Final report

Reduction of vitamin A in liver for pet food applications

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Abstract

Liver is a highly palatable ingredient used in pet food formulations, but its inclusion level is constrained to prevent vitamin A toxicity in cats and dogs, in practice generally limited as < 5 - 10 wt% DM. This project investigates whether a simple non-polar liquid extraction of vitamin A from liver might address this issue while also increasing the utility and value of liver for the red meat industry.

The potential to extract vitamin A from liver using common food grade solvents (hexane, vegetable oil and tallow) was explored at laboratory scale. The results indicate that extraction of vitamin A is technically feasible, with proof-of-concept experiments indicating that the vitamin A content can be reduced by 45 - 64% for fresh liver and 68 - 91% for dehydrated liver on a dry matter basis. If similar reductions are achievable at a commercial scale this could enable cat and dog food formulations to implement quite high inclusion levels.

Executive summary

Background

Liver is a highly palatable and nutrient-dense organ used as an ingredient in pet food formulations, and abundantly produced by the meat industry. However, liver can only be included into pet foods in limited quantities owing to its high vitamin A (VA) content to prevent toxicity in cats and dogs. Currently, pet food manufacturers do not know the VA content of their liver ingredients as it varies with the age and diet of the animal, and different regional sources of liver may vary substantially. As a result, manufacturers must maintain a relatively low inclusion rate (e.g., generally recommended to be < 5 - 10 wt% DM of the diet) to ensure the consumption of the final product will not lead to VA toxicity.

To enhance the overall palatability and nutritive value of pet food and treats, it would be advantageous to include liver in higher proportions, without concomitant increase in total VA in the final formulation.

The project findings will be primarily of use to the red meat and pet food industry, specifically red meat processors, and ingredient and pet food manufacturers, to determine whether such a process warrants further investigation.

Objectives

This project set out to determine whether simple non-polar liquid extraction of vitamin A from liver using hexane, vegetable oil or tallow is technically feasible.

Methodology

- The non-polar solvents hexane, tallow and vegetable oil were used along with ground sheep liver (fresh and dehydrated) to test the extent of vitamin A extraction at mild temperature.
- Vitamin A and proximate composition were determined for the liver products pre- and post-extraction.

Results/key findings

All the tested solvents reduced the vitamin A content in both fresh and dehydrated liver. On a dry matter basis, 45 - 64% reduction in vitamin A was observed for fresh liver, and 68 - 91% reduction for dehydrated liver. These results reflect our provisional extraction conditions, relating to liver-to-solvent ratios, temperature, time, agitation and separation method. At this point it is not possible to identify the conditions that optimise efficiency and consistency, or that might apply to scaled-up processes.

The oily solvents were not completed separated post-extraction and some remained in the liver residue. This increases processing variability (particularly between solvents) and confounds calculations of true yield. In terms of mass, the results were:

- A post-extraction liver product prepared from fresh liver contained 267 – 409 mg/kg DM vitamin A (initially 741 mg/kg DM vitamin A).
- A post-extraction liver product prepared from dehydrated liver contained 43 – 163 mg/kg DM vitamin A (initially 502 mg/kg DM vitamin A).

Benefits to industry

The experimental results show that vitamin A can be extracted from fresh or dehydrated liver using animal or vegetable lipids, and centrifuged post-extraction to produce a proteinaceous liver fraction with at least 45 - 68% less vitamin A (on a dry matter basis) and a recovered lipid fraction containing 1.8 – 23 mg/L vitamin A. The wide range is a consequence of the solvents and conditions used in this proof-of-concept project, as well as the initial liver vitamin A concentrations that varied by up to two-fold.

Current maximum levels for vitamin A in dog and cat food formulations are 75 and 99 mg/kg DM respectively, and this has limited the usefulness of liver as an ingredient. Based on the average results observed here in this proof of concept, and if similar reductions can be obtained through a commercial scale process, pet food formulations might be able to contain a post-extraction liver ingredient at quite high inclusion levels, assuming no other ingredients are contributing to vitamin A in the food formulation.

