerals		
Calcium	mg	9
Iron	mg	23.3
Magnesium	mg	18
Phosphorus	mg	288
Potassium	mg	273
Sodium	mg	87
Zinc	mg	5.76
Copper	mg	0.677
Manganese	mg	0.344
Selenium	µg	52.7
Vitamins		
Vitamin A	µg (IU)	6502 (21650)
Vitamin B1	mg	0.283
Vitamin B2	mg	3.005
Vitamin B3	mg	15.301
Vitamin B5	mg	6.65
Vitamin B6	mg	0.69
Vitamin B9	µg	212
Vitamin B12	µg	26
Vitamin C	mg	25.3

1.3.2.2 Enzymatic digestion and thermal treatment

Due to the high protein content of meat by-products, the enzymatic digestion contributes to release peptides and free amino acids, among others, largely considered as tasting compounds for humans. Meat by-products, and especially viscera, naturally contain endogenous proteolytic enzymes, mostly exoproteases but, to produce a high palatable digest, meat by-products are treated with selected enzymes, mostly endoproteases such as papain or serine proteases [108]. The digestion takes place under controlled conditions of pH and temperature which correspond to the optimal values or ranges for the enzyme activity.

Various patents described the recent processing advancements made to improve the efficiency of meat digest-based palatability enhancers based on the use of several enzymes and sequences on the digest [109–112].

Once the digest is obtained, sugars or others compounds may be added and enzymes are inactivated usually by heat treatment between 80°C and 120°C. The addition of sugars and the temperature rise are an essential condition for thermal reactions, such as Maillard reaction, contributing to flavour and taste of the final product [113]. The Maillard reaction also named as “non-enzymatic browning” is one of the main pathways for the generation of flavour compounds in meat and meat products [114]. This browning is the result of the reaction between the carbonyl groups of sugars and the amino groups of amino acids when exposed to heat.

Thus, an effective tissue digestion may lead to favour the Maillard reactions, increasing the number of small peptides available to react with sugars present in the mix. It was shown that the compounds resulting from the reaction between small peptides (1000-5000 Da) from soybean hydrolysate and xylose contribute to increase the intensity of human mouthfulness in umami solution [115].

The effects of the Maillard reaction on the nutritive value of pet foods have been discussed highlighting the no-nutritional value of early Maillard reaction products (MRP) derived from lysine. In fact, significant amount of lysine (up to 62%) is replaced by these early MRP which can be absorbed in the gastrointestinal tract but cannot be used by the pet body [116].

1.3.2.3 Preparation of liquid and powder forms

After the digestion, the final product is in liquid form. This hydrolysate may be used directly as palatability enhancers after stabilisation or can be spray-dried to obtain products in powder form (Figure 6). Before spray-drying, inactive brewer's yeast extract is added to the liquid form product as drying support. These yeasts also contribute to enhance the palatability since they contains nucleotides enhancing umami taste [117].

After extrusion and drying steps, kibbles are coated by fat and, then, palatability enhancers. The typical application rates over kibbles or other types of pet food are 1-3% for liquid-form and 1-2% for powder-form palatability enhancers [108].

Figure 6. Liquid and powder palatability enhancers

1.3.3 Palatability

In pet food industry, palatants are used to increase the palatability. Wet or dry palatant coating is the last processing step to improve smell and taste and thus, attractiveness of pet foods.

1.3.3.1 Definition

Palatability was first described as the characteristics or the conditions which stimulate a selective response by the animal considering that the palatability was inherent to each aliment [118,119] and would depend on taste, smell, appearance, temperature and texture. However, other criteria may modulate the palatability, such as the feeding experience and the metabolic state of the animal [120] and even the appetite [121]. Thus, the palatability can be defined as a multi-factorial parameter which characterizes the acceptability of food and takes in account physical and chemical factors.

1.3.3.2 Factors influencing the palatability

Palatable pet food is the result of four main factors: high quality ingredients, processing design, high quality palatants, and uniform application of palatants. In fact, the performance of an enhancer depends on raw material and process used to manufacture it. Meat by-products are commonly used as palatability enhancers' raw material especially liver, red meat and blood which are highly palatable [53]. Nevertheless, fish can be preferred to red meat or rejected by some cats highlighting animal factors effect. Thus, the palatability may be affected by sensory factors but also by other factors.

1.3.3.2.1 Sensory aspects

Smell

Cats have a high-developed sense of smell with 21 cm^2 of olfactory epithelia, more developed than humans (until 10 cm^2) and less than dogs (until 150 cm^2) [122]. Before

eating their food, cats always smell it intensively, especially unfamiliar foods, in order to discard any unhealthy food. Cats like not only to smell all available foods but also to taste them before deciding which to eat [53].

Taste

Obviously, the role of taste in a food acceptance is undeniable. Cats have an extended sense of taste. They can detect and respond to some amino acids which contribute to meaty and savoury aromas. They also respond to some nucleotides or fatty acids which enhance the meaty taste perception. Acidified foods, like dry foods, appeal to cats [53].

Texture/mouth feel

The mouth feel component is very important to take in account when manufacturing cat food. The size of kibbles in dry foods and chunks in wet food affect palatability. For the same formula, some cats can prefer one shape to another. In fact, cats prefer pieces having smooth rather than irregular surfaces and sharp edges. A recent study comparing the palatability of different shapes demonstrates that the "O" (disc) is the most preferred shape. Moreover, the coating of "O" kibbles with palatability enhancers results more homogenous than other shapes. Cats are highly sensitive to the homogeneity of palatability enhancers coating [123]. Mouth receptors and movement detectors located in the mouth allow cats to evaluate some characteristics such as elasticity, viscosity, cohesiveness and hardness. Cats prefer easy-to-chew foods and do not appreciate sticky foods [124].

Vision

This factor is mostly related with the wild hunting behaviour of cats. Concerning commercial pet foods, there is no evidence that colour can influence the acceptance. It certainly affects more owners' preference than pets' [53].

1.3.3.2.2 Other factors

On average, moist foods are preferred to dry foods and semi-moist foods are not really appreciated by cats. Moreover, cats prefer foods with a high protein level so the increase in protein content increases the palatability. Cooking also have a positive effect on palatability but overcooking decreases preference. As mentioned before in section 2.2.1., food experiences influence cat food acceptance. In fact, cats "print" the preference of their mother and show preference for foods they received in their early age [56,57].

1.3.3.3 Measurement of palatability

As said before, palatability depends of several factors such as physicochemical or nutrition factors. Intentionally, in this part, only palatability measurements by sensory analysis will be described.

Palatability measurement tests can be conducted on two types of animal panels: in pet centres with expert panels or in an in-home environment with owner's pets. Expert panels have a higher discriminative power but need to be trained before being exposed to a large diversity of foods. Quality tests are conducted frequently in expert panels to avoid any bias. In-home panels do not have any training and testing conditions are less controlled. Moreover, the feeding history of such panels can lack diversity and these palatability tests must include at least around 100 animals. Nevertheless, an in-home panel permit to obtain "real-life" data [125].

In general, the main objective of palatability tests is to quantify the hedonic value of a pet food [26]. Two tests are most commonly used to assess palatability in pet food: one-bowl test and two-bowl test. One-bowl test or acceptance test is used to measure the acceptability of a product. In this case, the animals have free access to one product during a determined period. Pet food intake is determined by weight difference of the

bowl before and after the test. This test is mostly used for product development validation. Two-bowl test is used to measure if cats have a preference for one diet through quantities of food eaten. For this kind of tests, two identical bowls are simultaneously presented to the cats. It compares two products and permits to establish a preference based on the difference of quantities eaten in a defined period of time. Thus, it is the most common test used in expert pet panels for the development of new products when attempting an improvement of a product over another.

Some complementary indicators provide additional information to the acceptance and preference tests described previously but they are mostly described for dogs [126–128]. Human or rat taste panels can also be used to optimize the palatability as describing flavours and texture of cat food [129]. Recently, the American company Opertech Bio Inc. has developed what it calls the Microtiter Operant Gustometer (MOG) which consists in an "automated, high-throughput system for rapid characterization of taste sensory properties" by rats [130]. Obviously, the use of humans or rats has limitations due to the differences in taste and flavour perceptions between these species.

1.4 Potential non-volatile tastants

Some molecules, called tastants, stimulate the sense of taste. Among these compounds contributing to the taste, non-volatile taste compounds are considered to be relevant. In this section, some of the potential non-volatile tastants for humans and/or cats are described as they may influence the palatability of food and/or pet food. These tastants are naturally present or are generated during industrial processes or ripening processes of human foodstuffs and maybe cat foodstuffs.

Focus is done on the influence of each group of molecules separately and potential interactions between groups are not described. Nevertheless, it is important to mention

that these interactions exist and may affect food and pet food palatability [131,132]. The taste sensation has been divided into five basic tastes based on human description: bitterness, sweetness, sourness, saltiness and umami. Cat taste buds are very similar to human taste buds even if differences in sensory performance related to nutritional requirements may exist [20,43] but only few studies of domestic cat taste perception have been published. Thus, most compounds described in this section are tastants for humans and, by extrapolation, are considered as putative tastants for cats even if cats do not necessarily experiment the same subjective sensation that humans do. As mentioned in section 1.1.2., cats are not sensible to sweetness, even if "sweet sensation" can activate neural group involved in taste perception, thus carbohydrates are not described in this section. The potential influence of organic acids on palatability is not discussed either.

1.4.1 Proteins and peptides

1.4.1.1 Proteins as potential tastants

Several plant and animal proteins, such as thaumatins [133], curculin [134] or hen egg white lysozyme [135], have been described as tasting sweet or presenting taste-modifying capacities for humans. The amino acid sequence has been presented as a putative reason for protein sweetness, especially the presence of lysine and arginine residues at specific sites [136–138]. Nevertheless, no tasting proteins have been described in pet foods or meat-based products. Even if the taste may not be directly affected by proteins, they may play an important role in texturing a product, affecting palatability.

1.4.1.2 Peptides as potential tastants

The knowledge on the taste of peptides was first reviewed in 1969 [139,140]. The same year, aspartame (Asp-Phe-OCH_3) was discovered [141]. Kirimura (1969) proposed a classification in three groups based on taste characteristics of peptides for humans. He also pointed the relation between peptide taste and amino acid sequence of the peptides. Thus, peptides in Group I had a sour taste and were rich in acidic residues, peptides in Group II had a bitter taste and were rich in hydrophobic residues, and peptides in Group III with a balanced composition had no or almost no taste. However, there are no simple relations between the taste of peptides and the taste of amino acids [140].

Many peptides with sweet, umami or bitter taste have been described in the literature. In opposite to the proteins, lots of tasting peptides have been identified in meat or in meat-based products [142] and may affect the taste of palatability enhancers for pet food. Others peptides such as γ-glutamyl peptides have no taste but appear to enhance sweet, salty and umami tastes. It was reported that glutathione (GSH) enhance beef flavour [143] and mouthfullness and may influence the intensities of basic tastes in humans [144,145]. GSH has also been described as a *kokumi* (featured as taste-enhancing, complex and long-lasting impression) peptide found in several foodstuffs and yeast extracts [146].

Umami peptides

Many peptides have been identified as umami in food especially in Japanese foodstuffs. Most of them are di- or tripeptides. Arai et al. [147] reported that α-glutamyl peptides with hydrophilic amino acids such as Glu-Asp, Glu-Thr and Glu-Ser show umami at neutral pH for humans.

Nevertheless, some authors have suggested that small peptides cannot be considered as umami compounds by humans since contradictory results have been obtained. For example, Noguchi et al. [148] and Tamura et al. [149] reported that peptides such as Asp-Asp, Glu-Asp, Lys-Gly or Glu-Glu-Glu elicited an umami taste. Van den Oord and van Wassenaar [150] re-examined these results and did not find any peptides eliciting umami taste. In this way, the existence of an independent class of peptides defined as umami is not persuasive. Maehashi et al. [151] isolated peptides from food protein hydrolysates and characterized their taste properties. They showed that even if the hydrolysate possessed umami taste, most of the main peptide components had no umami taste but a sour taste and, only a combination of some of them and IMP elicited a "full" umami taste for humans. Currently, no published results report the effect of umami peptides on food acceptance for cats.

Bitter peptides

Bitter peptides have been identified in a large range of foodstuffs. Bitter peptides have been found in Japanese products [152,153] and also in cured or fermented products such as cheese [154], ham or cured meats [155], since the enzymatic hydrolysis tends to produce bitterness [156]. The presence of bitter peptides in meat-based palatability enhancers for pet food has not been studied. Nevertheless, since their production is mainly based on proteolysis, the presence of these peptides must be taken into account when studying their taste characteristics.

Only few authors have identified bitter peptides and then, synthesized them in order to evaluate the taste characteristics of pure peptides. Matoba and Hata [157] suggested that the hydrophobicity and the amino acid sequence of the peptides were involved in their bitterness. For most of tested peptides, when arginine is contiguous to proline (such as in Arg-Pro or Gly-Arg-Pro), a strong bitter taste has been observed for humans [158]. However, models are based on human receptor and may not be adequate for cats

since response profiles of the cat bitter receptors are distinct from those of human bitter receptors [43].

Sweet peptides

Up to date, no natural sweet peptide has been identified [159]. Nevertheless, some synthetized di- and tri-peptides containing glycine and alanine residues have been described as sweet by humans [160]. The most used sweet peptide is the aspartame (Asp-Phe-OMe) discovered by accident in 1969 [141]. Logically, models built to understand the relationship between structure and sweet taste of peptides were largely inspired by the structure of aspartame until the discovery of the human sweet receptor and the modelisation of its active sites [161].

Sour peptides

Most of di- and tri-peptides described as sour by humans contain Asp and/or Glu residues which can liberate a proton able to react with the salty taste receptors [152].

Salty peptides

Some peptides have been reported as presenting a salty stimuli or a salty after-taste in humans such as the L-Ornithyltaurine [151,162,163]. Nevertheless, the existence of salty peptides has been discussed because of the presence of sodium ions in the samples which could be responsible of the detected salty taste [164].

1.4.2 Free amino acids

The contribution of amino acids to food taste has been widely described since the importance of monosodium glutamate in Japanese foodstuffs was demonstrated in 1909 by Ideka [165]. Several studies have been done to elucidate the taste of each amino acid and to understand their contribution to taste in humans. In 1938, the first amino acid isolated from gelatine, now known as glycine, was defined as sweet [166]. Later, sensory

tests were performed by several groups to evaluate the taste intensity of individual amino acid focusing on enantiomeric differences. Solms et al. [167] concluded that L-amino acids mainly elicit sweet or bitter taste while D-enantiomers have sweet taste in aqueous solution. Other studies evaluated the taste qualities of amino acid powders by rating them on semantic differential scales with 46 adjective descriptions such as repulsive/not repulsive or meaty/not meaty [168,169]. The contribution of amino acids to food taste was shown in chicken [170], cheese [171] and Japanese products [139].

Taste qualities of amino acids and their thresholds were also evaluated by humans using amino acids solutions. However, general relationships between structure and taste quality of amino acid were observed, and the authors minimised the influence of chirality on the taste of amino acids [172]. More recently, Kawai et al. [173] published a complete amino acid sensory characterization established by measuring the human gustatory intensity and quality in response to aqueous solutions of amino acids. They concluded that hydrophobicity, size, charge, functional groups in the side chain and chirality of the alpha carbon may influence the basic taste of each amino acid. Differences observed between studies are mainly due to sample preparation and test conditions. Latest research focuses on *in silico* and *in vitro* binding assays with mutated receptors to understand the amino acid taste perception by humans [174].

The taste qualities of the L-enantiomers of amino acids are summarised in Table 3.

Umami amino acids

They correspond to the sodium salts of aspartic acid and glutamic acid. The most famous is the MSG used by the food industry as a flavour enhancer to intensify the meaty and savoury flavour of food [175–177]. In cats, the response to umami amino acids has been related to the NaCl response [177][176][175].

Bitter amino acids

Tryptophan, phenylalanine, tyrosine and leucine were reported to elicit bitter taste in 0.3% aqueous solution tested by humans [140]. This concentration is relatively low suggesting that some other amino acids may have a high threshold and may elicit bitter taste at high concentration. Tryptophan activated TAS2R4, TAS2R39, TAS2R43 and TAS2R49 human bitter receptors. Phenylalanine activated TAS2R1, TAS2R39, TAS2R8 and TAS2R4 human bitter receptors [174]. Subsequent studies attributed bitter taste to a greater number of amino acids: isoleucine, leucine, valine, arginine, methionine, phenylalanine, tryptophan, histidine and lysine [152,173]. Authors concluded that bitterness was related to the hydrophobicity of the amino acids. Nevertheless, the taste of amino acid remains complex to describe by humans because of individual differences in bitter sensing in humans and because bitterness can also be detected for some salty or sweet amino acids and vice versa or disappear in solution [179]. Cats reject amino acids described as bitter by humans such as phenylalanine or tryptophan which inhibit amino acid tongue units [180].

Sweet amino acids

Glycine, alanine and proline were reported as sweet by several teams [140,152,168,173]. Glycine activated TAS1R2/TAS1R3 human sweet receptor [174]. Then, depending on the sample preparation (powder or solution; concentration), others amino acids were also described as sweet by humans: serine, glutamine, threonine [168], lysine [152], and asparagine [173]. Even if their sweet receptors are non-functional, cats are particularly attracted by amino acids described as sweet by humans especially proline and alanine [180]. Thus, mechanisms of perception may be different from those in humans.

Sour amino acids

Only aspartic and glutamic acids in dissociated form elicit a sour taste in humans which is due to the interaction between protons from the acid group with taste receptors [181]. Even if cats are attracted by acidic food, they reject stimuli that taste very sour to humans [182].

Salty amino acids

Salty taste was detected by humans for proline, glutamic acid, lysine-HCl and valine [168,183]. However, in another study, only glutamine was described as salty [179]. Apparently, the salty taste of amino acids is not well established and, as well as in the case of salty peptides, the differences between studies with humans is a consequence of the sample preparation conditions with regard to the presence of sodium ions. No published results report the effect of salty amino acids on food acceptance for cats.

Table 3. L-amino acid taste qualities determined by humans under different conditions

	Taste in aqueous solution	*Taste in crystal form*
Ser	flat[1], sweet[2,3], umami[3]	sweet[4,5]
Gln	sweet[2,3], umami[3]	sweet[4], meaty[4], salty[5], bitter[5]
Gly	sweet[1,2,3], umami[3]	sweet[4,5]
Thr	flat[1], sweet[2,3], sour[3]	sweet[4,5]
Ala	sweet[1,2,3], umami[3]	sweet[4,5]
Tyr	bitter[1]	flat[4,5]
Val	flat[1], bitter[2,3], sweet[3]	flat[4], salty[4], bitter[4,5]
Met	sulphurous[1], meaty[1], sweet[1,3], bitter[2,3]	bitter[4,5], sweet[5]
Trp	bitter[1,2,3]	bitter[4,5]
Phe	bitter[1,2,3]	bitter[4,5]
Ile	flat[1], bitter[2,3]	flat[4], bitter[5]
Leu	bitter[1,2,3]	flat[4], bitter[5]
Asp	flat[1], sour[2,3]	sour[4,5]
Glu	unique « glutamate »[1], sour[2,3], umami[3]	sour[5]
Asn	sour[2]	bitter[5], sour[5]
His	flat[1], bitter[2,3]	bitter[5]
Arg	flat[1], bitter[2,3]	spicy[4], bitter[4,5]
Lys	flat[1], sweet[2], bitter[2,3]	salty (with HCl)[4], bitter[4,5], sour[5]
Pro	flat[1], sweet[1,2], bitter[2,3]	salty[4], sour[4], sweet[4,5], bitter[5]
Cys	sulphurous[1], bitter[2,3], sweet[3]	sulphurous[4], bitter[5], sweet[5]

[1] [140]; [2] [173], low concentration; [3] [173], high concentration; [4] [168]; [5] [179]

1.4.3 Lipids and fatty acids

The interest for fat taste in humans increased in the last two decades, since it may be linked with fat food consumption involved in the development of obesity. Converging

data suggest that “fatty” may be a taste quality for humans and rodents [168,184–186] even if preliminary results for human are very unsatisfactory because of the difficulty in isolating a taste component [187]. In fact, fat is often linked with texture modalities and flavour generation but not directly with gustatory effect and there are no clear quality labels for fat. Since criteria for acceptance or rejection of taste primaries have not been clearly articulated, Mattes [188] proposed six minimal elements of a primary taste quality:

- Provide some adaptive advantage;
- Have a defined class of effective stimuli, and apparently free fatty acids varying in chain length and saturation are the stimuli responsible of fat taste [187] and triacylglycerol fatty acids are not an effective taste stimulus
- Have a unique transduction mechanism, involving receptors to convert the chemical signal into electrical signal. Three plausible receptors have been identified: the delayed-rectifiying potassium channel Kv1.5, the G-protein coupled receptor 120 and the receptor-like glycoprotein CD36 [189]. Understanding the regulation of this mechanism is also a big challenge for public health [190];
- Initiates peripheral signals conveyed by gustatory nerves [126];
- Is perceptible and independent from other taste qualities;
- Evokes a functional physiological and/or behavioural response.

Mattes [188] proposed evidence on “fat taste” related to each of these elements but the existence of a unique transduction mechanism and a unique perceptible sensation remained questionable. Recently, Running et al. [191] demonstrated that medium and long-chain nonesterified fatty acids elicited a unique and perceptible sensation in

humans. Short-chain nonesterified fatty acids produced a sour sensation. Medium-chain nonesterified fatty acid sensation was characterised as irritancy. Long-chain nonesterified fatty acids were unpalatable. They proposed the term *oleogustus* as a sixth taste refering only to the taste quality of long-chain nonesterified fatty acids and avoiding confusion with any textural sensation [191].

In 2008, Dransfield reviewed the taste of fat in meat products highlighting the fact that understanding the mechanism of fat taste may have important implications on the development of meat products impacting human and animal nutrition [192].

1.4.4 Nucleotides and derivatives

Nucleotides exist in 2'-, 3'- and 5'- isomer forms but only the 5'-nucleotides are taste active [193]. IMP and guanosine 5'-monophosphate (GMP) are widely associated to umami taste sensation in humans, being generally considered as taste and flavour enhancers [194]. They usually are present in large amount in meat and meat-products and contribute to their taste and flavour [195]. The degradation of IMP to hypoxanthine has been associated to an increase of bitterness in pork meat suggesting that the content of hypoxanthine may influence pork meat taste [196]. In the same way, inosine, product of the thermal degradation of IMP, has a bitter taste for humans [197]. Adenosine 5'-monophosphate (AMP) has been described as "bitter blocker" since it can hide the bitter taste by blocking the transduction of the signal on the mouse tongue bitter-responsive taste receptor [198]. The degradation and synthesis pathways of some nucleotides and nucleosides are presented in Figure 7.

Synergism has been observed between 5'-nucleotides and peptides or amino acids, especially between 5'-nucleotides and glutamate. The study of the mechanisms involved in these synergies has been started over the past twenty years. The taste of glutamate is

intensified by GMP thanks to an allosteric molecular mechanism at the T1R1 receptor level [199] and Kawai et al. [50] showed that umami taste enhancement occurred when IMP was added to amino acids such as Ala, Ser or Gly and tested by humans. McGrane et al. [42] showed that solutions of histidine and IMP or alanine and GMP were preferred over the individual amino acid or nucleotide confirming the synergism between amino acids and nucleotides on cat umami receptor.

Yeasts extracts, are rich in ribonucleic acid which is a natural source of 5'-nucleotides. Consequently, yeasts extracts enriched in IMP and GMP are currently used as taste and flavour enhancers in a wide range of foodstuffs including pet foods [117,200–202].