Based on the project results:

- A post-extraction liver product prepared from *fresh* liver could be incorporated in pet food formulations at 18 - 28% DM for dogs, and 24 - 37% DM for cats, before exceeding the maximum allowable value for vitamin A.
- A post-extraction liver product prepared from *dehydrated* liver could be incorporated in pet food formulations at at least 46% DM for dogs, and at least 61% DM for cats, before exceeding the maximum allowable value for vitamin A.

The ability to realise maximum inclusion rates will depend on industry users knowing the starting concentration of vitamin A in the liver and final values obtained. On-line quantitation of vitamin A is unlikely to be achievable in the near-term, therefore in practice a wide margin of error may still need to be applied to ensure pet safety. As liver becomes more frequently utilised and its variability measured, and the extracted products become optimised, the confidence in inclusions rates will improve.

Future research and recommendations

Further research could optimise liver preparation and extraction conditions, assess the quality and safety of the produced materials and refine the business case for the technique. This may include:

- Optimisation of the time-temperature treatment and agitation method for an appropriate set of edible oils to be used as non-polar solvents
- Establishment of a consistent extraction process that delivers known upper values of vitamin A in the final product irrespective of starting concentrations in the raw material, which may require a polishing stage to achieve
- Assessment of the quality and techno-functional properties of the liver protein fraction (e.g., gel strength, water/oil retention capacity and level of protein denaturation) and lipid fraction (e.g., peroxide value)
- Determination of recovery and extraction efficacy through pilot-scale trials
- Technoeconomic assessment to determine the viability of an industrial process at a range of commercial production capacities

- Assessment of protein and lipid digestibility, or other health benefits of the products for specific applications
- Characterisation of the nutritional profile of the recovered liver protein and lipid fractions
- Assessment of the palatability of both the liver protein and lipid fractions

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Background

Liver is a highly palatable and nutrient-dense organ used as an ingredient in pet food formulations, and abundantly produced by the meat industry. However, liver can only be included into pet foods in limited quantities owing to its high vitamin A (VA) content to prevent toxicity in cats and dogs. To enhance the overall palatability and nutritive value of pet food and treats, it would be advantageous to include liver in higher proportions, without concomitant increase in total VA in the final formulation.

Vitamin A is a lipid soluble vitamin present in the liver of all vertebrate animals. Its safe upper limits in pet foods stipulated by the Association of American Feed Control Officials for adult cats and dogs are 250,000 IU[*]/kg DM[†] (Dog) and 333,300 IU/kg DM (Cat) (The Association of American Feed Control Officials, 2020). Industry food composition data reports cattle and lamb liver sourced from Australia and New Zealand contains between 136,000 – 314,000 µg VA per kilogram (i.e., ~453,000 – 1,047,000 IU/kg) (Food Standards Australia New Zealand, 2014; The New Zealand Institute for Plant and Food Research Limited & Ministry of Health, 2018a, 2018b; U.S. Department of Agriculture, 2019a, 2019b). After accounting for liver's moisture content[‡], this equates to 1,510,000 – 3,490,000 IU/kg DM and would therefore limit the inclusion level in a diet to a maximum safe upper level of 7 – 17 wt% for the dog, and 10 – 22 wt% for the cat (provided no other ingredients were contributing VA).

Currently, pet food manufacturers do not know the VA content of their liver ingredients as it varies with the age and diet of the animal, and different regional sources of liver may vary substantially. As a result, manufacturers must maintain a relatively low inclusion rate (e.g., generally recommended to be < 5 – 10 wt% DM of the diet) to ensure the consumption of the final product will not lead to VA toxicity. In order to increase inclusion rates of liver in pet foods and realise the benefits (e.g., increased palatability and nutritive value), the VA content must be substantially reduced.