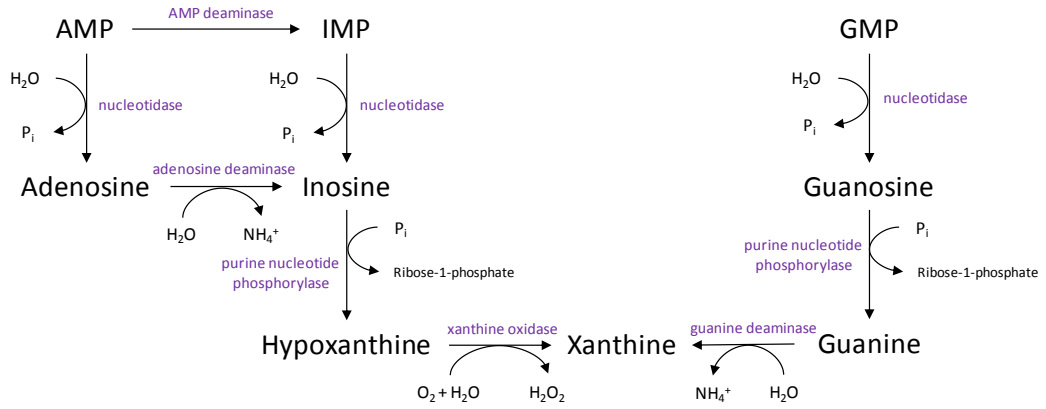

Figure 7. Degradation pathways of AMP, IMP and GMP

1.4.5 Minerals and ions

Ions are essentially associated to salty and sweet taste sensations in humans. Thus, sodium ion Na^+ is the lead compound involved in salty taste but other compounds such as potassium ion K^+ may also be responsible for salty sensation. These cations are naturally present in the form of salts, which means together with an associate anion

such as Cl^-. In general, the taste sensation elicited by a salt is essentially linked with the nature of the cation but the anion acts as a modulator [203]. Zinc salts are generally characterized by astringency, calcium and magnesium salts are often bitter and iron salts evoke a metallic sensation [204].

The influence of minerals on human taste has been widely studied in water. Tap water with high concentrations of HCO_3^-, Ca^{2+} and Mg^{2+} were preferred while high concentrations of Na^+, K^+ and Cl^- were scored low when tasted by humans [205,206]. Taste thresholds for humans have been established in tap water for minerals such as copper salts [207].

Many amphibians and rodents can detect the taste of calcium but the existence of an independent calcium taste quality is still controversial especially in mammals since studies have produced a lot of inconsistent results [208]. In fact, calcium taste sensation has sometimes been presented as a complex mix of bitterness, sourness and sweetness. Recent studies has suggested that mice and humans can detect specific calcium taste and that the receptor T1R3 (responsible of sweet and umami tastes) is involved in this perception [209,210]. A specific calcium receptor has also been described in rats and mice gustatory tissue [211]. Moreover, the bitterness of vegetables has been related with their calcium content [212].

Sodium and potassium pyrophosphate salts are often used as a pet food additive, especially in cat foods and treats where it is considered as a palatability enhancer. They have been used in a wide range of patented formulations for both wet and dry cat foods by different companies [109,213–216].

1.5 Analysis of potential non-volatile tastants

The objective of this section is to review the basic principles of the analytical procedures commonly used to analyse components listed in section 1.4 and considered as potential tastants for cat food. Examples of applications of these procedures for meat and meat-based product analysis (food and pet food) are listed in the next section (1.6).

The focus is done on compounds naturally present in liver used as raw material for cat food palatability enhancers, and on potential key tastants generated during the enzymatic digestion (sees section 1.3.2).

1.5.1 Proteins and peptides

1.5.1.1 Proteins

Proteins are one of the most relevant nutrients when talking about cat food. Thus, to ascertain the high protein content of raw material used for cat food palatability enhancers is crucial. Total protein can be quantified through methods for measuring protein nitrogen [217] or colorimetric protein assays [218–221] widely described in the literature.

1.5.1.2 Peptides

As described in sections 1.3.2.2 and 1.4.1.2, peptides are generated by enzymatic digestion of proteins during pet food palatability enhancer processing and generally do have a taste for humans [159]. Thus, the analysis of peptides from pet food palatability enhancers may allow the identification of potential tastants for cats. Peptides constitute a very complex fraction especially in processed foods and their analysis can be accomplished going through extraction, one or more fractionation/separation steps, identification and/or quantification [222].

1.5.1.2.1 Peptide extraction

To analyse peptides, the extraction is a crucial step. First, the sample has to be ground and homogenised with an extraction solution. This extraction solution can be bidistilled water, dilute saline solutions, acidic solutions, neutral phosphate buffer or water/organic solvents solutions. In the case of meat-containing samples, acidic solution is the most common extraction solution, especially 0.1 N hydrochloric acid [223,224]. After extraction, the homogenate is centrifuged and the supernatant is deproteinised by adding different deproteinising agents (organic acids or concentrated acid solutions). Sometimes, the extraction and the deproteinisation are made at the same time using trichloroacetic acid [225] or perchloric acid [226]. After these steps, the deproteinised sample contains soluble compounds such as peptides and amino acids.

1.5.1.2.2 Peptide fractionation

Even if smaller peptides can be directly analysed in the deproteinised extract, a fractionation is generally necessary before analysis [222]. Fractionation methods are based on size, charge or polarity, and they are described below.

Ultrafiltration

Ultrafiltration is a preparative technique which allows the peptide fraction of interest to be isolated based on size using a semi permeable membrane with an adequate pore size. It can also be used to concentrate peptides [227].

Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) is the leading method for the separation of proteins and peptides. Separation by one-dimension PAGE is based on molecular weight (in presence of sodium dodecyl sulfate, SDS as denaturing agent) while separation by two-dimensions PAGE is first based on isoelectric point and then on molecular weight. PAGE is usually used to separate proteins from 30 to 500 kDa but can also be used to separate smaller proteins and peptides from 1 to 30 kDa. The separation of peptides

requires an adjustment of acrylamide concentration and the presence of tricine in the buffer [228]. This system is also convenient for the isolation of hydrophobic peptides. Applications to meat peptides have been reported by Claeys et al. [229].

Size-exclusion chromatography (SEC)

This chromatography allows proteins and peptides to be separated by size. A solution containing molecules of various sizes is passed through a stationary phase consisting of a bed of porous beads. Smaller molecules diffuse further into the pores and therefore move through the bed slowly while larger molecules enter less and are excluded faster. Different types of stationary phase, in terms of pore size and solvent compatibility, exist and can be used depending on the peptide ranges to be separated [224,225]. The elution of peptides is usually made with 0.01 HCl or diluted phosphate buffers at low flow rate. Eluted fractions are monitored by ultraviolet absorption at 214, 254, 280 nm for the detection of peptide bonds, aromatic rings and proteins, respectively. Fractions are usually collected for further peptide identification and/or quantification, and for characterisation (sensory and bioactive potential analysis).

Reverse-phase high performance liquid chromatography (RP-HPLC)

This HPLC methodology is widely used to analyse peptide extracts since peptides are separated depending on their hydrophobicity which is directly related to their amino acid composition. Several types of reverse-phase columns are available but those based on silica support with octadecylsilane (C18) or octyl (C8) covalently bonded are the most often used. For RP-HPLC, the stationary phase is non-polar and the mobile phase is polar. The typically-used mobile phase is water containing acetonitrile as organic modifier and 0.1% trifluoroacetic acid or formic acid as volatile buffer [230]. Moreover, to optimize the separation of the peptides, the hydrophobicity of the mobile phase is progressively increased. Hydrophilic peptides elute first while hydrophobic peptides are retained in the column and elute later. Eluted peptides are monitored by ultraviolet

absorption at 214 and 254 nm for the detection of peptide bonds and aromatic rings, respectively. As an example, this technique allows the isolation of peptides to be further identified by other specific techniques that will be described later [231,232].

The analysis of glutathione (γ-Glu-Cys-Gly) is widely done by HPLC because of its convenience, specificity and satisfactory sensitivity. The previous derivatization of the GSH may be required to improve separation and detection and the agents used depend on the type of detection. GSH can be derivatized by 1-fluoro-2,4-dinitrobenzene [233] or iodoacetic acid [234] for UV/Vis detection and o-phthalaldehyde [235] or n-1-(pyrenyl) maleimide [236] for fluorimetric detection. GSH can also be analysed by mass spectrometry but also requires previous derivatization [237]

Ion-exchange chromatography (IEC)

This chromatography is complementary to the RP-HPLC and separates peptides based on their charge. Ionic functional groups are present on the stationary phase surface and can interact with opposite-charged sample ions. Thus, acid peptides are separated better in anion exchange columns [238] while neutral or basic peptides are separated better in cation exchange columns [232]. The best results are obtained when a non-volatile salt as NaCl is used but it may affect the latest mass spectrometry analysis by interfering with peptide ionization and adding chemical noise or background in the mass spectra. To separate salt from peptides, the ion-exchange separation is usually followed by RP-HPLC or hydrophilic interaction chromatography [239].

Hydrophilic interaction chromatography (HILIC)

Hydrophilic interaction liquid chromatography provides an alternative approach to separate small polar compounds on polar stationary phases with reversed-phase type eluents. HILIC has been used as a complementary method of RP-HPLC to separate small polar peptides in meat samples [240,241].

1.5.1.2.3 Peptides identification

Two different approaches have been developed to identify proteins: the peptide mass fingerprinting (PMF) and the identification of peptides after fragmentation to obtain the entire or partial amino acid sequence.

The PMF methodology consists of the previous protein hydrolysis with a known enzyme as trypsin and the determination of the list of peptide masses generated (called peptide fingerprint) using the mass spectrometer instrument in MS mode. The identified peptide masses are then compared with theoretical masses from protein databases, obtained using the same enzyme, and the identity of the protein is elucidated in a range of confidence previously fixed [242,243]. The correct identification of a protein supposes a high number of identified peptide masses which cover a part of the protein sequence from the database.

Several studies focus on the identification of peptides naturally generated by not-controlled proteolysis processes in complex matrices, such as dry-cured ham [244–246]. For this type of identification task, peptide mass fingerprinting cannot be obtained and cannot be compared to theoretical masses from database. Therefore, peptides are identified by elucidation of their amino acids sequence using tandem mass spectrometry (MS/MS).

Mass spectrometry methodologies

One of the first methodologies employed to elucidate the amino acid sequence of peptides was the Edman degradation which consists in a progressive liberation and identification of the N-terminal amino acids [247]. However, this method presents some limitations such as the impossibility to work if the N-terminal amino acid has been chemically modified or if the sequence to determine is a mix of two or more peptides [248].

Since the nineties, mass spectrometry is more and more used in biology and biochemistry, particularly as a consequence of the development of ionisation methods such as electrospray ionization (ESI) [249] or matrix-assisted laser desorption/ionisation (MALDI) [250]. These ionisation methods allow the conversion of polar and non-volatile macromolecules into ions in gas phase. Several combinations of ionization sources and mass analysers were developed but the MALDI source is usually coupled with a time-of-flight analyser (ToF) whereas ESI is usually associated to quadrupole mass analyser, an ion trap or hybrid instruments such as quadrupole ion trap, quadrupole-ToF and triple quadrupole.

A mass spectrometer allows the separation of ions present in a sample based on their mass/charge (*m/z*) ratio. It counts with, at least, one ion source, one mass analyzer and one detector. A tandem mass spectrometer is a mass spectrometer that has more than one analyser, usually two. Ions are selected in the first analyser and fragmented into the second analyser leading to several mass spectrums, one for each selected ion. These spectrums give information on the nature and the position of the amino acids in the peptidic chain. Thus, the determination of the complete peptide sequence is possible when a good fragmentation is performed. Several strategies and bioinformatics tools have been developed to interpret spectrums generated by MS/MS. These strategies can be *de novo* sequencing when spectrum is not contained in any of the existing protein databases, or by comparison of experimental MS/MS spectrums to the theoretical content of databases.

Main mass spectrometry systems

MALDI-ToF mass spectrometer

MALDI is a soft ionization method used in mass spectrometry. In this case, the peptides solution is deposed on a metallic slide and uniformly mixed with a large quantity of matrix, usually a low molecular weight aromatic acid such as α-cyano-4-hydroxycinnamic

acid or 2,5-dihydroxybenzoic acid, which absorbs the radiation of the nitrogen laser, helps the ionization and protects the peptides to be cut [250,251]. One of the biggest advantages of this technique is the generation of singly-charged ions $(M+H)^+$ (Figure 8). Charged ions of various sizes are generated on the sample slide.

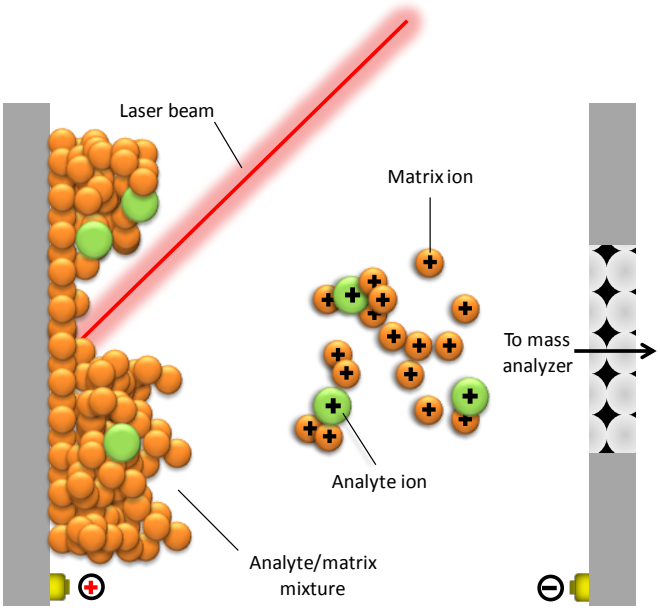

Figure 8. Matrix-assisted laser desorption/ionisation

A potential difference V_0 between the sample slide and ground attracts the ions to the drift space. The velocity of the attracted ions is determined by the law of conservation of energy. As the potential difference is constant with respect to all ions, ions with smaller *m/z* value (lighter ions) move faster through the drift space until they reach the detector (Figure 9).

The use of MALDI-ToF instrument can be adequate for a wide range of molecular masses (from 400 to 3000 Da), being mainly used in PMF approaches.

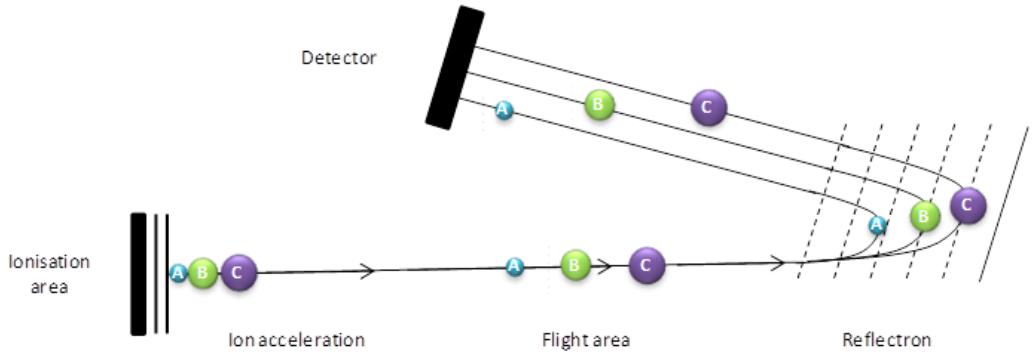

Figure 9. Schematic representation of a time-of-flight analyser

MALDI-ToF/ToF mass spectrometer

The analysers ToF/ToF give a better resolution and precision than ToF. In the first ToF, ions are accelerated at a low voltage (7 kV) in conditions favouring the metastable fragmentation. Then, selected ions are accelerated at higher voltage (20 kV). The second analyser allows ions to be separated based on their *m/z*. This type of instruments can be coupled to a liquid chromatography system (LC). After the separation of peptides by LC, each fraction is directly deposited in the MALDI slide. MALDI-ToF/ToF has been widely used to identify peptides in food matrices [252–255].

LC-MS/MS mass spectrometer

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is an alternative method to separate and identify peptides. In this case, peptides are usually purified and separated by HPLC. Then, the eluate is ionized and transferred to the mass spectrometer. The most common ionization method used for LC-MS/MS analysis is the electrospray ionization (ESI) (Figure 10).

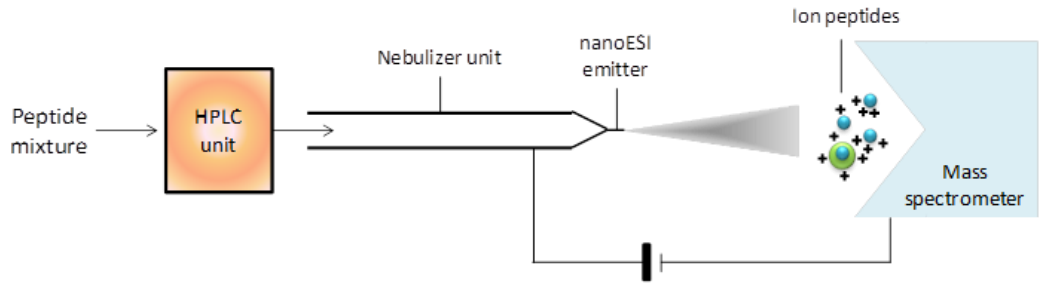

Figure 10. Schematic representation of a liquid chromatograph coupled to mass spectrometer

The liquid containing the analytes of interest is dispersed by electrospray into a fine aerosol (Figure 11). The sample passes through a capillary exposed to an electric field (5-10 kV/cm) and charged droplets are generated. The droplets (1 µm diameter) move to an electrode through an inert gas moving at counter current and causing solvent evaporation until the charged droplet becomes unstable. At this point, the droplet deforms due to the electrostatic repulsion of same sign-charges which becomes more powerful than the surface tension holding the droplet together. The original droplet 'explodes' generating smaller and more stable droplets. The new droplets undergo desolvation and pass to the analyser. A recent advance of this type of ionisation, named nanoelectrospray (nanoESI), allows the analysis of very low sample volumes (1-2 µL) optimizing the signal. The nanoESI improves the signal reaching a femtomole-order sensitivity [256].

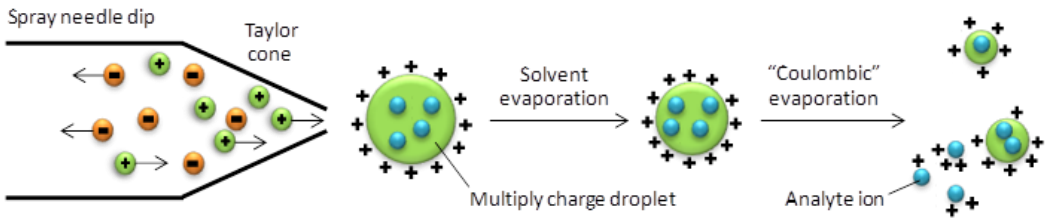

Figure 11. Electrospray ionization

The main difference between ESI and MALDI is that the ESI generates both single and multi-charged ions $(M + nH)^{n+}$. Consequently, different peaks of the mass spectrum can correspond to the same peptide sequence but differently charged.

Main types of LC-MS/MS analysers are Quadrupole/ Ion Trap (Q-ion trap) and Quadrupole/ Time of Flight (Q-ToF). Both types of instruments share a Quadrupole MS that is used to select the parent ion to be subsequently fragmented in the second MS analyser. It consists of four cylindrical rods, set parallel to each other. Each opposing rod pair is connected together electrically. Variable potentials of direct current (DC) and radio frequency (AC) are applied between one pair of rods and the other. Ions travel down the quadrupole between the rods in an oscillating movement depending on the applied voltage. Only ions of a defined *m/z* and oscillating moderately are able to pass completely through the canal and reach the detector. Thus, applying determined DC and AC potentials, ions of interest can be selected. In this case, the analyser acts as a filter and can be used to select only one m/z corresponding to one compound of interest (single ion monitoring) or as a ions sweep recovering all ions from a *m/z* range [257].

- *Quadrupole/ Ion trap mass spectrometer:* An ion trap MS is a quadrupole coupled to an ion trap that uses dynamic electric fields to trap charged particles. This analyser also uses an electric field for the separation of the ions by mass to charge ratios. The analyser is made with a ring electrode of a specific voltage and grounded end cap electrodes. The ions enter the area between the electrodes through one of the end caps. After entry, the electric field in the cavity due to the electrodes causes the ions of certain *m/z* values to orbit in the space. The quadrupole/ Ion trap usually runs a mass selective ejection, where the trapped ions are ejected in order of increasing mass by gradually increasing the applied radio frequency voltage [257]. Most of proteomics data available in literature have been provided by ion traps even if a disadvantage of ion traps is

their relatively low mass accuracy [258]. Shipkova showed that large peptides generated fewer fragments with higher relative abundance resulting in lower limits of detection on the ion trap as compared to those generated on triple quadrupole [259].

- *Quadrupole/ ToF mass spectrometer:* a triple quadrupole mass spectrometer with the final quadrupole replaced by a time-of-flight device is known as a quadrupole time-of-flight instrument (Figure 12). A selected ion is isolated in the first quadrupole (Q1) and fragmented in the collision cell (Q2). Then, the fragments are analysed in the time-of-flight analyser. Q-ToF MS/MS is generally used in metabolomics and for low molecular weight molecules. Q-ToF LC-MS/MS performance can exceed that of ion-trap systems for protein and peptide identification especially in terms of mass accuracy which permits to distinguish several peptides apparently having the same mass on ion traps [258].

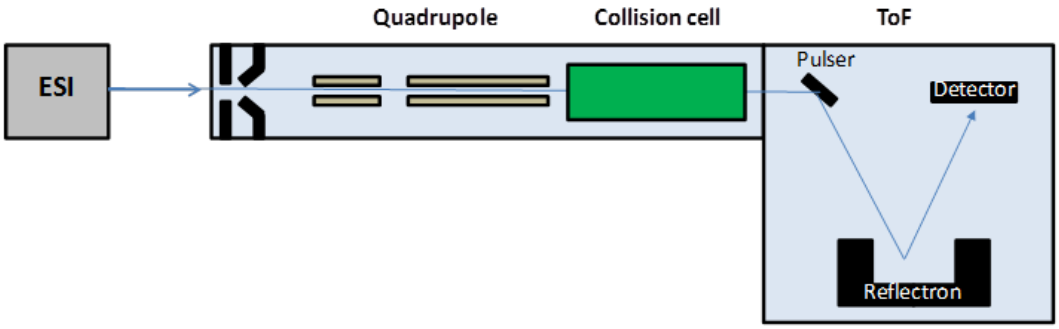

Figure 12. Schematic representation of Q-ToF analyser

1.5.2 Amino acids

Amino acids are the building blocks of peptides and proteins and their analyses can give essential nutritional information. As described in section 1.3.2.2, free amino acids are

generated by enzymatic digestion of proteins and peptides during pet food palatability enhancer processing and may affect the pet food palatability.

1.5.2.1 Sample preparation for free amino acid determination

Sample preparation for free amino acids includes two steps: extraction and deproteinization of the sample. The extraction consists in the separation of free amino acids from the insoluble part of the matrix and is usually achieved by homogenization in an appropriate solvent. Diluted hydrochloric acid is a typical extraction solvent for meat-based samples. In some cases, stronger acids such as trichloroacetic acid [260] or methanol-containing solutions [261] can be used. After centrifugation and filtration, amino acids are separated from extracted proteins and polypeptides. This separation is called deproteinization and can be achieved through chemical or physical methods. Chemical methods consist in mixing the extract with concentrated strong acids (trichloroacetic acid or perchloric acid among others) or organic solvents such as acetonitrile [262] or methanol to denature proteins. Physical methods consist in centrifuging through cut-off membrane filters to retain the largest compounds and recover amino acids.

1.5.2.2 Sample preparation for total amino acid determination - Hydrolysis of peptide bonds

The analysis of total amino acids requires previous total hydrolysis of proteins in the sample, generally using boiling 6 N hydrochloric acid [263,264]. In such acidic and oxidative conditions, some amino acids may be degraded so it is important to maintain an oxygen-free atmosphere in sealed vials and add some protective agent like phenol during the hydrolysis to minimize this degradation. Tryptophan is often completely destroyed by hydrochloric acid hydrolysis. Hydrochloric acid can contact directly with

the sample for liquid-phase hydrolysis or be used for vapour-phase hydrolysis, especially recommended when limited amount of sample is available.

Alkaline hydrolysis is recommended for tryptophan determination but te major drawback is the destruction of threonine, serine, cysteine and arginine [265].

Hydrolysis can be performed in a conventional oven or using microwave technology to reduce the duration of the treatment [266].

1.5.2.3 Separation and quantification by chromatography

Different methodologies are available for amino acid separation and quantification in food and pet foodstuffs. Some of them are presented below.

1.5.2.3.1 HPLC

Ion-exchange chromatography using post-column derivatization and reversed-phase chromatography using pre-column derivatization are the most commonly used techniques for separating and quantifying amino acids [265]. Derivatization has two functions: to increase the hydrophobicity of amino acids (useful for RP-HPLC separation) and to improve detection by allowing the use of ultraviolet or visible-absorbance or fluorescence.

Post-column derivatization

Three reagents have been usually employed for post-column derivatization:

Ninhydrin: this reagent was first used by Roth and Hampaĭ [267] for post-column derivatization. It reacts with primary amines, giving a blue reaction product with a maximum absorbance at 570 nm, and with secondary amines, giving a brownish product with a maximum absorbance at 440 nm.

Fluorescamine: this reagent was introduced for potential improvement of the derivatization using ninhydrin [268]. It reacts with primary amines to form a fluorescent derivative (λ_{ex} = 390 nm; λ_{em} = 475 nm). The reaction only takes place under alkaline conditions whereas the separation is through ion-exchange column. Consequently, a second post-column pump must be added to introduce an alkaline buffer before fluorescamine.

o-Phthaldialdehyde (OPA): this reagent reacts very fast with primary α-amino groups of amino acids (thus, it does not react with proline) in the presence of a thiol group, often 2-mercaptoethanol [269]. A major advantage of OPA is that it is much more sensitive than fluorescamine or ninhydrin [270]. The OPA-amino acid derivative fluoresces strongly (λ_{ex} = 350 nm; λ_{em} = 450 nm) but is unstable. OPA can also be used for pre-column derivatization followed by RP-HPLC.

Pre-column derivatization

The most common derivatizing agents for amino acids are described below. All amino acid derivatives are usually separated by using a C_{18} reversed-phase column.

Phenylisothiocyanate (PITC): this reagent was firstly used in protein sequencing as Edman's reagent and was found suitable for amino acid analysis [271]. It reacts with the N-terminal of amino acids to produce phenylthiocarbamyl-amino acids which have an absorbance maximum at 254 nm. The reaction is largely complete within 20 min and, after that, the sample must be dried to remove the excess reagent which may cause some damages to the chromatographic column. PTC derivatives are stable for several weeks if frozen but only for few hours at room temperature.

6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC): this reacts with primary and secondary amines from amino acids, peptides and proteins producing highly fluorescent derivatives (λ_{ex} = 250 nm; λ_{em} = 395 nm). UV detection at 254 nm can also be used. The

reaction is rapid, about 1 min at 55°C and AQC-amino acids are stable at room temperature for up to a week.

9-Fluorenylmethyl chloroformate (FMOC): this reagent was initially used as a blocking agent in peptide synthesis. It reacts with both primary and secondary amines to produce fluorescent derivatives (λ_{ex} = 260 nm; λ_{em} = 313 nm). The reaction is very fast (30 s to 1 min) and derivatives are stable at 40°C for at least a week. One drawback of FMOC derivatization is that the excess of reagent, hydrolysed or decarboxylated, can produce a fluorescent alcohol that coelutes with some amino acids [272]. To avoid interferences, FMOC/amino acids ratio and reaction time must be optimized very carefully.

OPA-FMOC: OPA is commonly used as derivatizing agent for amino acid analysis (see above) but is unable to react with secondary amine. To remedy the situation, a new method has been proposed consisting in a two-step derivatization with OPA and FMOC [273]. First, all primary amines are derivatized by reacting with OPA for 2 min. Then, the secondary amines are derivatized with FMOC for around 1 min. In the optimized conditions, FMOC derivatives (FMOC-proline and FMOC-hydroxyproline) elute after OPA derivatives and before unreacted FMOC avoiding the interference in the chromatogram mentioned previously. This method requires a fluorescence detector capable to switch wavelengths during the run since OPA and FMOC derivatives have different excitation and emission wavelengths.

1.5.2.3.2 Gas chromatography

Gas chromatography is a very high resolution technique and is suitable for amino acid analysis [274]. To allow their analysis, amino acids must be turned into volatile and thermostable derivatives using reagents such as ethylchloroformate [275] or isobutyl chloroformate with pyridine [276]. Amino acids can also be converted into their corresponding *N*(*O*)-trifluoroacetyl amino acid 1-propyl esters, *N*(*O*)-pentafluoropropionyl amino acid 2-propyl esters [277] or N(*O,S*)-

isobutyloxycarbonyl/*tert*-butyldimethylsilyl derivatives [278]. Nevertheless, GC is not extensively used compared to HPLC especially for food samples since it requires a time-consuming sample derivatization [279]. Currently, detection is mostly done by mass spectrometry and some examples involving samples from meat have been described [280–282].

1.5.2.3.3 Capillary electrophoresis

Capillary electrophoresis (CE) is based on the use of an electric field to separate charged compounds such as amino acids. This method offers high efficiency, sensitivity and requires a small amount of sample but is not commonly used especially for food analysis [283]. Moreover, structure of amino acids does not allowed good separation by CE. In fact, an amino acid extract contain basic, neutral and acidic compounds and a good separation of these three groups requires different pH conditions. Most amino acids should also be derivatized because of their lack of strong detectable properties. Derivatized amino acids can be detected by the same detectors used for HPLC.

1.5.3 Lipids and fatty acids

Lipids are among the major components of food of animal origin. Thus, precise analysis of lipids and fatty acids in foods is important for determining taste and nutritive values as well as for understanding the effects of fats on food palatability.

1.5.3.1 Lipid extraction

Several lipid extraction techniques are described in the literature and can be classified in three major categories depending on the use of organic solvents, non-organic solvents or no solvents [284]. The most used are described below.

1.5.3.1.1 Extraction by organic solvents

The most used method for total lipid extraction from meat and meat products is the Soxhlet method, even if it is not suitable for samples with very high lipid content [285]. Diethyl ether and petroleum are commonly used solvents for single-solvent extraction of lipids, especially for dairy products [286]. The drawback of these single-solvent methods is that polar lipids and free fatty acids may not be extracted.

To ensure a complete recovery of lipids, a solvent combination composed of varying proportion of polar and non-polar solvents may be used. The greatest improvement of the extraction of total lipids from animal tissues was made when Folch described his classical extraction procedure [287]. This method remains one of the most commonly used around the world. A typical Folch procedure uses a mixture of chloroform and methanol in a two-step extraction. First, the sample is homogenized with the solvent and the mixture is filtered to eliminate the residue. This step is usually repeated to recover about 95% of tissue lipids. The extract is then washed with water or a salt solution (KCl or NaCl) until the phases separate. The phase containing lipids is collected. Modifications of the method have been proposed. Bligh and Dyer [288] used a mixture of chloroforme and methanol but in different proportion. Other solvent mixtures containing hexane, isopropanol or dichloromethane [289,290] have been successfully used to extract tissue lipids. These solvents were used instead of chloroform to limit health hazards. In case of meat and meat products, the Folch method is the most appropriate for total lipid quantification since it is suitable for all ranges of lipid content [285].

With the advent of green chemistry, pressurized liquid extraction (PLE), also called accelerated solvent extraction, has been developed to increase the efficiency of conventional solvent extraction using lower volumes of organic solvents [291]. The solvent consumption can be decreased about 50% by using PLE compared to

conventional Folch extraction [292]. These techniques use classical solvent combinations, such as chloroform/methanol for meat and meat-based products, to extract lipids but close to their supercritical region (high pressure and temperature) where they show higher extraction performance [291,293]. However, in this region, the high temperature enables high solubility and high diffusion rate of analytes in the solvent. This technique is currently considered as a promising technology which could be automated, reducing time and solvents consumption [294].

1.5.3.1.2 Extraction by non organic solvents

Microwave-assisted extraction

Microwave-assisted extraction is an improvement of microwave digestion method based on heating a solvent [295]. For this purpose, a microwave oven is combined to a closed or open vessel containing a classical solvent mixtures and the sample. Microwave energy decreases the energy required to break hydrophobic associations, electrostatic forces and hydrogen bonding and thus helps to dissolve lipids. The heating speed is proportional to the dielectric constant of the solvent. Many organic solvents are characterized by a low dielectric constant whereas water is easily heated due to a high dielectric constant. Performance of microwave lipid extraction was quantitatively and qualitatively comparable to conventional Folch method for both vegetal and animal-based samples such as beef steaks, chicken breasts, peanuts or croissants [295,296].

Supercritical fluid extraction (SFE)

This process of extraction used supercritical fluid as extracting solvent. Several solvents have been used such as hexane, pentane or nitrous oxide but carbon dioxide is the most common because it is safe, readily available and low cost [297]. It allows supercritical operations at relatively low pressures (around 74 bar) and near-room temperatures (around 31°C). Co-solvents as ethanol or methanol are sometimes used [298]. The extraction efficiency of lipids in wet samples as meat was improved when samples were

previously lyophilized. The main drawback of SFE is the equipment cost and the extraction of unwanted non-fat compounds in addition to the fat.

Others

Some lipid extraction methods without solvents were described for determining fat content of fresh milk or oilseeds. These method are based on destabilizing or breaking up emulsion releasing fat (fresh milk), or on applying external compression forces (oilseeds) [286].

Before further analysis, the lipid extract storage and preservation is a very critical point since lipids can be oxidized by air and sunlight, especially polyunsaturated fatty acids. To prevent oxidation process, antioxidants such as tocopherol or butylated hydroxytoluene are added to the lipids extracts [284].

1.5.3.2 Fatty acid esterification

Fatty acids have low volatility owing to polar groups which makes problematic their direct analysis by gas chromatography (GC). In fact, they tend to absorb on the packaging of the column or to dimerize which can cause peak asymmetry, shouldering or tailing. Derivatization of fatty acids results in better separation on the GC columns as the ionization of hydroxyl group is blocked, making derivatives to differ more in their physiochemical properties than the original fatty acids. Fatty acids are usually converted into fatty acid methyl esters (FAME), but butyl or other derivatives are less used [299]. Esterification solutions can be divided into the following groups: acid-catalysed, base-catalysed and diazomethane.

Acid-catalysed esterification

Through this derivatization method, free fatty acids are esterified and *O*-acyl lipids transesterified by heating with excess of anhydrous methanol in the presence of an acidic catalyst. The most common and most frequently cited acid derivatization reagent

for the preparation of methyl esters is anhydrous hydrogen chloride in methanol. Boron trifluoride in methanol is another solvent mixture which has been highly used as a rapid means of esterifying free fatty acids [300,301].

Based-catalysed esterification

O-acyl lipids are transesterified rapidly in anhydrous methanol in the presence of a basic catalyst, unlike free fatty acids are not normally esterified in these conditions. Between 0.5 and 2 M sodium methoxide in anhydrous methanol is the most useful reagent but potassium methoxide or hydroxide have also been often used. Nevertheless, potassium containing solvents are less recommended due to artefacts formation or hydrolysis of lipids [284,302].

Diazomethane

Diazomethane is another esterification solvent. It reacts rapidly with free fatty acids in the presence of a little methanol to form methyl esters. However, diazomethane is highly explosive and both diazomethane and required intermediates are toxic and carcinogenic [299,303]. It is then used in a less extend.

1.5.3.3 Fatty acid separation, identification and quantification

Methods for fatty acid derivatives separation and identification were largely reviewed in the literature [284,304]. The most commonly used is gas chromatography (GC).

GC is the first technique that would be chosen for fatty acid analysis. By this technique, it is possible to obtain a complete quantitative analysis of fatty acid composition of a sample in a short time. Each known fatty acid can usually be identified by GC with certainty on the basis of its chromatographic behaviour (i.e. retention time). The main advantages of GC for fatty acid separation from a complex mixture are its separation efficiency, speed of analysis, the availability of various capillary columns and the sensitivity detectors. The choice of the column is determinant for the resolution of the

different fatty acids especially for minor fatty acids. Capillary columns are made of flexible fused silica characterized by a very high inertness and compatibility with spectroscopic detectors. They are classified in three categories: the porous layer open tubular (PLOT) columns, the support coated open tubular (SCOT) columns and the wall coated open tubular (WCOT) columns [305]. The choice of one or another type of column is conditioned by the complexity of the sample. Traditionally, FAME are separated using capillary columns (until 60 m) with polar polyesters as stationary phase [284]. Recently, new approaches have been evaluated, especially to improve the separation of isomers trans by using longer columns around 100 m and new stationary phases [306,307]. However, if the objective of the study is the whole fatty acid set, GC analysis may be coupled to other preparative separation techniques such as silver ion-HPLC.

The electron capture detector (ECD) and flame ionisation detector (FID) are commonly used for fatty acid analysis. FID is very sensitive for organic compound detection and is by far the most common GC detection system for FAME. Compounds are pyrolysed in a hydrogen-oxygen flame and produce ions in the process. The ECD has high sensitivity for fatty acid analysis but is not as selective as FID is. GC coupled to mass spectrometry makes a good combination for fatty acid analysis and is used especially for determination of double bonds on fatty acids [308,309].

1.5.4 Nucleotides and nucleosides

Nucleotides and nucleosides are present in significant quantity in meat and meat-based products and contribute to meat flavour [195]. Since palatability enhancers for pet food are mainly made of meat by-products (see section 1.3.2.1), modification of nucleotides and/or nucleosides may influence the palatability of pet foods.

1.5.4.1 Sample preparation

Nucleotides and nucleosides are typically extracted by homogenisation with ice-cold 0.6 N perchloric acid. The extract is then neutralized by adding solid potassium carbonate and stored ideally at -80°C to avoid degradation by enzymatic reactions.

1.5.4.2 Nucleotides and derivatives determination

Several chromatographic techniques including enzymatic assay [310], RP-HPLC, IEC , CE [311] and CE-MS [312] have been developed for nucleotide and nucleoside analysis. The relative high cost, the lack of sensitivity, reproducibility and the poor concentration sensitivity were the major limitation of the enzymatic assay and CE-MS. However, results obtained by IEC were not very conclusive [313,314]. Recently, ion-pairing reversed-phase high performance liquid chromatography (IP-RP-HPLC) methods have been developed improving separation and resolution for nucleotides analysis [315,316]. Even if this technique is more expensive than the previous cited ones, it is now the most commonly used for the separation of nucleotides in dairy products and food ingredients [317–321]. An ion pair reagent is added to the mobile phase and the analytes are separated in a reversed-phase column. Tetrabutylammonium hydrogen sulfate or phosphate is the ion pair reagent most used [319]. Depending on mobile phase pH, specific side groups of the analytes are ionized and carry positive or negative charges. The ion pair reagent acts as a source of counterions forming ion pairs with the analytes which then interact with the stationary phase during RP-HPLC. If di- or trinucleotides are not present in samples, RP-HPLC with a phosphate buffer/acetonitrile mobile phase is the most common technique. Detection is UV at 250 or 260 nm. Peak identification is then performed by comparing retention times and spectral characteristics with those of standards [322].

1.5.5 Minerals

The main source of minerals for cats is through dietary intake. As mentioned in section 1.2.2.3, cats have specific mineral requirements and a lack or an excess of one mineral can profoundly affect cats' health. Therefore, it is necessary to know the mineral contribution of each ingredient used to manufacture pet foods.

1.5.5.1 Sample preparation

Heterogeneous samples, such as raw meat or palatability enhancers in powder form, must be homogenized to obtain a representative sample and to prevent errors. Meat samples are usually cut, grinded and submitted to a mineralization to remove any organic material. This step is very decisive in the analytical process and can be performed by dry ashing and/or by wet decomposition [323–325].

Dry ashing procedure uses a muffle furnace capable of maintaining elevated temperatures around 450-550°C. First, samples are placed in a crucible made of porcelain or platinum. Then, due to the high temperature, water and volatile materials are vaporized and organic substances are burned in the presence of oxygen. Most minerals are converted to oxides, sulphates, phosphates, chlorides or silicates. Some minerals are volatile (mercury for example) or semi-volatile (iron or lead for example) and may form volatile compounds such as chlorides ($FeCl_3$; $PbCl_2$) at such temperatures. To minimize losses of these minerals, closed glass flasks must be used. Apart from the potential losses, the main disadvantage of dry ashing is the long time required, around 12-24 hours [326,327].

Wet decomposition is primarily used in the preparation of samples for subsequent analysis of specific minerals. It breaks down and removes the organic matrix surrounding the minerals so that they are left in an aqueous solution. Nitric, perchloric and sulphuric acids are the most commonly used reagents for complete digestion of meat products.

Wet decomposition can be carried out at atmospheric pressure in open systems or at higher pressures in a closed vessel by conductive or microwave heating [328]. Microwave digestion procedure is widely used in food analysis for practical reasons. It is a quick method, it provides high sample throughput and the microwave energy can be programmed ensuring good reproducibility [329,330].

1.5.5.2 Mineral separation, identification and quantification

Different methodologies are available for mineral analysis. Some of them are presented below.

Classic methods

Several gravimetric, titrimetric and colorimetric methods are reported for major element determination but they are very time consuming and, therefore, are confined to specific situations with a limited number of samples [331].

Ion chromatography

Ion chromatography connected with conductivity detectors is also used to measured concentrations of major anions, such as fluoride, chloride, bromide and iodide, as well as major cations, such as sodium, potassium, calcium and magnesium [332]. This special type of liquid chromatography requires a meticulous cleaning with bidistilled water of all laboratory glassware used to limit interferences. Ions are separated by passing through ion-exchange resins. This technique allows simultaneous determination of several ions [333]. Electric conductivity detectors are often used and sometimes, chemical suppression avoiding mobile phase conductivity is interposed between the column and the detector to increase sensitivity, especially for anion analysis. Other types of detection such as fluorescence or UV-Vis spectrometric detectors are also described. Examples of ion determination in food using ion chromatography have been reviewed [334,335].

Atomic spectrometry methods

Atomic spectrometry methods are often used for determination of minerals and trace elements in food [336,337]. They are based on the measurement of radiation absorbed (absorption spectrometry) or emitted (emission spectrometry) by the atoms of the element to be measured. They are more rapid, precise and accurate than classic techniques [338]. Among these methods, atomic absorption spectrometry has been widely used for food and pet food analysis [339–343] but one of its disadvantage is that only one element can be quantified at a time. Thus, when fast multi-element analysis is required, inductively coupled plasma optical emission spectrometry (ICP-OES) is preferred. Actually, if the objective is to quantify metals and trace elements in pet foods, wet ashing followed by inductively coupled plasma mass spectrometry is recommended because of its high sensitivity [344]. ICP-OES is widely employed for food and pet food analysis [345–348]. The ICP-OES equipment is divided in an ICP torch and an optical spectrometer (Figure 13). The plasma torch is composed of three quartz glass tubes surrounded by a radiofrequency (RF) generator. The RF signal produces an intense electromagnetic field where argon gas is ionised. The sample is delivered by a peristaltic pump to a nebulizer unit where it is changed into mist and then introduced directly inside the plasma. The molecules present in the sample break up into atoms which lose electrons and recombine repeatedly in the plasma emitting radiation at a specific wavelength. In the optical chamber, wavelengths from the emitted light are separated and analysed by an array of semiconductor photodetectors such as charge coupled devices (CDD). The intensity of the light emitted by each element is measured and compared to a standard analysed in the same conditions.

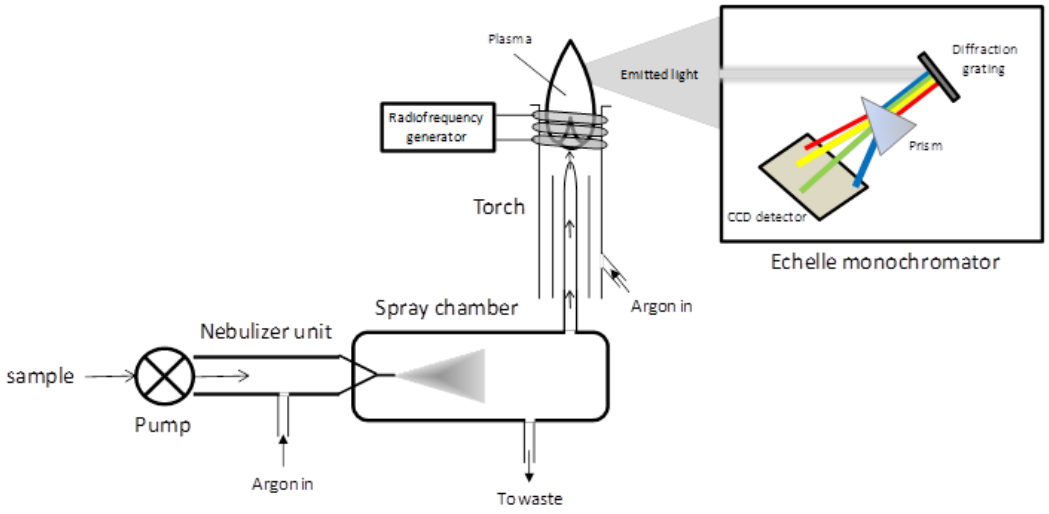

Figure 13. Schematic representation of an ICP-OES equipment

1.6 Analysis of potential key tastants in meat by-products used for pet food processing and in pet food products

The aim of this section is to present some applications of the methods presented in section 1.5, among others, focusing on the analysis of potential tastants and nutrients in meat by-products used as raw material for pet food palatability enhancer manufacturing (Table 4) and in pet foodstuffs (Table 5). The focus is done on pork liver since it is one of the most used by-products for cat food palatability enhancer manufacturing. Most pork liver analyses have been done to compare different genetic lines or for medical applications and little information is available about pork liver as ingredient for pet food. Analyses of pet foodstuffs are generally done to evaluate their nutritional quality by quantifying specific nutrients and little information is available about tastants in pet foodstuffs, previously described in section 1.4. In addition, most analyses are initiated by pet food companies and remained confidential.

Table 4. Analysis of tastants and nutrients in raw materials used for pet food manufacturing

Raw material	Tastant / Nutrient	Analytical method	Ref.
pork liver	proteome	IEC, RP-HPLC, ESI LC-MS/MS	[349]
	proteins	2D-electrophoresis, MALDI-ToF MS	[350]
	peptides	IEC, RP-HPLC, ESI Q-ToF MS/MS	[351]
	GSH	method of Sedlak and Lindsay (colour complex formation)	[352]
		LC-MS/MS (stabilization by N-ethylmaleimide)	[353]
	amino acids	RP-HPLC (OPA derivatization)	[354]
		automated amino acid analyser	[355]
	taurine	automated amino acid analyser	[356]
	fatty acids (FAME)	Folch extraction; GC	[357]
	K, Na, Mg, Ca, Zn, Fe, Cu, Mn, (P)	flame atomic absorption spectrometry (spectrophotometry)	[327]
	volatile aroma compounds	solvent-assisted flavour evaporation, GC/Olfactometry	[106]
pork lungs and kidneys	total protein	nitrogen analyser	[358]
	fat	Microwave drying with non-microwave solvent extraction (AOAC 985.15)	[358]
pork meat	hypoxanthine, inosine, IMP, AMP, ADP, ATP	IP-RP-HPLC	[317]
hog liver	amino acids	RP-HPLC	[359]
	fatty acids (FAME)	Folch extraction; GC-FID	[359]
bovine liver	GSH	HPLC	[144]
	taurine	automated amino acid analyser	[356]
	Al, Ca, Cu, Fe, Mg, Mn, Zn	ICP-OES	[360]
chicken viscera	total protein	nitrogen analyser	[358]
	fat	microwave drying with non-microwave solvent extraction (AOAC 985.15)	[358]

duck liver	fatty acids (FAME)	GC-FID	[361]
enzymatic protein hydrolysates	umami peptides	SEC, HPLC, protein sequencer	[362]
	amino acids	automated amino acid analyser	[355]
	free amino acids	IEC	[362]
yeast extract, beef-flavored broth	GMP, IMP	IP-RP-HPLC	[363]

Table 5. Analysis of tastants and nutrients from pet foodstuffs

Pet foodstuff	Tastant / Nutrient	Analytical method	Ref.
foods for dogs and cats	crude protein		[364,365]
	amino acids (lysine)	RP-HPLC (ninhydrin derivatization)	[366]
	taurine		[364,365]
	fat; LA; ArA		[364,365]
	Ca, P, Mg, Na, K, Cu, Zn, Mn, Fe, I, Se		[364,365]
	Cu, Ni, Pb, Fe, Mn, Cr, Cd	flame atomic absorption spectrometry	[342]
	Al, Ca, Cd, Cr, Cu, Ba, Fe, K, Mg, Mn, P, S, Sr, Z	ICP-OES	[345]
	iodine	alkaline ashing, titration method	[367]
	vitamin A	RP-HPLC	[368]
	ash, crude fibre, vitamins A, D, E, B1, B2, B3, B5, B6, B9, B12, choline		[364,365]
	vitamins A, E, B1, B2, B6, B12	RP-HPLC	[369]
	volatile aroma compounds	headspace- solid phase microextraction, GC-MS	[370]

2.**OBJECTIVES**

The main objective of this project is to fractionate and characterise interesting fractions of DIANA *Petfood* palatants by means of separation and analytical techniques, evaluate these fractions by means of palatability evaluation, and establish a correlation between product composition and animal preferences. The final goal is to achieve a better comprehension of the compounds involved in taste improvement of cat food.

The approach followed for this general objective includes:

1. The chemical and biochemical characterisation of different pork livers used as raw material for cat food palatability enhancers focusing on biochemical compounds that can constitute potential non-volatile tastants.