Industrial processes for defatting liver which concurrently extract VA are well established in the fisheries industry (Butler, 1948). These processes include high temperature rendering, enzymatic and alkali digestion methods and solvent extraction techniques, carried out at relatively high temperatures (>70 ^{o}C) and optimised for producing high-quality VA rich oils (e.g., cod liver oil), with the remaining protein dried (e.g., fish liver meal). The choice of method for producing fish liver oil depends on both the lipid and VA contents, which vary between species (Butler, 1948). For fish species with very high liver lipid content (e.g., cod, 20 – 60 wt%), the lipid and VA can be easily liberated by simple mechanical or thermal disruption of the cell membranes, although for species with higher VA content, more elaborate methods may be used to enhance VA recovery. By contrast, for fish species whose liver contain low (e.g., halibut, 8 – 21 wt%), or very low lipid content (e.g., salmon, 4 – 8 wt%), digestion of the diced or comminuted liver using acid, alkali or enzymes is required, or the use of lipid extraction techniques using non-polar solvents (Butler, 1948). In all these cases, relatively high temperatures are employed (< 70 – 100 ^{o}C) to partially denature or hydrolyse the liver proteins to maximise the liberation of lipid and recovery of VA.

From this, it can be speculated that simple particle size reduction-temperature combinations alone won't be sufficient for recovering the lipid fraction from cattle or sheep liver, which have a significantly lower lipid content (e.g., <10 wt%) than that of oily fish like cod. Digestion using acid,

[*] Vitamin A content is often expressed in International Units (IU), 1 IU is equivalent to 0.3 µg of vitamin A.
[†] Dry matter (DM).
[‡] Moisture content of liver is ~70 wt%.

alkali or enzyme at elevated temperature to help liberate tightly bound lipid could be technically feasible. However, it may lead to deterioration of the protein quality through thermal denaturation and hydrolysis, an undesirable outcome for pet food producers in premium product categories, where high palatability, digestibility and minimal processing (e.g., raw or low temperature cooking) ingredient labelling are expected. As such, in terms of technical feasibility, precedent and alignment with customer values, a solvent extraction approach using edible oils or pet food safe solvent carried out at milder temperatures may be the best strategy to explore for reducing VA in cattle and sheep liver.

To date there are no available reports which outline VA being extracted from terrestrial livestock liver for its later inclusion in pet food products. The purpose of this project is to address this gap by undertaking a proof-of-concept project to determine whether VA can be readily extracted from raw fresh or dehydrated liver through solvent extraction at mild temperatures, and physically separated to recover the proteinaceous and lipid phases. The project will trial solvents which should be acceptable in a manufacturing setting (e.g., hexane, tallow and vegetable oil), using sheep liver as a starting material. The efficacy of the solvents for reducing VA will be determined by measuring the vitamin A content in the recovered solvent (supernatant) and quantifying the VA and proximate composition of the remaining liver protein fraction (residue).

These project findings will provide preliminary evidence towards understanding the feasibility of this innovation, and used to support any rationale for continued investment by the red meat industry and pet food sector.

1. Objectives

The objective of this proof-of-concept project is to determine whether VA in the liver from livestock can be readily reduced through non-polar solvent extraction. The project objective was successfully completed.

2. Methodology

2.1 Materials

Frozen lamb liver was obtained from a central North Island New Zealand meat processor. The solvents were laboratory grade hexane, commercial retail soy oil, and sanitised edible beef tallow from Quality Approved Suppliers (Timaru, New Zealand). The liver and tallow were stored at -20°C until used.

2.1.1 Materials preparation for selecting a solid-to-solvent ratio

Livers from three individuals were thawed at 4°C overnight. Once thawed, each was separately diced and then minced, and stored chilled at 4°C until used (**Figure 1**). These materials are described as 'fresh' liver.

Figure 1. Photos of diced liver and minced liver immediately after processing (top) and after sitting at room temperature for 10 minutes (bottom).

Beef tallow was prepared in two formats: native intact ('Tallow') and a lower melting point fraction of tallow ('Low-tallow'). Intact tallow fully melts at 55 °C. To collect Low-tallow, tallow was liquified by microwave heating, then held in a water bath set to 45°C until its temperature stabilised. The still-liquid portion was decanted off of the solids for use in subsequent extraction experiments.

2.1.2 Materials preparation for vitamin A extraction efficacy

Thawed livers from three new individuals were cut into two pieces each of approximately 430 grams. One piece was prepared as above for fresh liver. The remaining piece was sliced into thin sections and dehydrated gently overnight at 65°C, with a moisture loss of 67% (from 430 to 142 grams). This dehydrated liver was then finely ground using a coffee grinder (**Figure 2**).