2. The chemical and biochemical characterisation and comparison of two different palatability enhancers focusing on biochemical compounds that can constitute potential non-volatile tastants, with a special emphasis on the proteomic study of peptides present in these palatability enhancers.

3. The evaluation of the two palatability enhancers based on their biochemical characterisation and their sensory evaluation by a new technology called Microtiter Operant Gustometer (MOG) using trained rats in order to establish a correlation between product composition and animal preferences.

3. MATERIALS AND METHODS

3.1 Raw materials and palatability enhancers

The raw materials and products were supplied by DIANA *Petfood* (Elven, France).

3.1.1 Pork liver homogenates

Raw materials consisted in three grinded pork liver homogenates (PLWL, PLW, L). Each homogenate was composed of three pork livers supplied by a slaughter house (Brittany, France). Livers were grinded in a cutting system (Karl Schnell, Winterbach, Germany) using a 3 mm hole plate, frozen stored and sent to the laboratory.

Differences between each liver homogenate were based on pork breed, diet and slaughter age. The diet of animals was controlled. A partial description is presented in Table 6.

Table 6. Characteristics of pigs used for pork liver homogenates

		PLWL	**PLW**	**L**
Breed		50% Pietrain/25% Large White/25% Landrace	50% Pietrain/50% Large White	100% Landrace
Age (days)		175	175	193
Diet	protein (%)	15	15	15
	fat (%)	2	2.6	2.5
	ashes (%)	4.1	4.49	4.4
	iron (ppm)	75	77.5	20
	copper (ppm)	12	12.4	10
	zinc (ppm)	90	93	50

3.1.2 Palatability enhancers

Two powders (NEp and OEp) were obtained from grinded pork livers which were submitted to two different enzymatic processes. Pork lungs were also used as raw material for OEp manufacturing (in addition to liver) but not for NEp. The general process curve is presented in [redacted]. The first process involved an enzyme which was called “Old enzyme”; the second process involved a “New enzyme”.

[redacted]

3.2 Analytical methods

Pork liver homogenates’ proximal composition was analysed. Then, biochemical analyses were performed for both pork liver homogenates (PLWL, PLW, L) and

palatability enhancers (NEp and OEp). Each analysis described in this section was performed in triplicate.

3.2.1 Proximal composition

3.2.1.1 Moisture

Moisture content of pork liver homogenates (PLWL, PLW, L) was determined by drying (method 24003 (a), [371]). Five grams of pork liver were homogenised with 15 g of washed sand and 5 mL of ethanol in a preweighed porcelain dish. Most of the ethanol was evaporated at room temperature for 10 min and the sample was dried in an oven at 100-102°C for 24 hours. Results are expressed in percentage.

3.2.1.2 Total proteins

Total protein content of pork liver homogenates (PLWL, PLW, L) was determined using the Kjeldahl method [217]. First, 0.5 g of sample was mixed with 3.5 g potassium sulfate and 0.4 mg copper sulphate 5-hydrate, and the mixture was homogenized with 10 mL of 96% concentrated sulphuric acid and 2 mL of 30% hydrogen peroxide in a digestion tube. The digestion tube was heated during 1h at 250°C followed by 2h more at 410°C. Then, the distillation and titration of ammonia were performed using a Kjeltech analyzer (2300 Kjeltech Analyzer Unit, FOSS, Denmark). Three digestions were performed for each sample. Protein content was estimated by multiplying the nitrogen content by a 6.25 factor. Results are expressed in percentage.

3.2.1.3 Fat

Total lipids were extracted from pork liver homogenates (PLWL, PLW, L) according to Folch *et al.* [287] using dichloromethane: methanol (2:1) and with 0.05% butylhydroxytoluene instead of chloroform: methanol (2:1). The sample was

homogenised by using an Ultra-turrax T 25 basic (IKA Werke GmbH & Co, Germany) at 15000 rpm for 1 min. Then, it was filtered by paper Whatmann N°1 under vacuum. The filter was cleaned using Folch mixture to recover all the lipids. The filtrate was recovered in a separatory funnel and 20 mL of 0.73% sodium chloride was added. The separation of the two phases was done overnight at 4°C. The organic phase was recovered and "clarified" by using acetone. The extract obtained was evaporated in a rotating vacuum evaporator and weighed to determine the total lipid content. Then, it was diluted in 5 mL of chloroform and stored at -20°C until fatty acids profile analyses. Results are expressed in percentage.

3.2.1.4 Ashes

The ashes content of pork liver homogenates (PLWL, PLW, L) was analysed using the international standard ISO/R 936. Five grams of pork liver were placed in a preweighed porcelain dish with 1 mL of 15% magnesium acetate solution. First, each dish containing sample was heated on a hot-plate until sample carbonization. Then, they were heated in a muffle furnace (L 9/11/B170, Nabertherm, Germany) at 550°C for 5 hours up to obtain white ashes. Results are expressed in percentage.

3.2.2 SDS-PAGE protein and peptide profiles

Protein and peptide profiles from pork liver homogenates (PLWL, PLW, L) and palatability enhancers (NEp, OEp) were obtained by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. First, two grams of sample were homogenized with 20 mL of 0.1 M disodium phosphate buffer pH 7.4 containing 0.7 M potassium iodide and sodium azide (0.02%) in a stomacher (IUL Instrument, Barcelona, Spain) for 8 min. The homogenate was centrifuged at 4 °C for 20 min and 12,000 *g*. The supernatant

containing proteins, peptides and free amino acids was filtered through glass wool and kept at 4 °C until further analyses.

Total proteins and/or peptides content in the extracts were quantified using a BCA Protein Assay Kit (Sigma-Aldrich, Saint Louis, MO, USA) using bovine serum albumin (BSA) as standard.

Proteins extract were diluted twice with a sample buffer (SB) composed of 50 mM Tris buffer, pH 6.8, containing 8 M urea, 2 M thiourea, 75 mM dithiothreitol, 3% (w/v) SDS and 0.05% bromophenol blue, submitted to 95°C for 4 min and put in ice rapidly. Protein concentration in samples was adjusted to 1 mg/mL with the SB and 10 µl each were loaded into a 12% polyacrylamide gel reserving one lane for a molecular markers mixture (SDS-PAGE molecular weight standards, Broad range, Sigma-Aldrich). The elution buffer was composed of 50 mM Tris, pH 8.6, 0.384 M glycine and 0.1% SDS. Electrophoresis was performed using computer controlled electrophoresis power supply Model 3000 Xi (Bio-Rad, Hercules, CA, USA) at a variable voltage (max. 120 V) and constant current (50 mA for two gels). Resulted bands were stained by silver nitrate using a ProteoSilver™ plus silver stain kit (Sigma-Aldrich). Each protein extract was analyzed in triplicate.

3.2.3 Soluble proteins, peptides and amino acid extracts

Extracts from pork liver homogenates (PLWL, PLW, L) and palatability enhancers (NEp, OEp) were prepared according to Escudero et al. [372]. Two grams of sample were homogenized with 20 mL of 0.01 N hydrochloric acid in a stomacher (IUL Instrument) for 8 min. The homogenate was centrifuged at 12,000 *g* and 4°C for 20 min. The supernatant contained soluble proteins, peptides and free amino acids. It was filtered through glass wool and kept at 4°C until further analyses.

3.2.4 Molecular mass fractionation

Deproteinised extracts from palatability enhancers (F0 NEp and F0 OEp) were fractionated by gel filtration (GFC) according to their molecular mass. For this purpose, soluble proteins and peptides extracts from palatability enhancers (F6 NEp and F6 OEp; section 3.2.3) were deproteinised by adding 3 volumes of ethanol and maintaining the sample 20 h at 4°C. After that, the sample was centrifuged (12,000 *g* for 20 min at 4 °C) and the supernatant was filtered under vacuum using a 0.45 µm nylon membrane filter (Teknokroma, Barcelona, Spain). The ethanol was eliminated using a rotary evaporator and the sample was dried under vacuum. The dried deproteinised extract was dissolved in 0.01 N hydrochloric acid to adjust its concentration at 10 mg/mL, filtered through a 0.45 µm nylon membrane syringe filter (Teknokroma, Barcelona, Spain) and stored at − 20°C until use.

Samples containing approximately 50 mg of peptides (5 mL) were injected into a Sephadex G25 column (2.6 × 70 cm, GE Healthcare, Uppsala, Sweden), previously equilibrated with 0.01 N hydrochloric acid. The separation was performed at 4 °C using 0.01 N hydrochloric acid as eluent, at a flow rate of 15 mL/h. Five millilitres fractions were collected during 50h using an automatic fraction collector and further monitored by ultraviolet (UV) absorption at 214 nm, 254 nm and 280 nm. Fractions were pooled together according to the elution profiles in four major fractions, lyophilised and stored at -20°C until analysis.

3.2.5 Amino acids analyses

Amino acids were analysed by RP-HPLC using pre-column o-phtalaldehyde/9-fluorenylmethyloxycarbonyl (OPA/FMOC) derivatization.

3.2.5.1 Samples preparation for total amino acids analysis

In order to analyse total amino acids, pork liver homogenates (PLWL, PLW, L) and palatability enhancers (NEp, OEp) were previously hydrolysed using 6 N hydrochloric acid in a Pico Tag® work station system (Waters, Millford, MA, USA). Thus, 200 mg of sample were put in a vial supplied with the system and dried under vacuum. Then, 2 mL of 6N hydrochloric acid containing 1% phenol were poured in direct contact with the samples. Vials were placed in the system and submitted to three cycles of vacuum/nitrogen to remove completely the oxygen from the vial. The vial was closed during the last vacuum step and placed into the oven at 110°C for 22 hours.

After complete hydrolysis, the sample was evaporated to dryness, diluted in 1.5 mL of bidistilled water and centrifuged at 14,000 *g* for 5 minutes. The amino acids in the supernatant were analysed by HPLC (section 3.2.5.3).

The same protocol was used to quantify total amino acids of major fractions from the GFC (F1-F4) using 50 µL of fraction, dried before adding 500 µL of 6N hydrochloric acid containing 1% phenol. After hydrolysis and evaporation of HCl, 2.5 mL of bidistilled water to dilute the sample after hydrolysis.

3.2.5.2 Samples preparation for free amino acids analysis

Free amino acids were analysed in the peptides extracts (section 4.2.3.) after deproteinisation as described by Aristoy and Toldrá [262]. Thus, extracts were mixed with 2.5 volumes of acetonitrile and kept for one hour at room temperature. Then, the samples were centrifuged at 14,000 *g* for 5 minutes and free amino acids from the supernatants were analysed. Fractions from GFC were directly analysed without deproteinisation.

3.2.5.3 Derivatization by o-phtalaldehyde/9-fluorenylmethyloxycarbonyl (OPA/FMOC) and high-performance liquid chromatography analysis

The derivatization was done as described by Schuster [373]. To this end, the reagents for the derivatization were prepared as follows: (1) 50 mg de OPA was dissolved in 1 mL of methanol. 50 µL of 3-mercaptopropionic acid (MPA) was added and the mixture taken to 10 mL with borate buffer (0.4 M boric acid with 0.6% of Brij (35%) adjusted at pH 10.4 with potassium hydroxide). This solution was stored in an amber vial during a maximum of one week adding MPA every 2 or 3 days. (2) The FMOC reagent was prepared in acetonitrile at 6.25 mg/mL. This reagent was prepared daily. Derivatization reaction was automatised in the HPLC autosampler device as follows: 1 µL of sample was mixed with 5 µL of the OPA reagent and let to react for 2 min. Then, 1 µL of the FMOC reagent was added to the mix and injected into the chromatograph.

Calibration curves were generated for each amino acid ranking from 10 µM to 100 µM.

An Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) with autosampler and fluorescence detector was used. Derivatized amino acids were separated in a Hypersil ODS (250 x 4.0 mm; 5 µm) column (Agilent Technologies) conditioned at 45°C. The solvents and the gradients used are detailed in Table 7. The flow rate was fixed at 1 mL/min. The column was stabilised for 8 min at initial conditions before each new injection. The detection of the eluted amino acids was done by fluorescence in two different conditions. First, the excitation was fixed at 230 nm and the emission at 455 nm. Then, at t=32 min, the excitation was turned to 266 nm and the emission to 315 nm for the FMOC-proline and hydroxyproline detection.

Sarcosine and Norvaline (0.1 mM) were used as internal standards for quantification of proline and the other amino acids, respectively. The results were expressed in mg/100 g of product as the mean of three replicates.

Table 7. Mobile phases and gradient for the analysis of amino acids by RP-HPLC

Time (min)	Mobile phase (%)	
	A	**B**
1	100	0
10	91	9
41.5	40	60
42	0	100
51	0	100

A: 20 mM Sodium Acetate in water with 0.018% triethylamine at pH 7.2 and 0.3% tetrahydrofuran (v/v)
B: Acetonitrile: methanol: 100 mM Sodium Acetate (40:40:20)

3.2.6 Glutathione and cysteine analysis

Concentrations of glutathione (GSH), its oxidised form (GSSG) and cysteine were determined by UPLC-MS/MS (Ultrahigh Performance Liquid Chromatography and Mass Spectrometry in tandem) after alkylation by N-ethylmaleimide (NEM) [374].

Two grams of palatability enhancer (NEp, OEp) or pork liver homogenate (PLWL, PLW, L) were homogenized in 20 mL of 5 mM NEM (Phosphate-buffered saline, PBS) and centrifuged for 20 min at 12,000 *g* and 4°C. Supernatants were filtered through glass wool. Extracts from pork livers were diluted 20 times in 5 mM NEM (PBS); extracts from powders were diluted 100 times in 5 mM NEM (PBS). Then, perchloric acid (PCA) 70% was added to each sample in order to obtain a final concentration of 6% of PCA. Samples and standards were stored at -80°C before analyses.

Calibration curves were generated for each analyte, ranging from 25 nM to 1000 µM for GSH, from 1.25 nM to 50 µM for GSSG, from 0.5 nM to 20 µM for Cys and from 0.48 nM to 19.4 µM for Cis. GSH standard solution was prepared in 50 mM NEM (PBS 10x). GSSG and Cys standards solutions were prepared in PBS 10x. Cis standard solution was prepared in bidistilled water.

Samples were injected in an Acquity UPLC coupled to a Xevo TQD from Waters. Chromatographic separations were carried out at 30°C using an Acquity UPLC BEH C18 (2.1 x 50 mm, 1.7 µm) from Waters. The solvents and the gradients used are detailed in Table 8.

Table 8. Mobile phases and gradient for the analysis of GSH, GSSG, cysteine and cystine content by RP-HPLC

Time (min)	Mobile phase (%)	
	A	B
0	100	0
2.5	100	0
4.4	35	65
6	35	65
6.1	100	0
10	100	0

A: 0.1 % formic acid in water

B: Acetonitrile

Positive ion electrospray MS/MS was recorded using the conditions detailed in Table 9.

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Table 9. Mass spectrometry operating parameters for GSH, GSSG, cysteine and cystine analysis

Capillary voltage (kV)	33.5
Source temperature (°C)	120
Cone gas flow (L/h)	25
Nebulization gas flow (L/h)	700

3.2.7 Lipid analyses

Total and free fatty acids must be converted into volatile compounds for its analysis by gas chromatography. In this case, fatty acids were methylated to be converted in fatty acids methyl esters (FAME).

3.2.7.1 Total lipids extraction

Total lipids were extracted as described in section 3.2.1.3.

3.2.7.2 Sample preparation for total fatty acids analysis

Total fatty acids (TFA) were extracted from 10 mg of total lipids and methylated according to Berry et al. [375]. Heneicosanoic acid (C21:0) was used as the internal standard (540 µg). Samples were dried under nitrogen, dissolved in 5 mL of Berry solution methanol: 12 N HCl: 2,2-dimetoxipropane (25:2.5:1) and heated at 70°C during 4 hours. Two millilitres of hexane was added to the mix and this organic phase was cleaned by using water (1 mL x 3). The hexanic phase was dried under nitrogen and total fatty acids were dissolved in 300 µL of hexane. Samples were stored at -20°C until analysis of FAME by GC-FID.

3.2.7.3 Sample preparation for free fatty acids analysis

The free fatty acids (FFA) were separated from the total lipid fraction by ion-exchange resin (Amberlyst A26 OH, Dow Chemical, Midland, MI, USA) as described by Needs et al. [376]. One millilitre of total lipids extract and 540 µg of C21:0 were dried under nitrogen and dissolved in acetone: methanol (2:1). The adsorption of FFA on the resin was done with the aid of magnetic stirrer during 30 minutes. Then, the sample was filtered under vacuum. The filter was cleaned by acetone: methanol mixture (5 mL x 5). FFA were converted into FAME using 1.5 mL of boron fluoride-methanol (Sigma-Aldrich) and agitating for 10 min. Then, 3 mL of hexane was added to the mix and this organic phase was cleaned with water (1.5 mL x 3). The hexanic phase was dried under nitrogen and later dissolved in 300 µL of hexane. Samples were stored at -20°C before analysis of FAME by gas chromatography (GC-FID).

3.2.7.4 Chromatographic conditions for FAME analysis

Analysis of FAME from TFA and FFA was carried out using a gas chromatograph (GC) (7890A, Agilent Technologies) equipped with a flame ionization detector (FID) and a split injector (split ratio 100:1) according to Olivares et al. [377]. The separation was performed in a capillary column CP-SIL88 (Agilent Technologies; 100 m, 0.25 mm i.d., 0.2 µm film thickness). The oven temperature program began at 140 °C for 10 min, ramped to 190 °C at 4 °C/min, held at 190 °C for 10 min, ramped to 220 °C at 2 °C/min, and finally held at 220 °C for 5 min. Helium was used as carrier gas at a flow rate of 17.7 cm/s. Detector and injector temperatures were 240 and 220 °C respectively. FAMEs were identified by comparing their retention times with those of standard fatty acid methyl esters (Supelco® 37 Component FAME Mix, Sigma-Aldrich). For quantification, the response factors of the standard FAME respect to the internal standard were used. The results were expressed in mg/100 g of product as the mean of three replicates.

3.2.8 Lactic acid analysis

Sour taste is commonly associated with the presence of acids in foods and cats are very sensitive to sour taste. Among organic acids, lactic acid is metabolised mostly in the liver. Thus, some organic acids were analysed as potentially contributing to sour taste in pet foodstuffs but only lactic acid was quantified.

Lactic acid in pork livers homogenates (PLWL, PLW, L) and palatability enhancers (NEp, OEp) was quantified by a Lactate Assay Kit® from Sigma-Aldrich. In this assay, lactate concentration is determined by an enzymatic assay which results in a colorimetric product that is proportional to the lactate present in the sample. The kit contains four reagents: lactate assay buffer, lactate probe, lactate enzyme mix and L(+)-Lactate standard.

Fifty milligrams of sample were homogenised in 1 mL of lactate assay buffer and centrifuged at 13.000 *g* for 10 min. The supernatant was recovered and filtered using a centrifugal filter device 10 kDa (Millipore) at 10.000 *g* for 30 min. The enzymatic reaction was performed in a 96-wells plate. To each well containing 50 µL of the sample, was added 50 µL of a reaction mixture (lactate assay buffer/ lactate enzyme mix/ lactate probe 46/2/2). After 30 min of incubation at room temperature, the absorbance was measured at 570 nm. The results were expressed in mg/100 g of product as the mean of three replicates.

3.2.9 Nucleotides and derivatives analyses

Nucleotides and derivates were analysed in pork liver homogenates (PLWL, PLW, L) and in palatability enhancers (NEp, OEp) by HPLC. Pork liver homogenates were previously lyophilized. To prepare the extracts, 2.5 grams of sample were homogenized in 15 mL of 0.6 M perchloric acid in a stomacher for 10 min at 4°C. The homogenate was centrifuged

for 20 min at 4 °C at 10,000 g. The supernatant was filtered through glass wool, neutralized by adding carbonate potassium and stored at -20°C until use.

Calibration curves were generated for adenosine monophosphate, hypoxanthine, xanthine, uridine, inosine, guanosine ranking from 30 µM to 150 µM. Standards solutions were prepared in neutralized 0.6 M perchloric acid.

An Agilent 1200 Series HPLC (Agilent Technologies) with diode array detector (λ = 254 nm) was used. Separation was performed at 45 °C using a Gemini-NX C18 column (4.6 × 150 mm, 3 µm) from Phenomenex (Torrance, CA, USA). The mobile phase consisted of two solvents: solvent A, 100 mM potassium phosphate buffer, pH 4.5 or pH 3; solvent B, methanol 75%. The flow rate was 0.8 mL/min. The separation was initiated with 100% solvent A for 5 min followed by a gradient to 50% B in 10 min. The column was washed with 100% B for 7 min and returned to the initial conditions for a new injection after 7 min of equilibration. The results were expressed in µmol/g of product as the mean of three replicates.

3.2.10 Minerals analysis

Minerals were analysed by two different methods: ion chromatography and ICP-OES analysis.

3.2.10.1 Ion chromatography analysis

Soluble molecules from pork liver homogenates (PLWL, PLW, L) and palatability enhancers (NEp, OEp) were extracted. Three grams of sample (pork livers or powders) were homogenised with 30 mL of bidistilled water in a stomacher (IUL Instrument), for 10 min. The homogenate was centrifuged at 12,000 *g* for 20 min at 4°C. The supernatant was recovered through glass wool and diluted 5 times with bidistilled water. Samples

were stored at -20°C until use. Cations analysis (Na^+, K^+, Ca^{2+}, Zn^{2+}) was performed in an ion chromatograph 861 Advanced compact IC (Metrohm, Schiedam, the Netherlands) using a Metrosep C3 250 (Metrohm; 250 x 4.0 mm; 5 µm) column and 3 mM nitric acid as mobile phase. The flow rate was fixed at 1.2 mL/min. Anions analysis (F^-, Cl^-, $(HPO4)^{2-}$) was performed in an ion chromatograph 761 Compact IC (Metrohm) using a Metrosep A Supp 5 (Metrohm; 250 x 4.0 mm; 5 µm) column and 1 mM sodium bicarbonate/ 3.2 mM sodium carbonate as mobile phase . The flow was fixed at 0.7 mL/min. Ions were identified by comparison of their retention time with standards ones. In both cases, conductivity detection was used but, in the case of anions, chemical suppression before detection was performed. The concentration of each ion was determined from its respective calibration curve (ranking from 0.5 to 50 ppm), using a set of standard solutions (Sigma-Aldrich). The results were expressed as mg/100 g of product as the mean of three replicates.

3.2.10.2 ICP-OES analysis (Inductively coupled plasma optical emission spectrometry)

Minerals were quantified in pork liver homogenates (PLWL, PLW, L) and palatability enhancers (NEp, OEp) after acid digestion based on Vázquez et al. [378]. Thus, 200 mg of sample were homogenized with 4 mL of concentrated nitric acid and 1 mL of hydrogen peroxide in a Teflon tube. The digestion was carried out in a microwave oven at 800 W and 180°C for 45 min. The extract was recovered in a test tube and diluted to 15 mL with bidistilled water. Samples were stored at 4°C until analyses by ICP-OES. The results were expressed as mg/100 g of product as the mean of three replicates.

An ICP-OES (Optima 5300 DV, Perkin Elmer, Shelton, CT, USA) equipped with a cross-flow nebulizer and a Scott spray chamber was used for determining the concentrations of the elements. The optical system was purged with argon, and the operating conditions are listed in Table 10. Rhenium (197.248 nm) was used as internal standard.

Table 10. ICP-OES operating parameters for minerals quantification in pork liver homogenates (PLWL, PLW, L) and palatability enhancers (NEp and OEp)

RF generator	1300 W
Nebulization gas flow	0.8 L min^{-1}
Auxiliary argon flow	0.2 L min^{-1}
Plasma argon flow	15 L min^{-1}
Sample flow rate	1.0 L min^{-1}
Wavelengths (nm)	396.153 (Al); 249.772 (B); 233.527 (Ba); 315.887 (Ca); 327.393 (Cu); 239.562 (Fe); 766.490 (K); 279.077 (Mg); 257.610 (Mn); 589.592 (Na); 214.914 (P); 407.771 (Sr);

3.2.11 Enzymatic activity

Peptidase activity was assayed exclusively in pork livers homogenates (PLWL, PLW and L).