Figure 2. Photos of thinly sliced and chopped liver in the dehydrator (left) and dehydrated liver ground to a fine powder (right).

2.2 Selecting a solid-to-solvent ratio

Selection of an appropriate solid-to-solvent ratio followed a factorial design using the solvents hexane, tallow and soy oil each at 1:2, 1:3, and 1:5 (minced fresh liver:solvent, w/v). The aim was to determine suitable ratios for each solvent type to aid both physical separation of the solvent from the solid (lamb liver), as well as vitamin A reduction. Treatments were evaluated on their ability to form a decantable liquid phase, the amount of solvent retained in the liver residue, accumulation of vitamin A in the solvent, and reduction of vitamin A content in the liver residue.

For the purpose of the ratio experiments, individual livers were not tested separately. Instead, a homogenate was prepared from an equal blend of their minced material and which was thoroughly mixed by hand. For each factorial iteration (done in duplicate), 5 g of homogenate was weighed into a 50 mL tube and solvent added at the required ratio. The mixtures were incubated for 60 minutes with continuous shaking at 150 rpm. Temperature was maintained at 55°C for the soy oil and tallow treatments and at room temperature for hexane treatments. Following incubation, hot water at 50°C was added to the soy oil and tallow tubes (not to hexane) to rinse and separate the oily and solid phases, this was done as a single step prior to centrifugation. All tubes were centrifuged at 2,000 × *g* for 10 minutes at 25°C and their resulting supernatants decanted off into fresh tubes. The solid liver residue (‘the pellet’) from hexane-treated samples (not soy oil and tallow) were kept in a fume hood for 1 hour at 25°C to allow some of the entrapped solvent to volatilise. Finally, all extracted liver residues were stored until tested for their vitamin A content.

2.3 Vitamin A extraction efficacy

To determine the relative efficacy of each solvent for reducing vitamin A in fresh and dehydrated liver, three independent trials (one per liver) were carried out in duplicate for each solvent type (**Figure 3**).

For fresh liver samples, 25 grams was weighed into a 300 mL pottle for the extraction followed by the addition of 75 mL of solvent. Note that increased volumes were used here compared to those in *Section 2.2 Selecting a solid-to-solvent ratio*. For the dehydrated liver powder, 15 grams was used with the addition of 45 mL of solvent.

For the hexane and soy oil treatments, the mixtures were incubated at room temperature with continuous stirring by a magnetic bar. The tallow treatments made use of native intact tallow or its

lower melting fraction ('low-tallow'). These were incubated in water baths at 55°C and 45°C respectively, with continuous shaking at 150 rpm. All incubations lasted 60 minutes, during which the samples were covered to protect them from light exposure.

Following incubation, hot water at 50°C was added into the soy oil and tallow tubes (not to hexane) to rinse and separate the oil and solid residue phases, this was done as a single step prior to centrifugation. For fresh and dehydrated samples, 10 mL and 5 mL of water was added, respectively. The entire mixture was transferred to a centrifuge tube, then immediately centrifuged at 2,000 × g for 10 minutes at 25°C. The resulting supernatants were carefully decanted into fresh tubes. The solid residues from hexane-treated samples were kept in the fume hood at 25°C for 1-2 hours to allow the residual solvent to volatilise. Samples of the unextracted fresh and dehydrated liver and the post-extraction residues were measured for their proximate composition and vitamin A content. The vitamin A concentration in the supernatant was also measured.

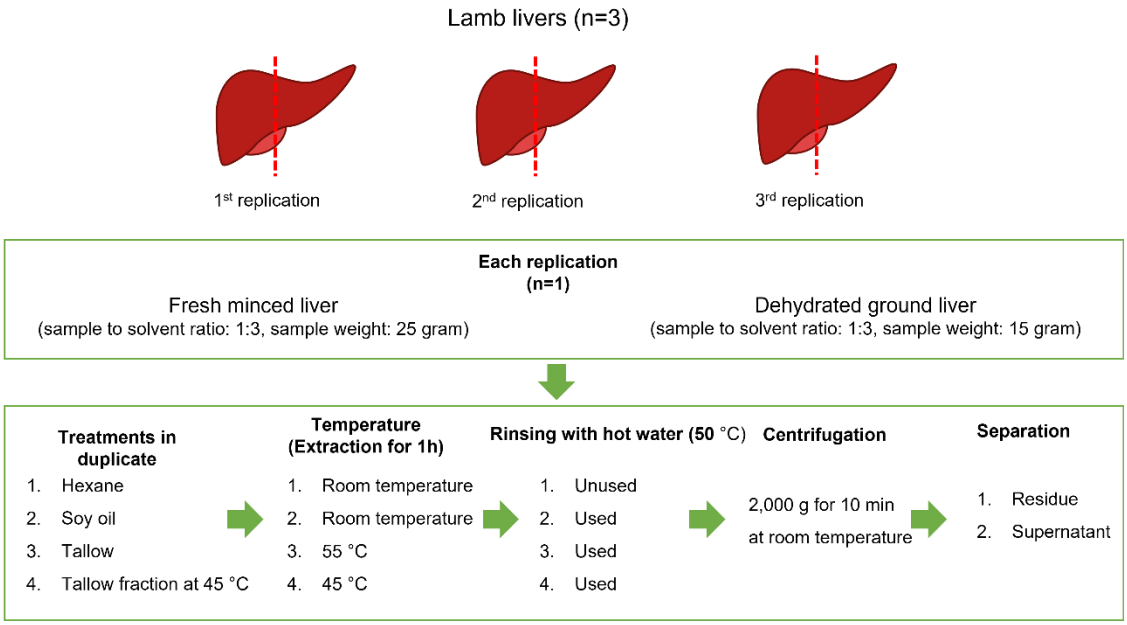

Figure 3. Schematic diagram of the experimental design.

2.4 Yield calculations

Yields were calculated as weight of residue or volume of solvent before and after extraction.

$$\%\ recovery\ residue = \frac{\text{mass of liver residue after extraction}}{\text{initial mass of liver}} \times 100$$

$$\%\ recovery\ supernatant = \frac{\text{volume of solvent supernatant after extraction}}{\text{initial volume of solvent}} \times 100$$

Note: The initial solvent volume includes both solvents and water, such as 45 mL solvent and 5 mL water for 15 g dehydrated sample and 75 mL solvent and 10 mL water for 25 g fresh minced sample.

2.5 Chemical analysis

2.5.1 Proximate composition

Proximate composition was determined through an IANZ accredited laboratory, measured using the following methods: moisture and ash were determined using AOAC methods AOAC 930.15/925.10/942.05, crude protein was measured using AOAC method 968.06 (Dumas method), and fat was determined using AOAC method 991.36 (Soxhlet method) for fresh liver products and AOAC method 922.06 (Mojonnier method) for dehydrated liver products. A portion of each liver and post-extracted liver residues were assessed for proximate composition.

2.5.2 Vitamin A analysis

Vitamin A was measured by liquid chromatography with diode array detection. Two variations of the method were used, the first for experiments in Section 3.1 and the second, which had better quantitation, for experiments in Section 3.2.

For *Section 3.1 Selecting a solid-to-solvent ratio*, to assess liver, liver residues and tallow, 0.5 g of sample was weighed into a 50 mL plastic centrifuge tube. The sample was homogenised with 16 mL of 2% Pyrogallol in ethanol using an Ultra-Turrax at the lowest setting for about 20 seconds, followed by the addition of 8 mL of 50% potassium hydroxide solution, then flushed with nitrogen before sealing. The tubes were covered with aluminium foil and shaken at 200 rpm overnight. To the mixture, 30 mL of 0.01% BHT in hexane was added and the tube centrifuged at 3,000 rpm for 5 minutes. The hexane supernatant was transferred into a glass sample tube and evaporated to dryness with nitrogen at 40°C. The residue was then redissolved in 500 µL of methanol and transferred to an amber vial for HPLC analysis.