3.2.11.1 Exopeptidase activity

Exopeptidase activity was measured as described by Toldrá and Flores [379]. Four grams of sample (PLWL, PLW, L) were homogenized with 20 mL of 50 mM disodium phosphate buffer pH 7.5 containing 5 mM ethylene glycol tetraacetic acid (EGTA), using a Polytron® PT 2100 (Kinematica, Luzern, Switzerland) for 3x20 s at 27,000 *g*. The sample was centrifuged at 10,000 *g* and 4°C for 20 min and filtrated through glass wool. The supernatant (enzymatic extract) was recovered. Then, three reaction buffers were prepared: (1) for alanyl aminopeptidase (AAP) activity, 100 mM phosphate buffer pH 6.5 containing 2 mM 2-mercaptoethanol and 1 mM alanine-amido-4-methylcoumarin (AMC); (2) for arginyl aminopeptidase (RAP) activity, 50 mM phosphate buffer pH 6.5

containing 0.2 M NaCl, 0.1 mM arginine-AMC and 0.25 mM puromicine; (3) for methionyl aminopeptidase (MAP) activity, 100 mM phosphate buffer pH 7.5 containing 10 mM dithiothreitol, 0.15 mM alanine-AMC and 0.05 mM bestatine. The enzymatic activity was measured by mixing 250 µL of reaction buffer and 50 µL of enzymatic extract. For AAP and RAP measurements the enzymatic extract was previously diluted 5 and 2 times, respectively. A Fluoroskan Ascent Fl from Thermo Scientific (λ_{ex} = 355 nm, λ_{em} = 460 nm) was used. The activity was measured at t=0 and after 15 min of incubation at 37°C. Results are the mean of four measures and are expressed in µmol/hour*g of sample.

3.2.11.2 Endopeptidase (cathepsins) activity

Cathepsins activity was measured as described by Toldrá and Etherington [380]. In order to prepare the enzymatic extract, 2.5 g of sample (PLWL, PLW, L) were homogenized with 25 mL of 50 mM sodium citrate containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.2% Triton X-100, pH 5.0 using a Polytron for 3x20 s at 27,000 *g.* The sample was centrifuged at 10,000 *g* and 4°C for 20 min and filtrated on glass wool. The supernatant was recovered. Then, three reaction buffers were prepared: (1) for cathepsin B activity, 40 mM phosphate buffer pH 6.0 containing 0.4 mM EDTA, 10 mM cysteine and 0.05 mM Z-arginyl-arginine-AMC; (2) for cathepsin B+L activity, 40 mM phosphate buffer pH 6.0 containing 0.4 mM EDTA, 10 mM cysteine and 0.05 mM N-CBZ-L-phenylalanyl-L-arginine-AMC; (3) for cathepsin H activity, 40 mM phosphate buffer pH 6.8 containing 0.4 mM EDTA, 10 mM cysteine and 0.05 mM arginine-AMC. The enzymatic activity was measured by mixing 250 µL of reaction buffer and 50 µL of enzymatic extract. The activity was measured at t=0 and after 15 min of incubation at 37°C using a Fluoroskan Ascent Fl from Thermo Scientific (λ_{ex} = 355 nm, λ_{em} = 460 nm). Results are the mean of four measures and are expressed in µmol/hour*g of sample.

3.3 Proteomics tools

3.3.1 Peptides extraction and molecular mass fractionation

Peptides were extracted from palatability enhancers (NEp, OEp) and separated by gel filtration (GFC) as described in sections 3.2.3 and 3.2.4. Thus, after complete drying, the peptides extract was diluted in 20 mL of HCl and 5 mL were injected into the Sephadex G25 column (2.6 × 70 cm, GE Healthcare). The eluted fractions were collected using an automatic collector in 5 mL fractions. They were pooled together according to the elution profiles in four major fractions (F1, F2, F3, F4). Each major GFC fraction was dried under vacuum, resuspended in 1 mL of 0.1% trifluoroacetic acid (diluted in bidistilled water) and stored at -20°C until use.

3.3.2 Fractionation by RP-HPLC

Each major fraction from GFC was analysed by RP-HPLC (Agilent Technologies) using the method described in Escudero et al. [372] with modifications. Samples to analyse were filtered through syringe nylon filters (0.45 µm) and the filtrate (50 µL) was injected in a Symmetry C18 (250 x 4.6 mm; 5 µm) column (Waters) at 30°C. Solvents and gradient used are detailed in Table 11 and the flowrate was fixed at 1 mL/min. Eluted components were detected in the UV range at 214 nm, 254 nm and 280 nm. Each major fraction from GFC was injected three times in HPLC and two eluated fractions were collected per injection (from 0 to 10 min and from 10 to 20 min). In total, 48 fractions (4 fractions from GFC x 3 injections x 2 times) were obtained from HPLC. Each fraction was lyophilised, diluted in 200 µL of trifluoroacetic acid 0.1% (samples “dilution 1:1”) and stored at -80°C until analysis.

Table 11. Mobile phases and gradient for the analysis of GFC fractions (F1-4) from palatability enhancers (NEp and OEp) by RP-HPLC

Time (min)	Mobile phase (%)	
	A	**B**
0	100	0
65	72.7	27.3
66	0	100
76	0	100
80	100	0

A: 0.1% trifluoroacetic acid in water
B: Acetonitrile: water (60:40) with 0.085% trifluoroacetic acid

3.3.3 Peptides profile using MALDI-ToF analysis

The fractions obtained by RP-HPLC (section 3.3.2) were analysed by MALDI-ToF mass spectrometry. To optimize the concentration of the sample, two different concentrations were tested:

- Samples "dilution 1:1" in 0.1% trifluoroacetic acid.
- Samples from "dilution 1:1" were diluted 10 times in 0.1% trifluoroacetic acid and called "dilution 1:10".

After optimization of the methodology, each sample was analysed by MALDI-ToF mass spectrometry to obtain its peptide profile. Analyses were performed using a 5800 MALDI-ToF/ToF (AB Sciex Instruments, MA, USA). MALDI plates were prepared as follows. First, 1 µL of sample was located on the plate. Then, 0.75 µL of matrix (5 mg/mL α-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid – acetonitrile/H_2O (7:3, v/v)) was added to each µL of sample. The mix sample-matrix was air-dried and analysed for two mass ranges from 150 to 800 *m/z* and from 800 to 3500 *m/z*. The plate and the

acquisition method were calibrated using 1 µL of the calibration mix CM5 (AB Sciex Instruments, MA, USA) for 13 positions.

3.3.4 Peptides identification by nanoliquid chromatography and mass spectrometry in tandem (nanoLC-MS/MS)

Deproteinised extracts from NEp and OEp, and major fractions from GFC (F1-F4) were analysed by nanoLC-MS/MS. The nanoLC-MS/MS was performed using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to a Q-ToF TripleTOF® 5600+ system (AB Sciex Instruments, MA, USA) with a nanoelectrospray ionisation source.

For the analysis of deproteinised extracts from NEp and OEp and major fractions from GFC (F1-F4), lyophilised samples were diluted at 0.1 mg/mL with 0.1% trifluoroacetic acid. A total of 5 µL of each sample were injected and preconcentrated on an Eksigent C18 trap column (350 x 0.5 mm; 3 µm) at 3 µL/min for 5 min and using 0.1% trifluoroacetic acid as mobile phase. Then, the trap column was automatically switched in-line onto a nano-HPLC capillary column C18-CL (120 x 0.075 mm; 3 µm, Nikkyo Technos, JPN) equilibrated with 0.1% formic acid – acetonitrile/H_2O (5:100, v/v). Peptides elution was carried out using a liner gradient from 5% to 35% of solvent B in 120 min at 30°C and 0.30 µL/min. The column outlet was directly coupled to a nanoelectrospray ionisation system. The Q-ToF was used in positive polarity and data-dependant acquisition mode, in which a 0.25-s TOF MS scan in the range 150 – 1250 m/z was performed followed by 0.05-s product ion scans in the range 100 – 1250 m/z on the 50 most intense 2 – 5 charged ions (or 1 – 5 charged for mono-charged ions analysis).

3.3.5 Data analysis

For MALDI-ToF data analysis, spectral comparison was performed using mMass v5.5.0 software (Martin Strohalm). For MS/MS data analysis, ProteinPilot v5.0 search engine (AB Sciex Instruments, MA, USA) default parameters were used to generate a peak list directly from TripleTOF® 5600 system wiff files. The Paragon algorithm of ProteinPilot was used to search in NCBInr protein database with the following parameters: no enzyme specificity, no cys-alkylation and taxonomy Metazoa. The ProteinPilot false discovery rate (FDR) analysis tool algorithm provided a global FDR of 1% and a local FDR at 5% in all cases. The identification of protein of origin of peptides was done with a significance threshold $p < 0.05$ and a tolerance on the mass measurement of 100 ppm in MS mode and 0.3 Da for MS/MS ions.

3.4 Sensory analysis

Palatability and taste qualities of soluble protein and peptide extracts (F6 NEp and F6 OEp; see section 3.2.3), deproteinised extracts (F0 NEp and F0 OEp) and the four major fractions from GFC (F1-4 NEp and F1-4 OEp; see section 3.2.4) from both OEp and NEp were evaluated at Opertech Bio Inc. (PA, USA) using the rMOG technology detailed in Annex 1 (http://www.opertechbio.com/). [redacted]

[redacted]

[redacted]

[redacted]

3.5 Statistical analyses

All results are expressed as the mean of three replicates and standard deviation. The differences among pork liver homogenates used for the manufacture of palatability enhancers were studied. Also the effect of different processing conditions used to obtain palatability enhancers (new enzyme *vs.* old enzyme) was determined. The samples were compared in terms of composition and biochemical characterization by analysis of variance (ANOVA) using the statistic software XLSTAT, 2011, v5.01 (Addinsoft, Barcelona, Spain). Significant effects ($p < 0.05$) were compared using Fisher's least significant difference test ($p < 0.05$).

4. RESULTS AND DISCUSSION

4.1 Characterisation of pork livers used as raw material for palatability enhancers processing

Pork liver is one of the most common by-products for pet food industry. It is known as a good source of proteins and, as a consequence, very palatable for cats. Nevertheless, very little information relative to key tastants in pork liver as ingredient for pet food industry has been reported [381].

The purpose of this section was to achieve Objective 1 by characterising and comparing three pork liver homogenates used as raw material in pet food industry with regard to their proximal composition and key tastants (amino acids, glutathione, fatty acids, lactic acid, nucleotides and minerals) as well as proteolytic activity since this may significantly affect amino acid composition.

4.1.1 Proximal composition

The proximal composition of pork livers PLWL, PLW and L was analysed by the methods described previously in section 3.2.1. No significant differences were found among pork liver homogenates in protein, fat and ashes levels as shown in Table 12. Carbohydrates were not analysed. The proximal composition of pork liver homogenates were consistent with U.S. Department of Agriculture databases [107] and were not affected by breed or farming conditions. Nevertheless, many different studies have shown that the diet and the breed of pigs affect the composition of pork meat [382–388]. In addition, several studies showed the negative impact of slaughter age on meat sensory quality [389,390]. The constant composition of liver as main raw material ensures, to DIANA *Petfood*, a constant quality of its products.

Table 12. Proximal composition of pork liver homogenates PLWL, PLW and L (expressed in percentage)

Item	PLWL		PLW		L		$P^{\#\#}$
	M	SD	M	SD	M	SD	
Moisture	70.3 [b]	± 0.1	70.0 [c]	± 0.1	70.5 [a]	± 0.1	***
Protein	22.6	± 0.2	22.5	± 0.4	22.6	± 0.6	*ns*
Fat	5.8	± 0.6	6.1	± 1.3	5.6	± 0.5	*ns*
Ashes	1.3	± 0.0	1.3	± 0.0	1.3	± 0.0	*ns*

Results are expressed as means (M) and standard deviation (SD).

[a-c] Means in the same row with the same letter do not differ significantly at the 0.05 level of probability

[##] *p value* : *ns* , not significant; *** P < 0.001

Cats need a high protein intake [10]. The high protein content of pork liver makes it an interesting raw material to be used as palatable enhancer in formulations for cat feed. Moreover, based on amino acids bioavailability, the quality of pork liver proteins were described as very high compared to beef or chicken by-products [391]. Proteins of PLWL, PLW and L were separated by electrophoresis to obtain the protein profile of each homogenate. The three pork liver homogenates had the same protein profile: several high molecular proteins and polypeptides from 25 kDa to 200 kDa, high concentration of 60 kDa proteins, few peptides from 15 to 25 kDa, several peptides from 10 to 14 kDa and very low quantity of peptides below 8 kDa (Figure 16).

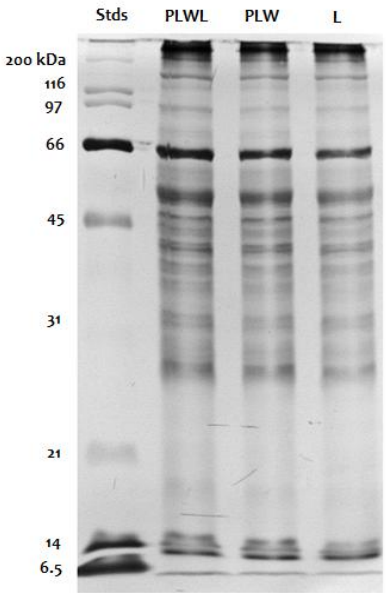

Figure 16. Electrophoretogram of proteins and peptides in pork liver homogenates PLWL, PLW and L. *(Stds) molecular weight standards; on sodium dodecyl sulfate-polyacrylamide gel (12%)*

4.1.2 Potential key tastants

Water-soluble compounds such as free amino acids, peptides, some free fatty acids, nucleotides and derivatives and inorganic ions have an important role in cat food acceptance and nutrition [53]. Before the evaluation of these free key tastants in the liver homogenates, total amino acid and fatty acid were quantified to discard differences among the raw material used.

4.1.2.1 Amino acid content

Total and free amino acids were analysed and quantified in the three liver homogenates PLWL, PLW and L, and results are presented in Table 13 and Table 14.

Total amino acid content of pork liver homogenates was quantified and no significant difference was observed which confirms the similar total protein content of pork liver homogenates (Table 13). Only few significant differences in the content of several amino acids (Ser, Thr, Arg, Tyr, Ile and Lys) were observed although these differences were low.

Table 13. Total amino acid content (mg/ 100 g product) in pork liver homogenates PLWL, PLW and L

	PLWL		PLW		L		*P* [##]
	M	SD	M	SD	M	SD	
Amino acids							
Asx[1]	1480.0	± 77.7	1443.0	± 5.9	1445.2	± 37.2	*ns*
Glx[1]	2012.3	± 114.4	1890.6	± 31.7	1942.5	± 41.3	*ns*
Ser	590.7 [a]	± 26.1	546.2 [b]	± 18.1	561.6 [a]	± 14.4	*
His	294.7	± 91.4	367.8	± 64.5	316.5	± 53.8	*ns*
Gly	1222.9	± 50.6	1160.6	± 24.7	1181.9	± 36.4	*ns*
Thr	746.1 [a]	± 27.7	687.7 [b]	± 28.9	716.8 [a]	± 20.1	**
Ala	1031.9	± 41.2	996.5	± 17.9	994.8	± 21.1	*ns*
Arg	1173.7 [a,b]	± 41.3	1093.3 [b]	± 37.5	1123.6 [a]	± 41.2	*
Tyr	673.7 [a,b]	± 22.6	610.4 [b]	± 89.2	673.2 [a]	± 17.9	*
Val	1043.1	± 33.4	1027.5	± 21.1	1016.4	± 25.8	*ns*
Met	468.5	± 15.6	520.2	± 88.5	455.3	± 13.0	*ns*
Phe	924.7	± 32.5	895.5	± 4.4	888.1	± 21.2	*ns*
Ile	869.2 [a,b]	± 27.8	827.7 [b]	± 28.0	838.5 [a]	± 20.9	*
Leu	1649.5	± 60.0	1550.7	± 99.7	1591.6	± 40.0	*ns*
Lys	1833.1 [a,b]	± 66.9	1730.3 [b]	± 14.2	1760.2 [a]	± 54.3	*
Pro	704.7	± 29.6	671.8	± 10.1	731.4	± 111.1	*ns*
Total	***16718.6***	***± 639.6***	***16169.7***	***± 85.9***	***16237.5***	***± 383.4***	***ns***

Results are expressed as means (M) and standard deviation (SD).

[1] Asx = Asp + Asn; Glx = Glu + Gln

[a;b] Means in the same row with the same letter do not differ significantly at the 0.05 level of probability

[##] *p value* : *ns* , not significant; * P < 0.05; ** P < 0.01

Several amino acids were exclusively identified and quantified as free amino acids (Asn, Gln and Trp) since they are degraded during acid hydrolysis treatment for total amino acid analysis. Free amino acid content in pork liver homogenates was higher in PLWL than in PLW and L ($p < 0.01$) (Table 14): free amino acids represented 0.48% of total amino acids in PLWL whereas in PLW and L they represented 0.38% of total amino acids. These results indicate a low *post mortem* exopeptidase activity which is lower in PLW and L than in PLWL. Significant differences were observed for several of the free amino acids especially for Gln, Ser, Ala, Arg, Ile, Thr and Pro.

The *post mortem* storage of pork meat affects the metabolism in muscles. Morzel et al. [392] reported that the storage at 4°C during 72h caused changes in protein content of Large White *Longissimus Lumborum*. These changes were due to sarcoplasmic proteins proteolysis. The high enzymatic activity in liver [393] suggests that these *post mortem* changes occur in liver too and could affect protein content of pork livers during transport (even short time) from the slaughterhouse to Diana *Petfood*. All samples received the same treatment, so the difference in free amino acid content observed must be due to the intrinsic nature of the pigs and must be correlated with different proteolytic activity.

Table 14. Free amino acid content (mg/ 100 g product) in pork liver homogenates

Item	PLWL		PLW		L		*P* ##
	M	SD	M	SD	M	SD	
Amino acids							
Asp + Glu	10.38 [a]	± 0.35	7.88 [b]	± 1.32	8.05 [b]	± 0.73	**
Asn	1.27 [a]	± 0.12	1.01 [b]	± 0.10	1.02 [b]	± 0.08	**
Ser	3.26 [a]	± 0.30	2.27 [b]	± 0.25	2.10 [b]	± 0.22	***
Gln	2.69 [a]	± 0.25	2.05 [b]	± 0.25	0.96 [c]	± 0.17	***
His	ND		ND		ND		
Gly	5.70 [a]	± 0.61	4.04 [b]	± 0.42	4.13 [b]	± 0.48	**
Thr	2.36 [a]	± 0.22	1.65 [b]	± 0.22	1.70 [b]	± 0.20	**
β-Ala	0.41	± 0.03	0.42	± 0.03	0.33	± 0.03	*ns*
Ala	5.88 [a]	± 0.61	4.36 [b]	± 0.45	4.10 [b]	± 0.35	***
Tau	0.26	± 0.01	0.20	± 0.05	0.24	± 0.04	*ns*
Arg	4.90 [a]	± 0.49	3.48 [b]	± 0.64	2.40 [c]	± 0.40	***
Tyr	2.70	± 0.18	2.28	± 0.29	2.31	± 0.22	*ns*
Val	4.23 [a]	± 0.33	3.44 [b]	± 0.36	3.75 [a]	± 0.28	*
Met	2.95	± 0.20	2.54	± 0.34	2.62	± 0.20	*ns*
Orn	1.25	± 0.07	1.12	± 0.14	0.99	± 0.05	*ns*
Trp	2.34	± 0.09	2.09	± 0.22	1.92	± 0.21	*ns*
Phe	6.64	± 0.31	5.86	± 0.77	6.10	± 0.65	*ns*
Ile	4.24 [a]	± 0.23	3.34 [b]	± 0.42	3.39 [b]	± 0.25	**
Leu	11.75	± 0.55	10.02	± 1.42	9.87	± 0.78	*ns*
Lys	3.43	± 0.17	2.69	± 0.61	2.44	± 0.43	*ns*
Pro	3.27 [a]	± 0.38	2.55 [b]	± 0.22	2.94 [a]	± 0.30	**
Total FAA	***79.92 [a]***	***± 5.09***	***62.28 [b]***	***± 7.98***	***61.70 [b]***	***± 6.04***	**

Results are expressed as means (M) and standard deviation (SD).

[a-c] Means in the same row with the same letter do not differ significantly at the 0.05 level of probability

p value : *ns* , not significant; * P < 0.05; ** P < 0.01; *** P < 0.001

ND, not detected

4.1.2.2 Proteolytic activity

The proteolytic activity was studied for endopeptidases (cathepsins B, B+L and H) and exopeptidases (alanyl, argynil and methionyl aminopeptidase) and results are shown in

Table 15. Cathepsins B and B+L activities were higher in PLW followed by PLWL and L while cathepsin H activity was higher in PLWL than in the other liver homogenates. These results suggest that PLW and PLWL would be richer in peptides, which consequently would be substrates for further exopeptidase activity. Moreover, *post mortem* exopeptidase activity was higher in PLWL than in PLW and L ($p < 0.001$). These exopeptidase activities can explain the highest amount of free amino acids found in PLWL. Especially AAP and RAP would be responsible for the high release of amino acids such as Ala and Arg, respectively [394,395]. In this way, enzyme preferences and differences in proteolytic activities may affect the profile of amino acids that would be released during the industrial process of palatants affecting the palatability of the final product. In the tested pork liver homogenates, the most abundant free amino acids were Leu, the sum of Asp and Glu, and Phe which have been characterized by humans as bitter, sour/umami and bitter, respectively [396]. However, cats are particularly attracted by amino acids described as sweet by humans (Pro or Ala) and reject amino acids described as bitter (Phe,Trp or Arg) or very sour stimuli which inhibit amino acid tongue units [46,180]. In the analysed pork liver homogenates most of the free amino acids quantified were described as bitter by humans (43.6% of free amino acids in PLWL, 37% in PLW and 48.6% in L). However, the total amino acid content of proteins is a source of amino acid described as sweet by humans (36.7% of total amino acids in PLWL, 31.7% in PLW and 36.6% in L without taking into account the glutamine converted in glutamic acid during the sample preparation) which are liberated during the process.

Table 15. Exopeptidase and endopeptidase activity (µmol/hour*g product) in pork liver homogenates[#]

Item	PLWL		PLW		L		*P* [##]
	M	SD	M	SD	M	SD	
Exopeptidase activity [1]							
AAP	6.72 [a]	± 0.28	6.06 [b]	± 0.20	6.01 [b]	± 0.20	***
RAP	3.66 [a]	± 0.07	2.58 [c]	± 0.04	2.74 [b]	± 0.16	***
MAP	1.18 [a]	± 0.04	0.96 [c]	± 0.03	0.98 [b]	± 0.03	***
Endopeptidase activity							
Cathepsin B	1.53 [b]	± 0.07	1.71 [a]	± 0.16	1.44 [c]	± 0.10	***
Cathepsins B+L	2.37 [b]	± 0.12	2.69 [a]	± 0.20	1.98 [c]	± 0.12	***
Cathepsin H	0.85 [a]	± 0.03	0.74 [b]	± 0.05	0.73 [b]	± 0.04	***

Results are expressed as means (M) and standard deviation (SD).

[1] AAP, alanyl aminopeptidase; RAP, arginyl aminopeptidase; MAP, methionyl aminopeptidase

[a-c] Means in the same row with the same letter do not differ significantly at the 0.05 level of probability

[##] *p value* : *** P < 0.001

4.1.2.3 Glutathione content

The glutathione (GSH) is a natural tripeptide that may have an impact in aroma and taste. This compound can be hydrolysed to generate its aminoacid components; Glu, Gly and Cys which is a sulphur-containing aminoacid. GSH and sulphur-containing aminoacids (Cys and Met) can act as precursors of “meat flavour”. Jung et al. [143] reported that adding GSH could reinforce “beef flavour” of beef soup. It can also contribute to the kokumi taste (savoury taste), be involved in the generation of volatile compounds which can act as antioxidant and prevent rancidity [144,397,398]. Moreover, addition of glutathione Maillard reaction products (GMRP) in beef soup also reinforce “beef flavor” but metallic and astringent notes appeared [399]. GSH/ glucose Maillard reaction products presented higher roasted flavour than cysteine/glucose Maillard reaction products [397].

GSH was analysed in PLWL, PLW and L by HPLC after derivatization by UPLC-MS/MS. Strong differences among them were found. GSH content was higher in PLWL than in PLW and L (Table 16). PLW and L GSH contents were similar to GSH content in several foodstuffs reported by Ueda et al. [144] and Balogh et al. [352]. Oxidised glutathione (GSSG) was higher in PLWL than in PLW and L.

The high differences of GSH content in pork liver homogenates can influence the taste and the volatile compound composition of manufactured products, and may be diet-related. For instance, a methionine or cysteine-supplemented diet resulted in an increase of GSH concentration in plasma and tissues [400]. The feeding of the pigs used to obtain the pork liver homogenates was the same in terms of protein content (Table 6) but it has been reported that glutathione synthesis in liver can be regulated by metabolic pathways or cysteine transport in hepatocytes and not only by diet [400].

Table 16. Glutathione content (mg/ 100 g product) of pork liver homogenates

Item	PLWL		PLW		L		*P* [##]
	M	SD	M	SD	M	SD	
GSH	41.25 [a]	± 0.60	6.50 [b]	± 0.49	5.37 [c]	± 0.24	***
GSSG	0.41 [a]	± 0.04	0.35 [b]	± 0.02	0.30 [b]	± 0.01	***

Results are expressed as means (M) and standard deviation (SD).

[a-c] Means in the same row with the same letter do not differ significantly at the 0.05 level of probability

[##] *p value* : *** P < 0.001

4.1.2.4 Fatty acid content

Fatty acid composition in animal foods has been shown as essential for their effect on flavour [401]. Thus, free (FFA) and total (TFA) fatty acids were extracted from pork liver hydrolysates PLWL, PLW and L and analysed by GC-FID. Results are presented in Table 17 and Table 18.

No significant difference was observed for TFA (Table 17). Nevertheless, differences had been observed among the different classes of fatty acids. PLW contained the greatest amount of monounsaturated fatty acids (MUFA) ($p < 0.05$) while L contained the greatest amount of polyunsaturated fatty acids (PUFA) ($p < 0.001$) but no significant difference was observed for saturated fatty acids (SFA) among liver homogenates. The calculated PUFA:SFA ratios for PLWL, PLW and L were 0.92, 0.90 and 1.03, respectively. These PUFA:SFA ratios are consistent with those reported in the literature and highlight the high PUFA content of liver when compared with muscle or subcutaneous tissue [357]. Several studies have shown the impact of diet and breed on fat composition in pork meat. In term of diet, the addition of flax in the pork diet resulted in an increase of liver linolenic acid content (30%) and a decrease of linoleic acid (13%) [357]. Also it produced an increase of n-3 polyunsaturated fatty acids in pork-based manufactured products like sausages or paté [402]. However, the decrease of the daily protein intake resulted in a decrease of polyunsaturated fatty acids in liver and in semimembranosus muscle [403]. On the other hand, the genetic line affected the fat composition in the *Longissimus lumborum* muscle [404] as it was observed a higher saturated and monounsaturated fatty acid contents in Large White pigs than in Pietrain pigs.

The ratio between monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) content in PLWL (0.57), PLW (0.61) and L (0.42) was lower than the ratio MUFA/PUFA in Iberian pigs' liver (1.09) [405]. This lower PUFA percentage described by Parra et al. can be due to a low protein diet.

Table 17. Total fatty acid content (mg/ 100 g product) in pork liver homogenates

Item	PLWL		PLW		L		P ##
	M	SD	M	SD	M	SD	
Fatty acids							
C12:0	1.4	± 0.7	2.1	± 0.9	1.1	± 0.1	*ns*
C14:0	23.1	± 9.8	31.5	± 11.3	20.6	± 1.6	*ns*
C15:0	4.2 [b]	± 1.0	5.6 [a]	± 1.0	5.7 [a]	± 0.1	*
C16:0	687.1	± 104.2	783.2	± 114.9	736.5	± 32.1	*ns*
C17:0	26.0	± 4.6	29.9	± 4.8	30.6	± 0.9	*ns*
C18:0	926.6 [b]	± 32.1	978.6 [a]	± 36.8	906.5 [b]	± 25.4	**
saturated	**1668.4**	**±** 150.4	**1830.8**	**±** 167.6	**1701.1**	**±** 59.9	***ns***
C16:1	60.0 [a,b]	± 18.5	76.7 [a]	± 21.1	50.8 [b]	± 2.9	*
C17:1	7.8	± 2.5	10.3	± 2.7	7.6	± 0.8	*ns*
C18:1 n9t	7.2 [a]	± 0.5	7.3 [a]	± 0.3	3.6 [b]	± 0.6	***
C18:1 n9c + C18:1 n7	771.4 [a,b]	± 119.8	896.5 [a]	± 140.6	650.3 [b]	± 49.8	**
C20:1 n9	19.2 [c]	± 2.2	21.8 [b]	± 2.7	34.8 [a]	± 0.9	***
monounsaturated	**865.6 [a,b]**	**±** 142.8	**1012.9 [a]**	**±** 167.0	**747.0 [b]**	**±** 54.3	*****
C18:2 n6t	2.9	± 0.5	3.3	± 0.4	2.7	± 0.2	*ns*
C18:2 n6c	585.2 [c]	± 67.5	664.8 [b]	± 80.8	792.2 [a]	± 22.4	***
C18:3 n6	2.8	± 1.1	3.7	± 1.2	2.3	± 0.1	*ns*
C20:2 n6	13.0 [b]	± 1.2	14.5 [b]	± 1.4	23.9 [a]	± 1.0	***
C20:3 n6	6.3 [c]	± 0.3	6.8 [b]	± 0.3	9.5 [a]	± 0.2	***
C20:4 n6	671.8 [b]	± 37.7	707.9 [a]	± 23.5	630.5 [c]	± 15.1	***
C22:4 n6	29.3 [b]	± 1.5	31.7 [a]	± 1.8	18.6 [c]	± 0.4	***
C18:3 n3	9.7 [b]	± 0.4	10.7 [a]	± 0.4	8.7 [c]	± 1.0	***
C20:5 n3	25.6 [a,b]	± 5.2	23.9 [b]	± 4.8	30.5 [a]	± 0.6	*
C22:5 n3	148.5 [b]	± 12.9	150.5 [b]	± 9.4	190.2 [a]	± 3.2	***
C22:6 n3	34.4 [c]	± 1.8	36.6 [b]	± 1.3	51.2 [a]	± 1.1	***
polyunsaturated	**1529.5 [c]**	**±** 85.0	**1654.3 [b]**	± 86.2	**1760.4 [a]**	**±** 41.5	*****
Total	***4063.6***	***± 397.7***	***4498.0***	***± 463.4***	***4179.7***	***± 107.3***	***ns***

Results are expressed as means (M) and standard deviation (SD).

[a-c] Means in the same row with the same letter do not differ significantly at the 0.05 level of probability

p value : *ns* , not significant; * P < 0.05; ** P < 0.01; *** P < 0.001

Free fatty acids content of pork liver homogenates was higher (about double) in PLW than in PLWL or L ($p < 0.001$) (Table 18). This result indicated a higher lipolitic activity in PLW and a more active fat metabolism.

During the storage at 4°C, free fatty acid content increases in Large White and Pietrain *Longissimus lumborum* due to an increase of lipolysis [404]. The high enzymatic activity

in liver suggests that these postmortem changes occur in liver too and could have affected protein and fat contents of pork livers during transport from the slaughterhouse to Diana *Petfood,* as it could have affected free amino acid content. Moreover, further degradation of these FFA generated by lipolysis through lipid oxidation reactions produces volatile compounds as aldehydes which can modify meat products flavor [406].

Cats rejected diet containing medium-chain triglycerides and caprylic acid [54] but this fatty acid was not detected in PLWL, PLW or L. In contrast, cats are very dependent on dietary sources of arachidonic acid (20:4 n6), and possibly eicosapentaenoic acid (20:5 n3) and docosahexanoic acid (22:6 n3), thus the rate of Δ6 desaturase activity in feline liver is limited [407]. Pork liver homogenates are a suitable source of fatty acids for cats since the arachidonic acid represented 16.5%, 15.7% and 15.1% of total fatty acids in PLWL, PLW and L, respectively.

Table 18. Free fatty acid content (mg/ 100 g product) in pork liver homogenates

Item	PLWL		PLW		L		*P* [##]
	M	SD	M	SD	M	SD	
Fatty acids							
C12:0	0.35 [b]	± 0.02	1.18 [a]	± 0.12	0.38 [b]	± 0.03	***
C14:0	5.69 [b]	± 0.40	17.12 [a]	± 0.70	6.28 [b]	± 0.55	***
C15:0	3.51 [a,b]	± 2.15	5.52 [a]	± 3.11	0.98 [b]	± 0.11	**
C16:0	126.29 [c]	± 4.43	269.28 [a]	± 14.90	140.85 [b]	± 12.32	***
C17:0	3.11 [b]	± 0.44	6.84 [a]	± 0.39	3.37 [b]	± 0.41	***
C18:0	95.32 [b]	± 1.98	141.80 [a]	± 7.05	81.40 [c]	± 10.61	***
saturated	**234.16 [b]**	**± 6.75**	**441.73 [a]**	**± 22.60**	**233.25 [b]**	**± 23.80**	***
C16:1	12.44 [c]	± 1.35	35.85 [a]	± 1.07	15.12 [b]	± 1.26	***
C17:1	1.16 [c]	± 0.16	3.93 [a]	± 0.23	1.40 [b]	± 0.15	***
C18:1 n9t	1.46 [b]	± 0.16	2.64 [a]	± 0.14	0.82 [c]	± 0.20	***
C18:1 n9c + C18:1 n7	136.24 [b]	± 6.06	309.41 [a]	± 8.11	121.03 [c]	± 14.99	***
C20:1 n9	4.39 [c]	± 0.45	8.84 [b]	± 0.31	11.70 [a]	± 1.00	***
monounsaturated	**155.69 [b]**	**± 8.10**	**360.68 [a]**	**± 8.61**	**148.14 [b]**	**± 16.56**	***
C18:2 n6t	0.78	± 0.41	0.64	± 0.17	0.83	± 0.06	*ns*
C18:2 n6c	2.42 [b]	± 5.58	4.50 [a]	± 4.02	1.58 [c]	± 12.07	***
C18:3 n6	0.44 [c]	± 0.06	1.61 [a]	± 0.06	0.69 [b]	± 0.05	***
C20:2 n6	2.77 [c]	± 0.11	5.24 [a]	± 0.17	4.39 [b]	± 0.51	***
C20:3 n6	0.74 [b]	± 0.06	1.14 [a]	± 0.03	1.19 [a]	± 0.11	***
C20:4 n6	50.56 [b]	± 5.85	70.88 [a]	± 2.43	55.00 [b]	± 4.52	***
C22:4 n6	3.10 [b]	± 0.20	4.97 [a]	± 0.15	2.39 [c]	± 0.24	***
C18:3 n3	2.42 [b]	± 0.21	4.50 [a]	± 0.23	1.58 [c]	± 0.25	***
C20:5 n3	2.57 [b]	± 0.37	2.78 [b]	± 0.12	3.86 [a]	± 0.32	***
C22:5 n3	11.88 [b]	± 0.95	16.67 [a]	± 0.54	16.10 [a]	± 1.53	***
C22:6 n3	2.32 [c]	± 0.20	3.91 [a]	± 0.12	3.22 [b]	± 0.33	***
polyunsaturated	**148.20 [c]**	**± 13.34**	**273.39 [a]**	**± 7.55**	**220.02 [b]**	**± 19.63**	***
Total FFA	***526.06 [b]***	***± 28.87***	***1054.29 [a]***	***± 39.34***	***583.84 [b]***	***± 61.85***	***

Results are expressed as means (M) and standard deviation (SD).

[a-c] Means in the same row with the same letter do not differ significantly at the 0.05 level of probability

[##] *p value*: *ns*, not significant; * P < 0.05; ** P < 0.01; *** P < 0.001

4.1.2.5 Lactic acid content

As mentioned in section 1.4.2, cats are very sensitive to sour taste associated to organic acids which they reject if too concentrated. Among organic acids, lactic acid is metabolised mostly in the liver [408,409]. Thus, lactic acid was quantified in PLWL, PLW and L using an enzymatic kit. There was no significant difference among PLWL, PLW and L in term of lactic acid content (Table 19) which was in the same range as in an aqueous extract of beef muscle [410].

Table 19. Lactic acid content (mg/ 100 g product) in pork liver homogenates

Item	PLWL		PLW		L		*P* [##]
	M	SD	M	SD	M	SD	
Lactic acid	296.8	± 44.0	296.5	± 41.1	264.8	± 21.2	*ns*

Results are expressed as means (M) and standard deviation (SD).

[##] *p value* : *ns* , not significant

4.1.2.6 Nucleotide and derivatives content

Pork liver is known to contain a large amount of purines which are often associated to umami taste [411]. Thus, nucleotides and derivatives were quantified by RP-HPLC. GMP and IMP are the key components of umami taste in humans whereas they inhibit the amino acid units of cat's tongue [33] and thus can be considered as unpalatable by cats. None of these compounds were present in the liver homogenates while their derivatives AMP, Ino, Hx, X, U, and G were found. Their contents are given in Table 20. AMP was the only nucleotide detected in our samples unlike meat where ATP, ADP, IMP and GMP are present [317]. Significant differences were observed for hypoxanthine ($p < 0.05$), xanthine, AMP ($p < 0.01$), uridine and inosine ($p < 0.001$) but the guanosine content was similar for all three samples. Hypoxanthine and xanthine are the final products of ATP

degradation and were the most abundant purine derivatives found in pork liver homogenates. The hypoxanthine accumulation that occurs in pork meat aging, that was related to the postmortem pH since it affects metabolism, was associated to an enhancement of bitter taste [196]. However, the bitter taste is likely not appreciated by cats, even if it can be counter-balanced by other tastants.

Table 20. Nucleotide and derivatives content (µmol/ 100 g product) in pork liver homogenates

Item	PLWL		PLW		L		*P* ##
	M	SD	M	SD	M	SD	
AMP	8.6 [b]	± 0.4	6.0 [c]	± 0.8	9.5 [a]	± 0.1	**
Hypoxanthine	230.6 [a,b]	± 0.9	218.2 [b]	± 1.5	220.3 [a]	± 4.0	*
Xanthine	217.5 [c]	± 0.9	224.5 [b]	± 2.0	223.9 [a]	± 1.7	**
Uridine	26.8 [a]	± 0.4	19.6 [b]	± 0.5	16.1 [c]	± 0.4	***
Inosine	75.6 [a]	± 0.0	59.3 [c]	± 0.5	59.1 [b]	± 0.2	***
Guanosine	15.8	± 0.2	16.5	± 0.0	13.8	± 1.1	*ns*

Results are expressed as means (M) and standard deviation (SD).

[a-c] Means in the same row with the same letter do not differ significantly at the 0.05 level of probability

p value : *ns* , not significant; * P < 0.05; ** P < 0.01; *** P < 0.001

4.1.2.7 Mineral content

Minerals are also potential tastants for cats since some inorganic salts like NaCl or KCl are associated to meat taste and can stimulate their taste system at low concentration [33]. Minerals were analysed by two methods. First, the most relevant ions were quantified in the pork liver homogenates (PLWL, PLW and L) by ion chromatography (Table 21). Then, minerals were quantified by ICP-OES in order to complete the analysis (Table 22). The abundance of each mineral in the pork liver homogenates was in accordance with the results published by Tomović et al. [327] except for Zn and Fe. Moreover, according to Tomović et al. [327], ashes content in liver do not change from one pig genetic line to another.

Table 21. Mineral content in pork liver homogenates PLWL, PLW and L (mg/ 100 g product) quantified by ion chromatography

Item	PLWL		PLW		L		*P* ##
	M	SD	M	SD	M	SD	
Na^+	52.6	± 0.5	54.7	± 1.6	58.7	± 6.1	*ns*
K^+	281.4 [a]	± 3.2	284.7 [a]	± 4.9	265.9 [b]	± 11.5	*
Ca^{2+}	6.0	± 0.3	7.6	± 0.9	7.2	± 0.0	*ns*
Zn^{2+}	ND		ND		ND		
Mg^{2+}	7.0 [b]	± 0.5	10.6 [a]	± 0.6	9.2 [a]	± 0.5	*
F^-	26.7 [a]	± 1.4	24.6 [a]	± 2.2	20.9 [b]	± 1.0	**
Cl^-	81.0 [a]	± 0.4	77.4 [b]	± 2.3	84.1 [a]	± 2.0	***
$(HPO_4)^{2-}$	583.2 [b]	± 25.2	693.1 [a]	± 38.3	675.0 [a]	± 22.3	***

Results are expressed as means (M) and standard deviation (SD).

[a;b] Means in the same row with the same letter do not differ significantly at the 0.05 level of probability

p value : *ns* , not significant; * P < 0.05; ** P < 0.01; *** P < 0.001

ND, not detected

Table 22. Mineral content in pork liver homogenates PLWL, PLW and L (mg/ 100 g product) quantified by ICP-OES

Item	PLWL		PLW		L		*P* ##
	M	SD	M	SD	M	SD	
Na	57.6 [b]	± 5.1	55.6 [b]	± 6.9	73.0 [a]	± 3.6	*
K	280.2	± 29.0	263.6	± 37.3	311.5	± 13.9	*ns*
Ca	8.4	± 2.4	4.7	± 1.2	ND		*ns*
Zn	0.7	± 0.1	0.7	± 0.1	0.8	± 0.1	*ns*
Mg	19.4 [b]	± 2.3	18.2 [b]	± 2.6	30.4 [a]	± 7.3	*
P	324.7	± 32.0	315.9	± 34.5	378.5	± 4.4	*ns*
Cu	0.09 [b]	± 0.0	0.12 [a]	± 0.0	0.14 [a]	± 0.0	**
Fe	1.5	± 0.2	1.9	± 0.3	1.7	± 0.1	*ns*

Results are expressed as means (M) and standard deviation (SD).

[a;b] Means in the same row with the same letter do not differ significantly at the 0.05 level of probability

[##] *p value* : *ns* , not significant; * P < 0.05; ** P < 0.01

ND, not detected

As shown in Table 21, the most abundant mineral in the liver homogenates was phosphorous (25.0%, 24.3% and 29.1% of total ash for PLWL, PLW and L, respectively) followed by potassium and sodium. Phosphorous is the most needed mineral for cat's body after calcium [412].

Five elements (Na, K, Ca, Zn, Mg) were analysed by both methods. No significant difference was observed between the results from the two methods for Na and K. However, the standard deviations obtained for K quantified by ICP-OES were higher than those obtained by ion chromatography analyses; this can be the reason for not detecting differences among liver homogenates in K content by ICP-OES.

Concerning Ca and Mg, results obtained by ICP-OES were different from those obtained by ion chromatography. Ca from L sample was not detected by ICP-OES. This method seems not to be suitable to analyse Ca. Mg content was twice higher in PLWL and PLW

when analysed by ICP-OES, and more than three times higher for L. However, no conclusion could be made about which of the two methods is better for Mg quantification. In contrast, Zn could not be quantified by the ion chromatographic method for coeluting with Ca.

Fe, Zn and Cu were quantified by ICP-OES. Concerning Fe and Zn content, there were no significant differences among liver homogenates. Cu content was significantly higher in PLW and L than in PLWL. Cu content decreases in pork liver all along their growth [413]. It has been reported that Cu, Fe and Zn concentrations increase when diet is complemented with these minerals [414,415]. Nevertheless, diets supplied to PLWL and to PLW animals were richer in Fe, Cu and Zn than the diet supplied to L animals (Table 6). Thus, the diet did not have any effect on these mineral contents in the analysed livers.

Mineral content of bovine liver has been reported by Trevizan et al. [360]. Ca content was similar in pork liver homogenates PLWL, PLW and L and in bovine liver but Cu, Fe, Mg and Zn were higher in bovine liver than in pork liver homogenates. Mineral content differences may be due to species specificity but may also vary before and after slaughtering or depending upon the liver section from which the sample was collected [416].

4.2 Characterisation of two palatability enhancers used in cat food processing

Palatability enhancers are essential ingredients for pet food industry. They improve smell and taste and thus, attractiveness of pet foods. Nevertheless, very little information relative to key tastants in palatability enhancers has been reported. In fact, analyses of pet foodstuffs are generally done to evaluate their nutritional quality by quantifying protein, minerals or vitamins (Table 5, section 1.6).

The purpose of this section was to achieve Objective 2 by characterising and comparing two meat-based palatability enhancers for cat food focusing on their potential key tastants.

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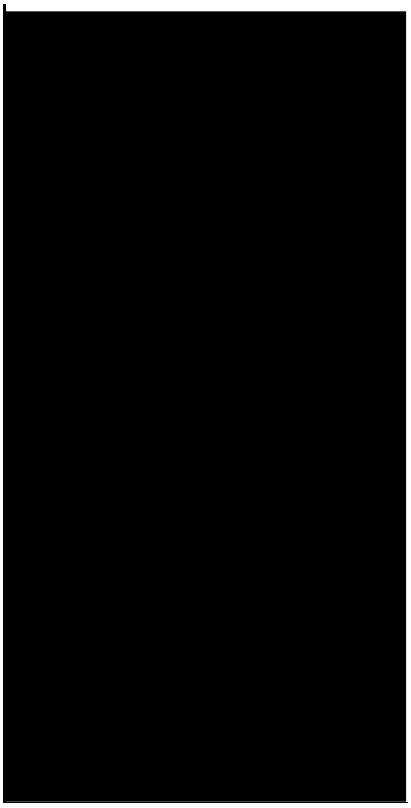

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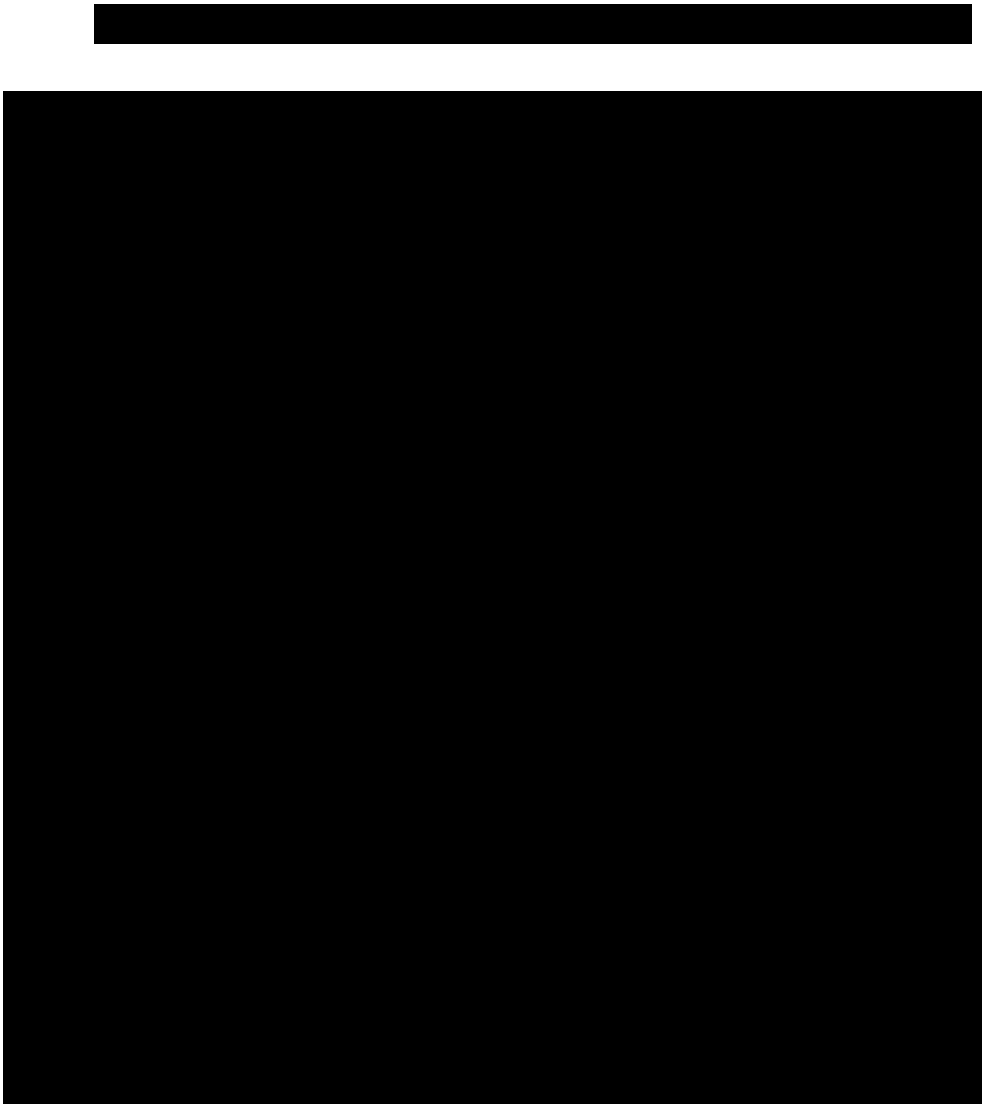

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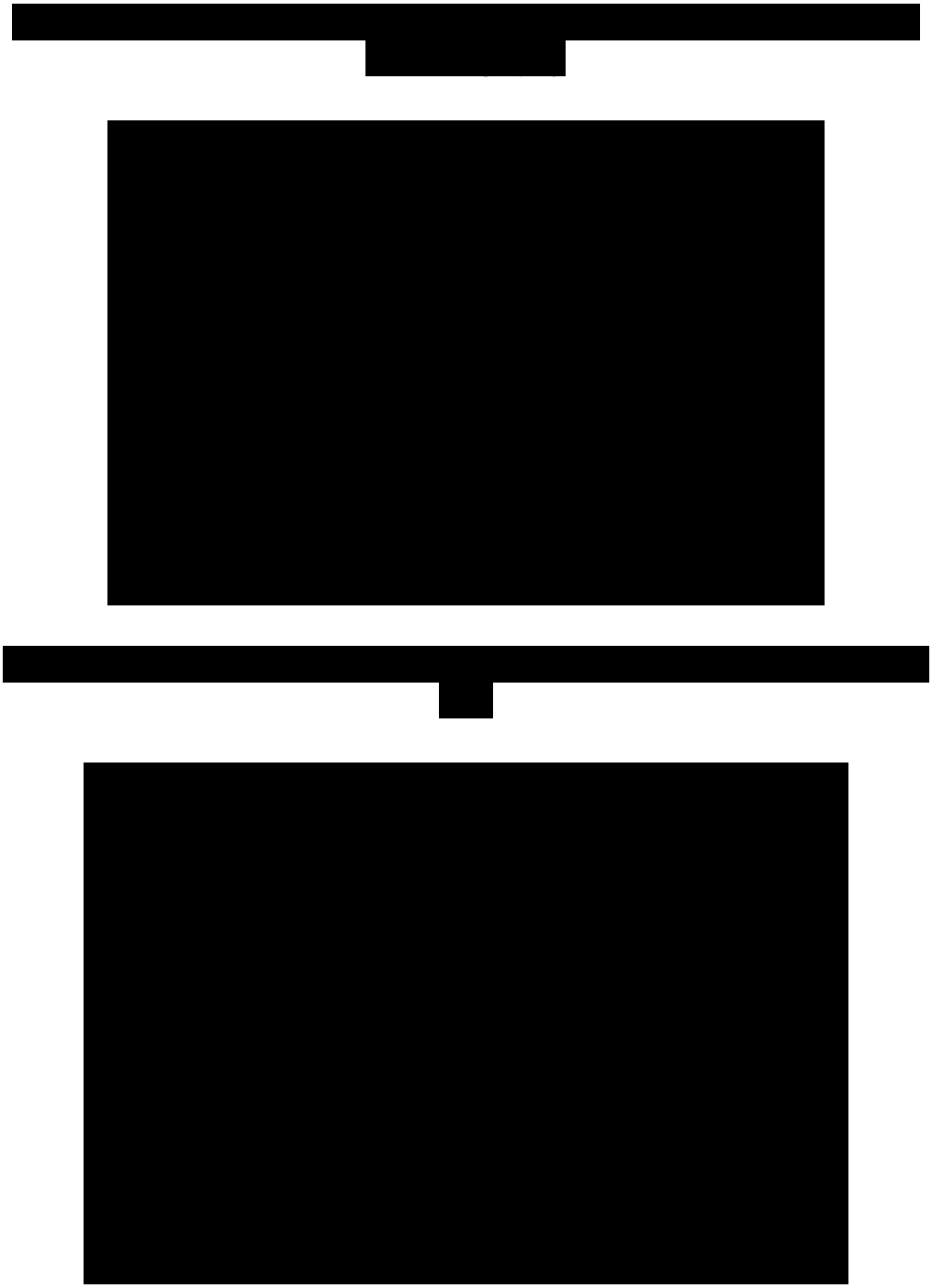

4.3 Fractionation and identification of peptides of interest in palatability enhancers

As shown in section 4.2.1, peptides from NEp and OEp seemed quantitatively and qualitatively different. Thus, the aim of this section was to complete Objective 2 by fractionating NEp and OEp using GFC in order to study those fractions showing the highest palatability-enhancing potential for cats. Selected key-tastants like amino acids and sodium were analysed in NEp and OEp fractions and peptides were identified using proteomic tools (see section 3.3).

4.3.1 Fractionation of NEp and OEp

Deproteinised extracts of NEp and OEp (F0 NEp and F0 OEp) were fractionated by gel filtration chromatography. Fractions of 5 mL were collected. The absorbance of each fraction was measured by UV spectrophotometry and data was recorded at 214, 254 and 280 nm. The elution profiles of the NEp and OEp deproteinised extracts are presented in Figure 19 and Figure 20, respectively. Similar GFC profiles have been reported for water-soluble raw pork meat extract [142].

Between the excluded volume (V_e) and the permeation volume (V_{perm}), the molecules were preferently eluted according to their molecular mass. The largest peptides were eluted earlier than the smallest. The exclusion volume of the column used was 5000 Da according to the specifications of the manufacturer (section 3.2.4).

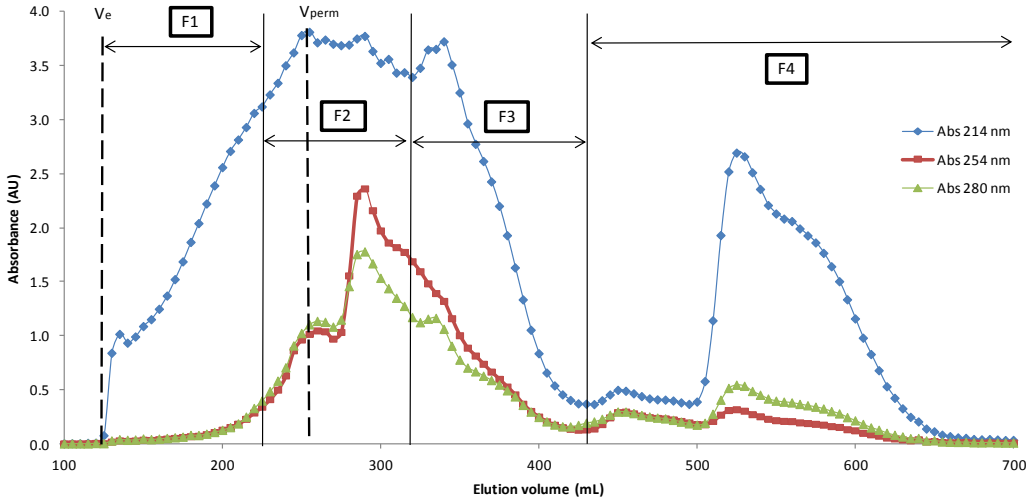

Figure 19. Elution profile of F0 NEp fractionation by GFC

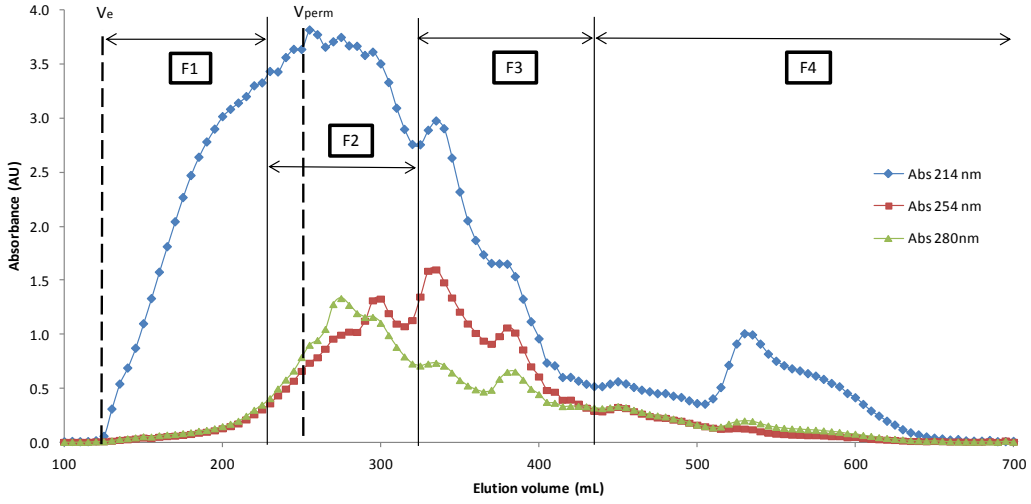

Figure 20. Elution profile of F0 OEp fractionation by GFC

Differences in the range of molecular mass were observed between samples from OEp and NEp. NEp presented a smaller amount of peptides than OEp in the initial fractions up to 225 mL of elution, corresponding to the peptides between 1 kDa and 2.5 kDa, as

shown by the lower intensity of the signal on the elution profile of these fractions. Nevertheless, after 225 mL of elution, the absorbance value corresponding to the product NEp was higher than that of OEp due to eluted peptides and free amino acids in the permeation volume (250 mL). The peptides which were eluted afterwards, were retained in the column by unspecific interactions due to aromatic amino acids like tyrosine, phenylalanine or tryptophan which were present either in peptides or as free amino acids. This absorbance could correspond to other compounds, like nucleotides that also contain aromatic moieties and absorb at the same wavelenght. In any case, the sample NEp was richer in this type of compounds than the sample OEp, especially in aromatic amino acids. These results agreed with results of section 4.2.1 ([redacted] and [redacted]). According to the elution profiles, fractions were pooled in 4 major fractions called F1, F2, F3 and F4 (corresponding to elution volumes from 120 mL to 225 mL; from 225 mL to 315 mL; from 315 mL to 405 mL and from 405 mL to 750 mL) for a separate study in both OEp and NEp products.

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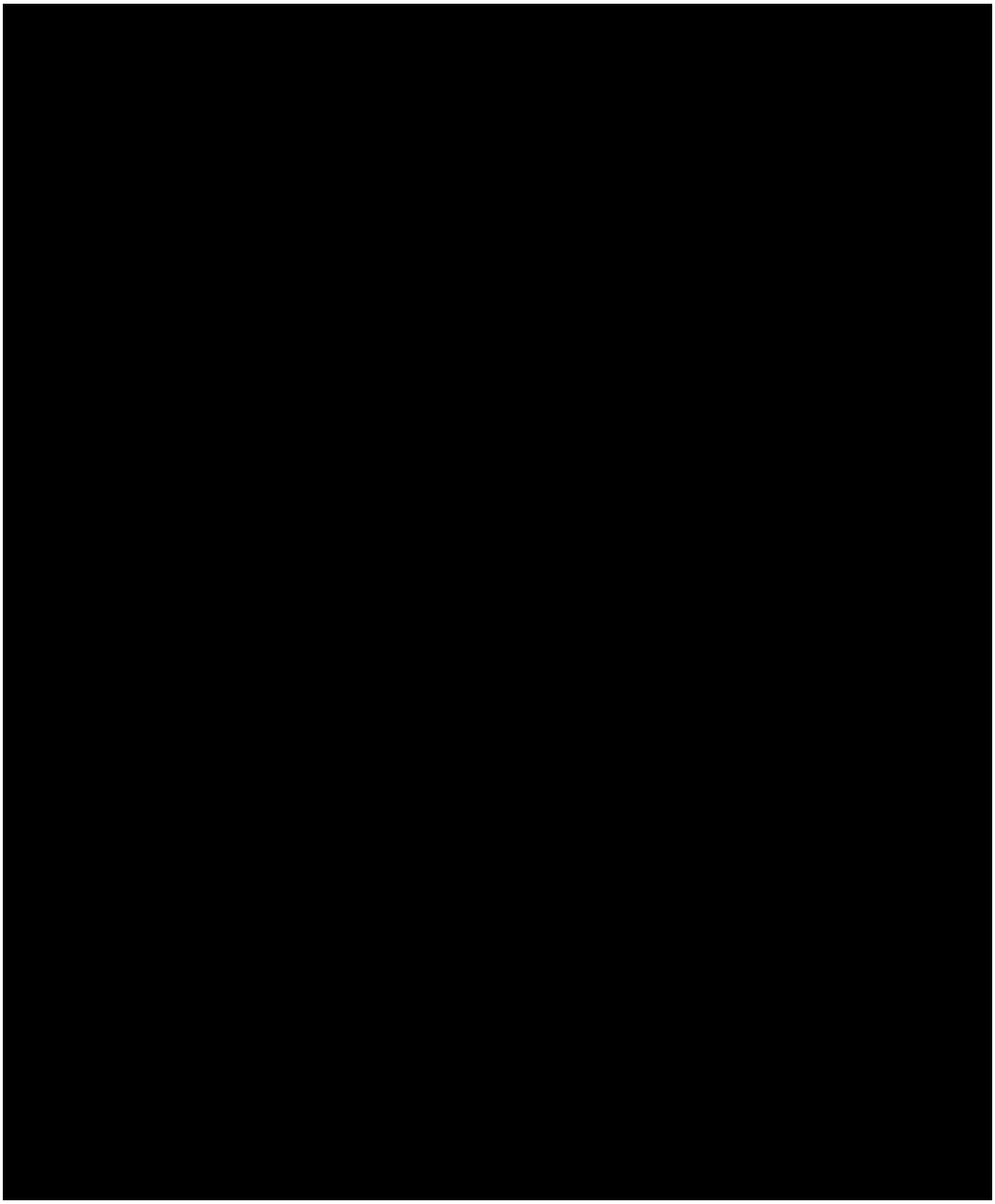

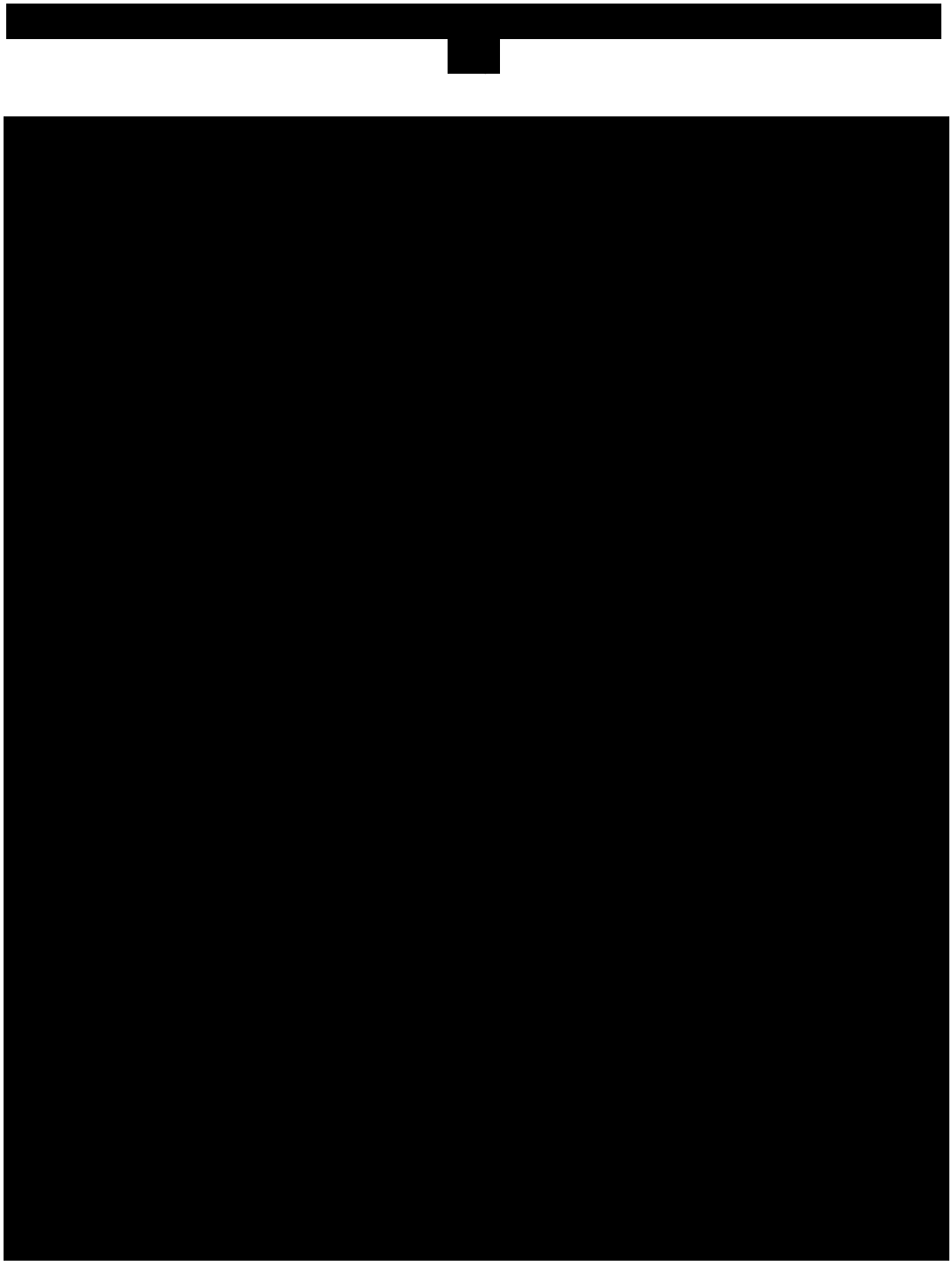

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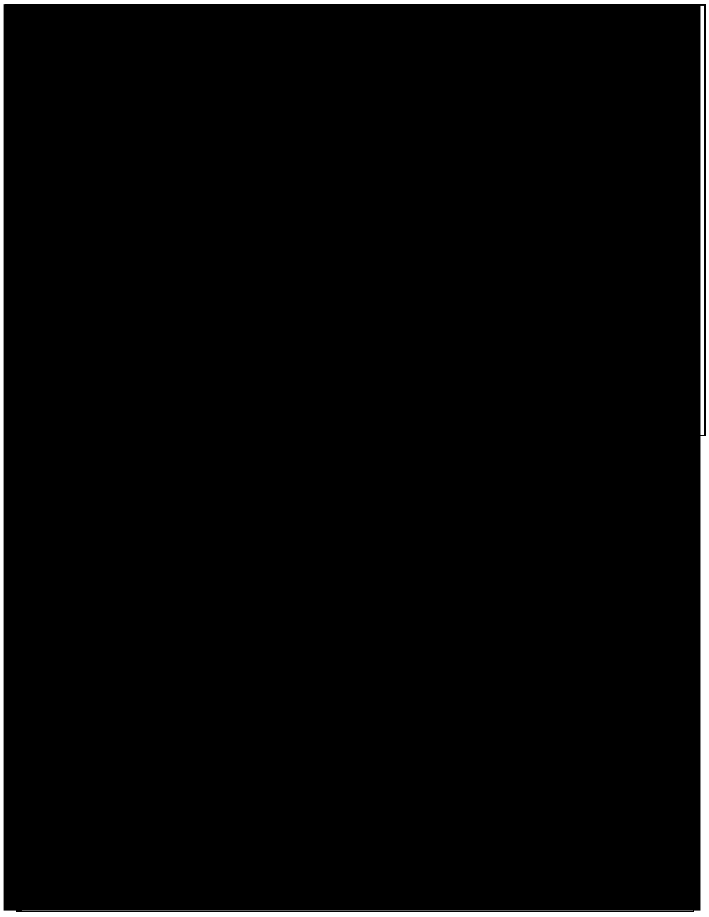

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4.4 Evaluation of fractions' palatability using a rat panel

The purpose of this section was to achieve Objective 3. Sensory analysis to evaluate the palatability of cat foodstuffs is generally performed using a trained-cat panel with a minimum of 30 cats to ensure statistical robustness. This type of analysis supposes very time-consuming training and replicate tests [125]. Moreover, this type of evaluation has been designed to measure palatability or taste quality as independant assays. Thus, the needed quantity of sample is preferably obtained at pilot-plan scale since preparation at laboratory scale would not provide enough quantity. In our study, the palatability and the taste quality of each fraction (F0, F1-4, F6) were evaluated by rats using the MOG technology allowing high efficient assays with a very low amount of samples (around

200 µL per well).

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CONCLUSIONS

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ANNEXES

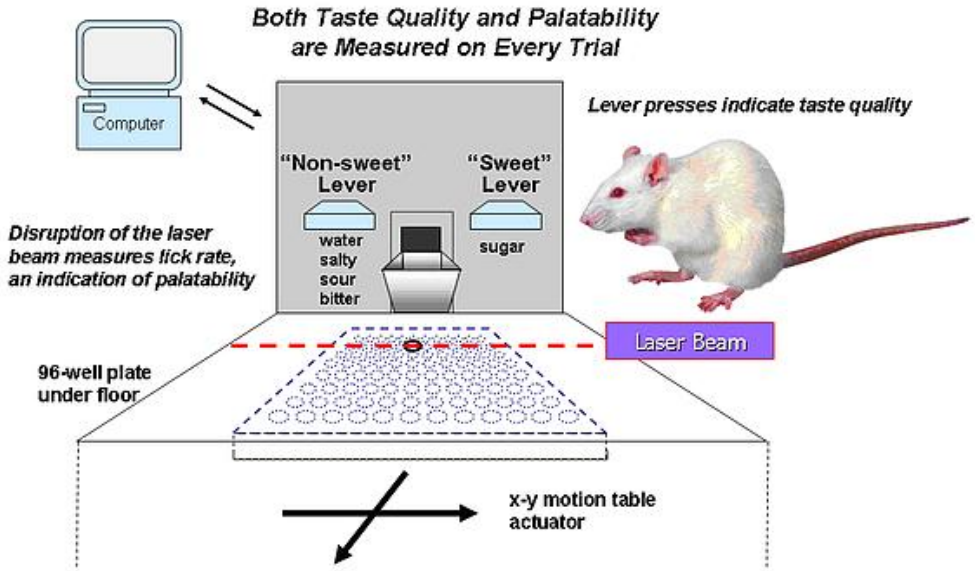
Annex 1. Details of the rMOG technology developed by Opertech Bio Inc.

All these details and more are available on http://www.opertechbio.com/#!rmog/lh5zf.

Taste quality measurement is achieved through the experimental paradigm of operant taste discrimination. Rats are trained to press two levers for a food pellet reward after they have tasted sample solutions presented to them in a 96-well plate. To receive the reward, the rats must press the right lever if the solution is a standard (for example, a sweet sugar solution) and the left lever if the solution presented has any other taste. By comparing the percentage of the presses on the right (standard) lever, the degree of similarity between a novel taste stimulus and the taste standard can be quantified.

Palatability of the sample solutions in the 96-well plate is determined by a laser beam counting the number of times a rat licks the sample. The more licks the more palatable.

Because of its ability to measure both taste quality and palatability in a high throughput capacity, the rMOG has proven particularly useful in the discovery of new flavor ingredients. MOG-trained rats are exceptionally efficient at screening large collections of natural products or other compounds for desirable taste properties. They also provide the ability to evaluate compounds not yet approved for use by humans



Schematic diagram depiction of the central components of the Microtiter Operant Gustometer (MOG), the first high throughput chemosensory system for in vivo testing.

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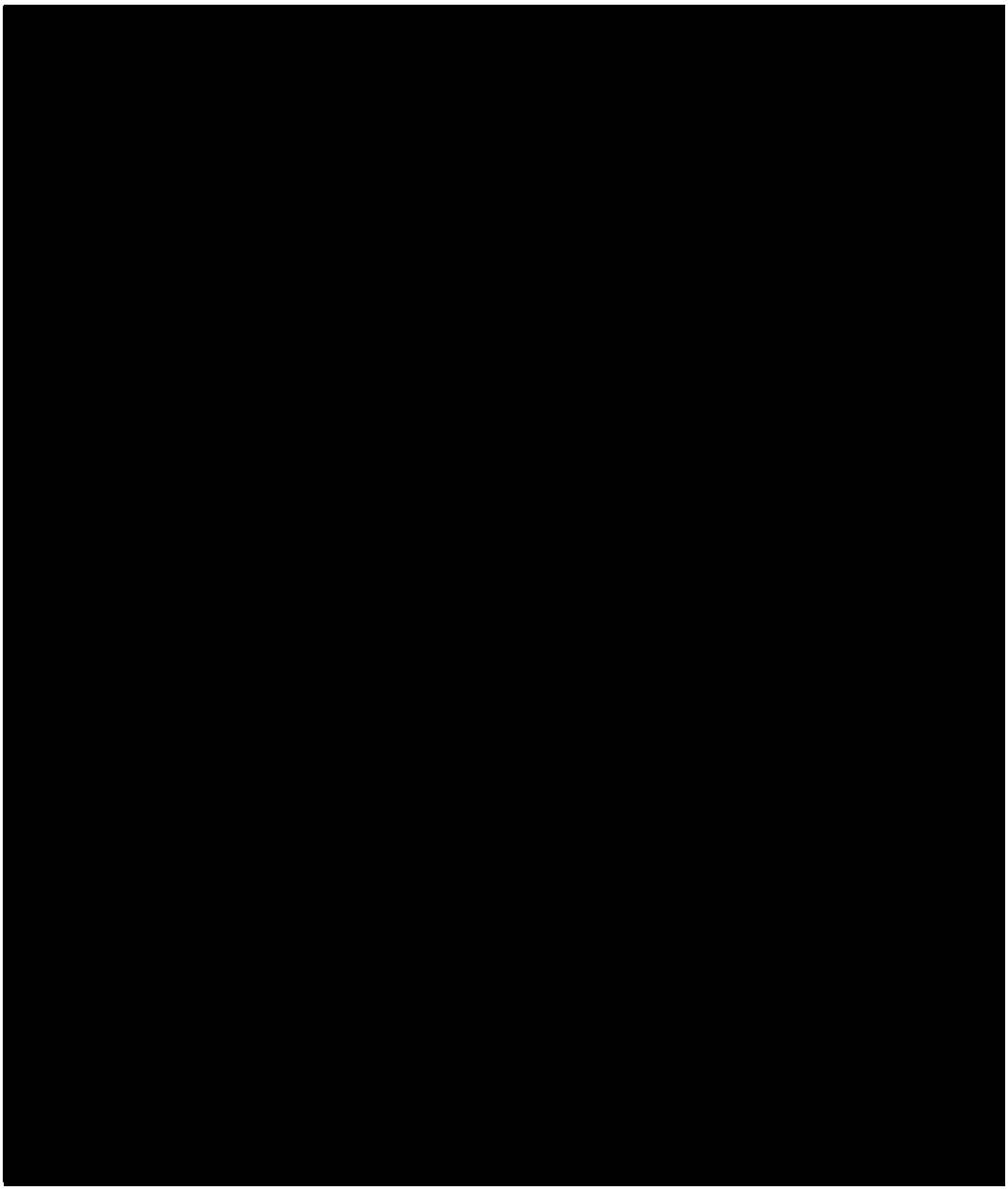

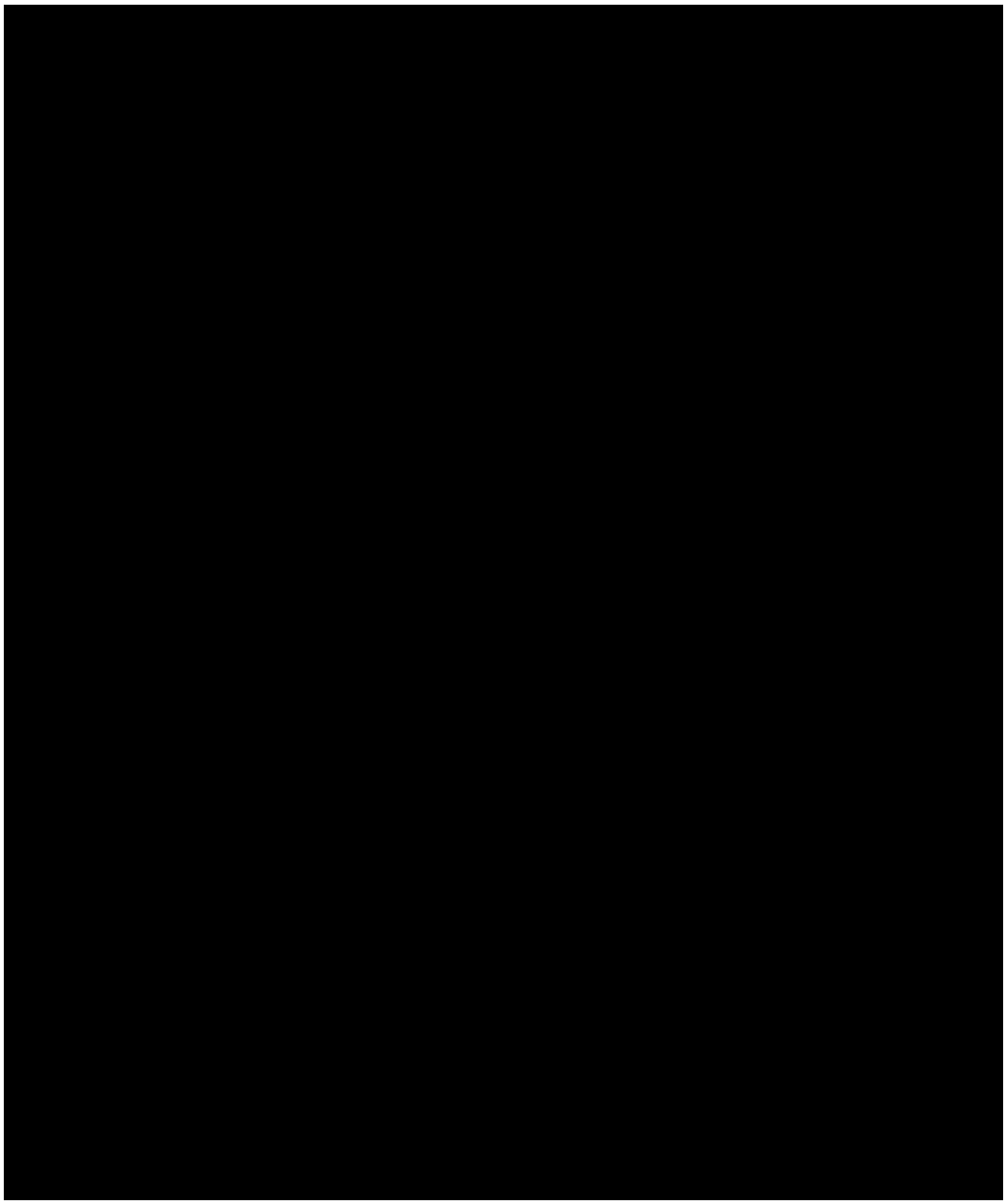

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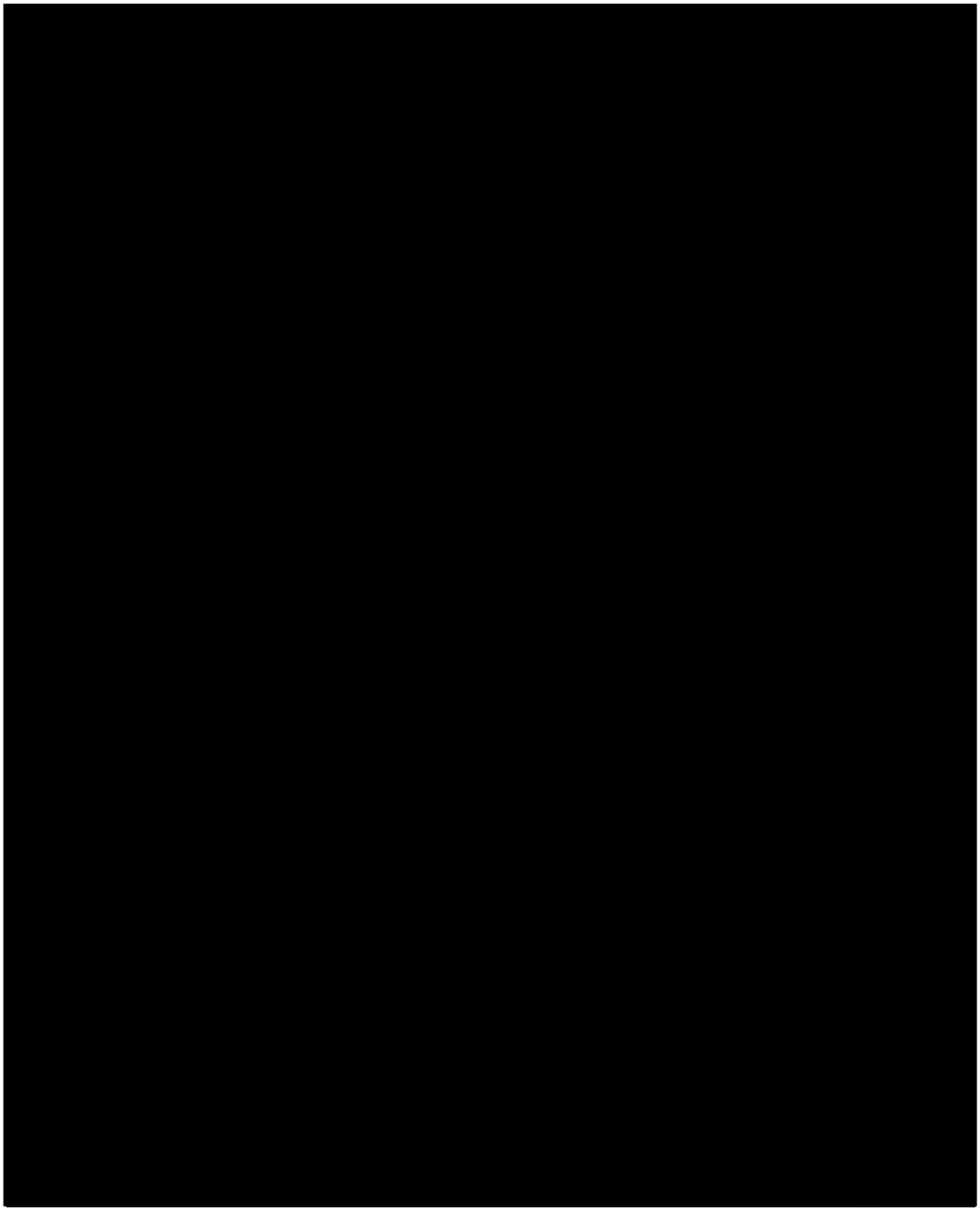

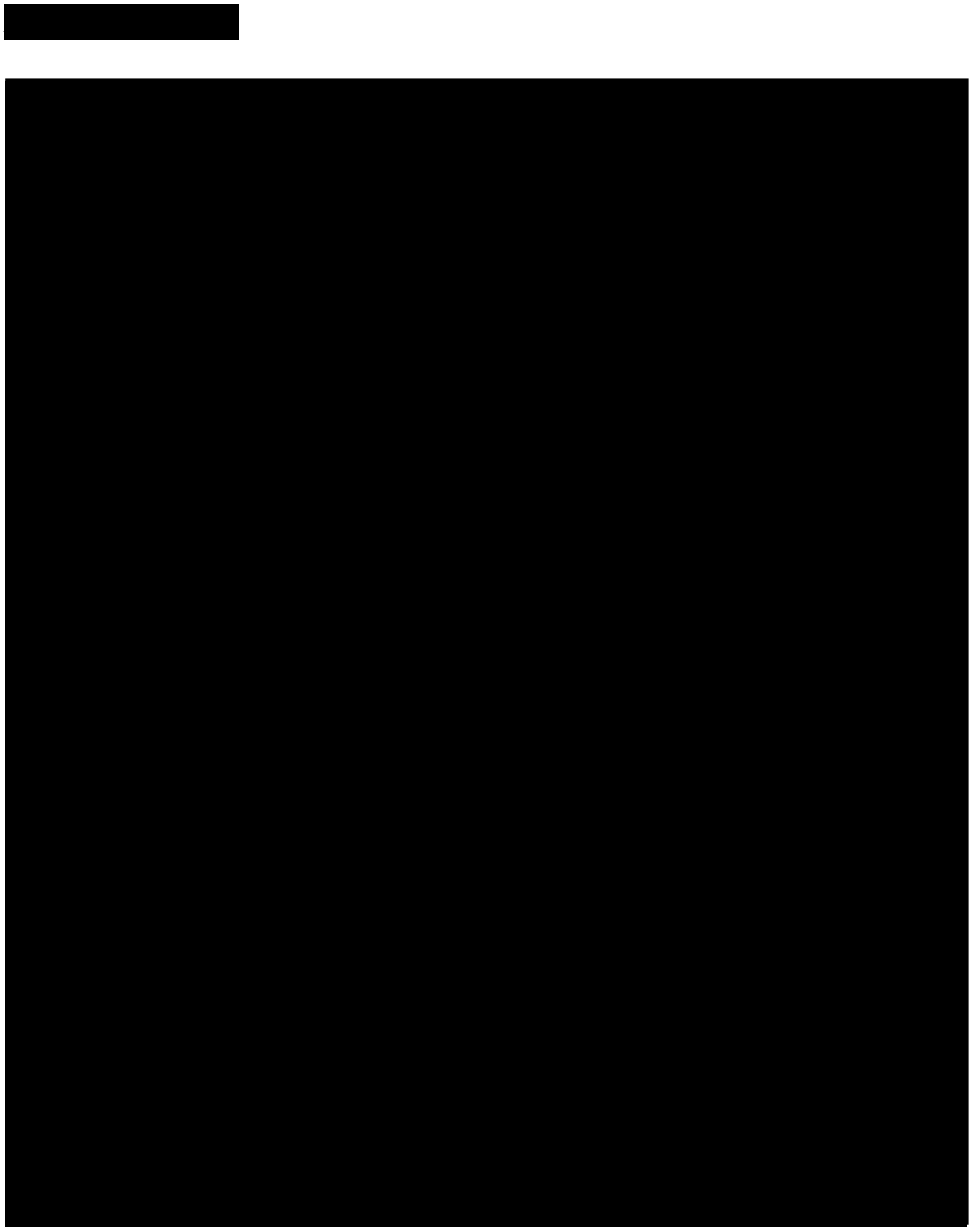

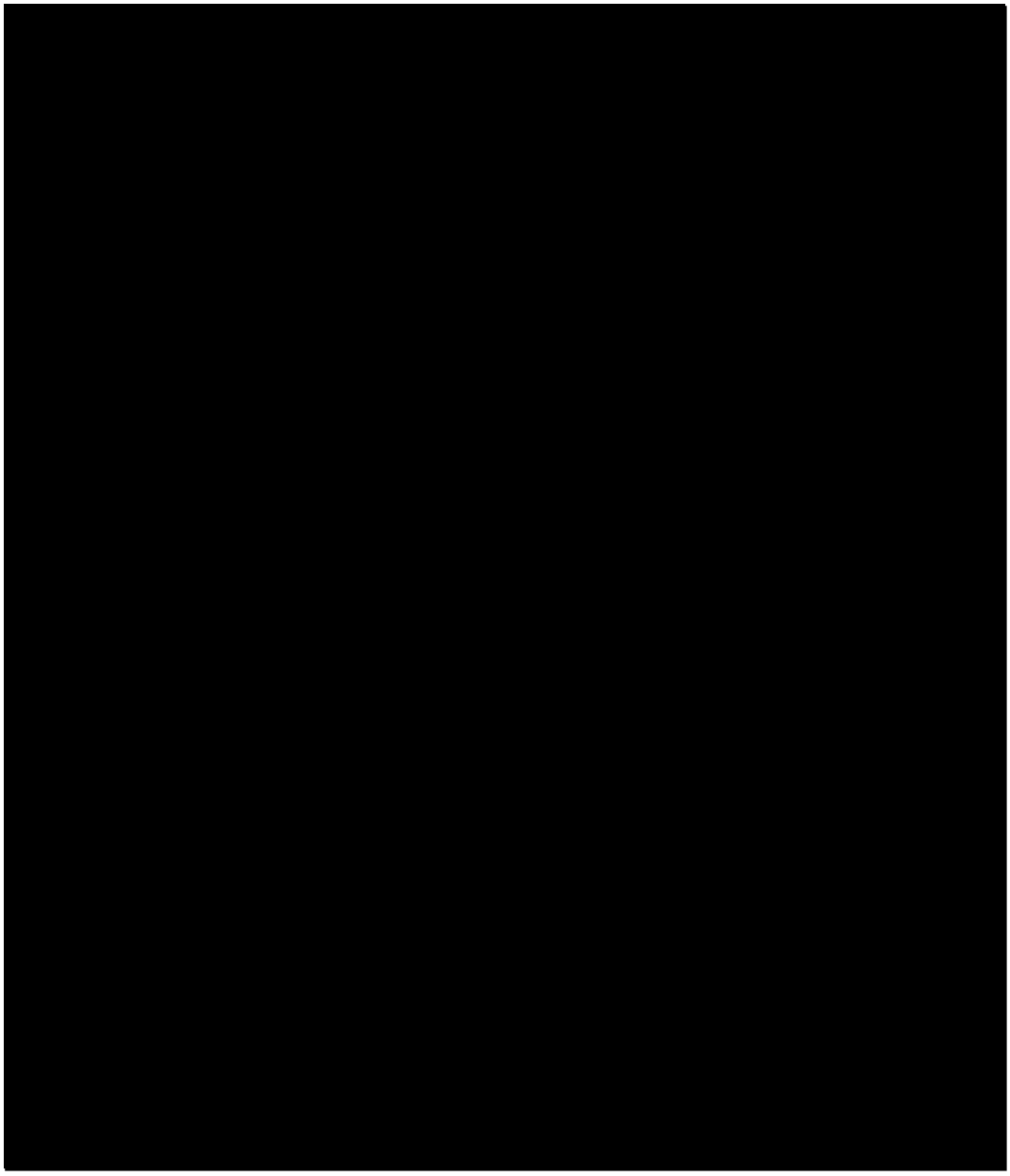

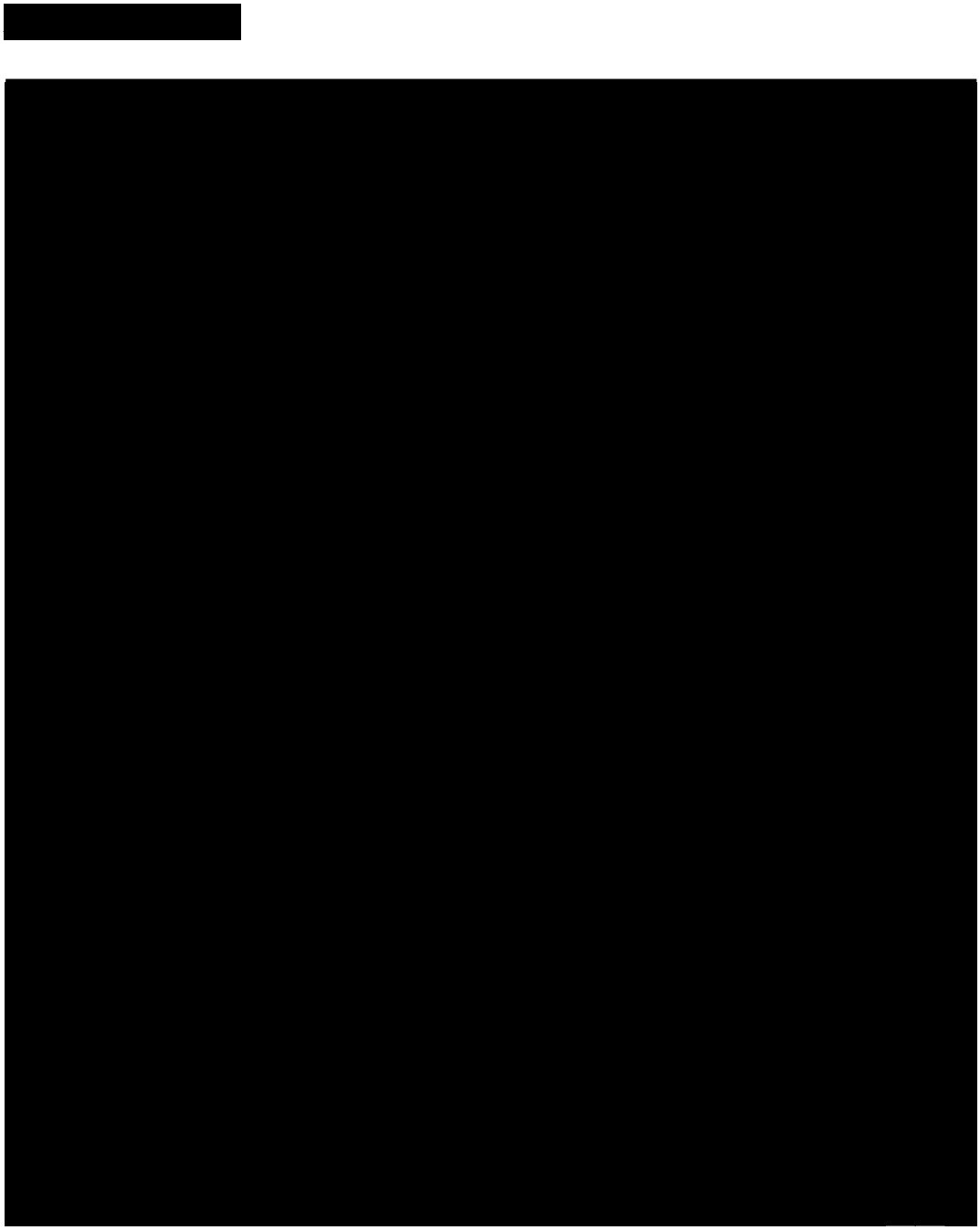

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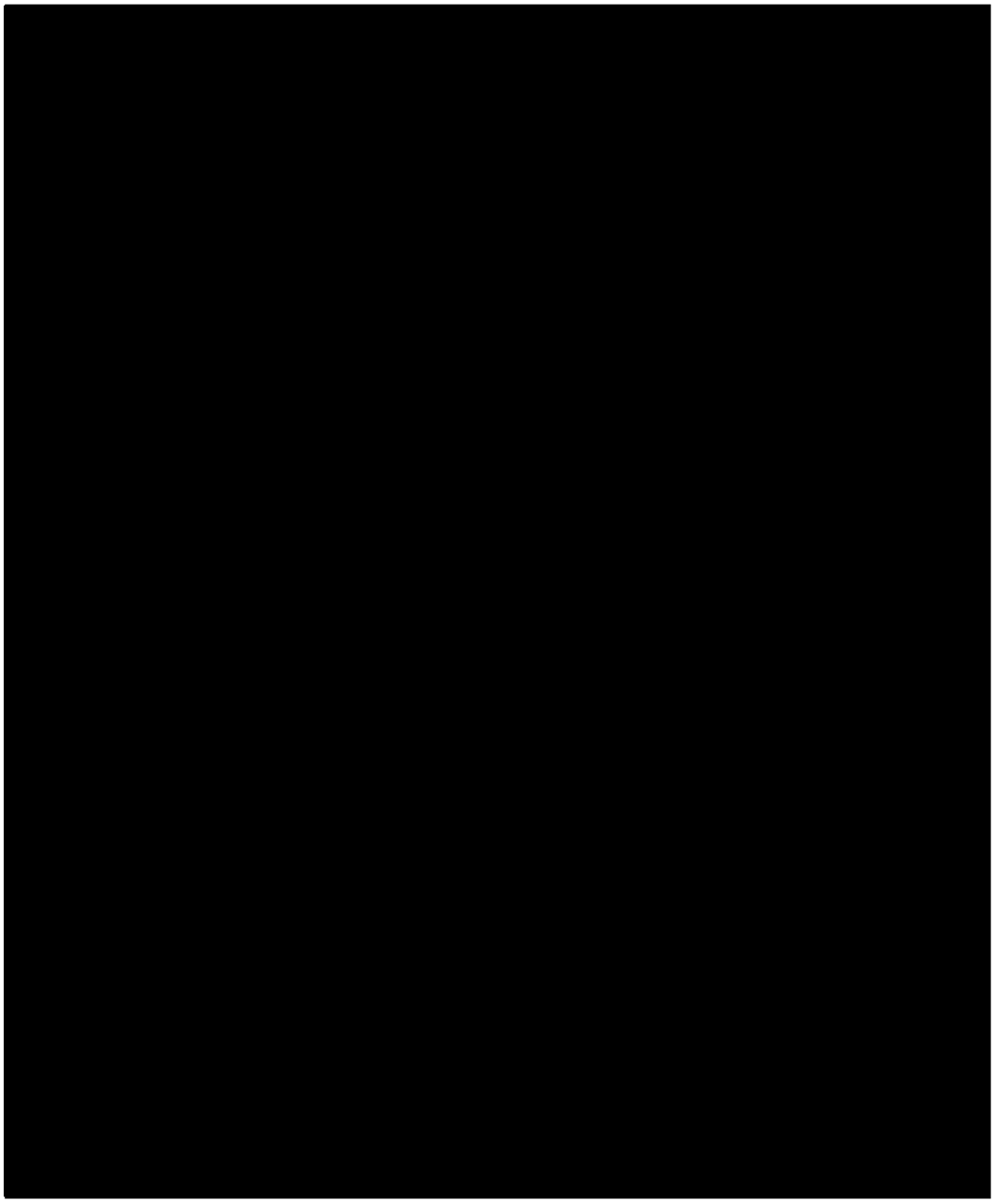

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