The chromatography system was an HPLC (Shimadzu LC-30, Shimadzu Corporation, Kyoto, Japan) equipped with a ThermoFisher Accucore C30 column and a Diode-Array detector (DAD). The isocratic elution was carried out using 90% methanol: 10% water (mobile phase A) and methyl tert-butyl ether (mobile phase B), with a flow rate of 0.3 mL/minute and 8% mobile phase B. Retinol (CAS No: 68-26-8, Sigma) detected by absorbance at 325 nm was used as the standard.

For Section *3.2 Vitamin A extraction efficacy*, a method was adapted from Hosotani and Kitagawa (2003) (Hosotani & Kitagawa, 2003). For liver and liver residues, 1 g of material was homogenised with 10 mL of water using an Ultra-Turrax, and 0.3 mL of the homogenate was then mixed with 0.5 mL of 25% sodium ascorbate, 0.7 mL of water and 2.0 mL of ethanol. The homogenate was saponified with 1 mL 50% KOH and heated at 70 °C for 30 minutes. After cooling, 4.0 mL of hexane was added to the mixture. The tubes were covered with aluminium foil and shaken for 10 min in a flat-bed shaker at 200 rpm. The mixture was then centrifuged at 3,000 rpm for 5 minutes. The hexane supernatant was transferred into an amber glass sample tube and evaporated to dryness using nitrogen at 40°C. The residue was redissolved in 500 µL of methanol and transferred to an amber vial for HPLC analysis. Alternatively, supernatant samples were directly analysed without the homogenization step, where 100 µL of the sample was used for the extraction process.

As before, the HPLC system used a ThermoFisher Accucore C30 column and a DAD. The isocratic elution was carried out using methanol:water and methyl-tert-butyl ether as the mobile phase, with a flow rate of 0.3 mL/min. Retinol was the standard.

2.6 Percentage vitamin A reduction calculation

The percentage reduction for vitamin A was determined for fresh or dehydrated liver on an 'as is' or 'dry matter' basis, as follows.

$$\%\ reduction = \frac{\text{[Vit A] unextracted liver - [Vit A] extracted sample}}{\text{[Vit A] unextracted liver}} \times 100$$

$$\%\ reduction\ (DM\ basis) = \frac{\text{[Vit A] unextracted liver, DM basis - [Vit A] extracted sample, DM basis}}{\text{[Vit A] unextracted liver, DM basis}} \times 100$$

3. Results

3.1 Selecting a solid-to-solvent ratio

3.1.1 Chemical composition

The proximate composition of three lamb livers prior to combining into a homogenate is shown in **Table 1**. Variability among them is low, which is to be expected for animals of similar livestock class and age. The results are comparable with the proximate composition of lamb liver reported in the Australian and New Zealand food composition databases (Food Standards Australia New Zealand, 2014; The New Zealand Institute for Plant and Food Research Limited et al., 2018a).

Table 1. Composition of three fresh lamb livers (wt %).

	Moisture	Ash	Crude protein	Fat
Liver 1	71.9	1.5	22.0	0.7
Liver 2	71.3	1.5	19.7	1.0
Liver 3	70.2	1.4	22.3	0.7
mean ± SD	71.1 ± 0.9	1.5 ± 0.1	21.3 ± 1.4	0.8 ± 0.2

The vitamin A content of the three livers and three sub-samples of bulk tallow is shown in **Table 2**. For liver, the average concentration was 139±74 mg/kg with large variability occurring between livers. This is not unexpected, as liver vitamin A content reflects an individual animal's diet and age, and it is possible these lambs came from different farms or feed management schemes. In a survey conducted by Scotter *et al* (1992), the vitamin A concentrations of lamb livers were assessed across multiple years, including 1960, 1978, 1982, and 1990. The study reported a range of vitamin A concentrations, with a minimum value of 30 mg/kg and a maximum value of 1,100 mg/kg (Scotter, Thorpe, Reynolds, Wilson, & Lewis, 1992). The average vitamin A concentration for the retail samples was found to be 310 mg/kg. The results here are in line with what is typically seen in New Zealand reared sheep grazing green pasture. The beef tallow, on the other hand, had very little vitamin A content, which is ideal for a starting solvent.

Table 2. Vitamin A concentration in three fresh lamb livers prior to combining into a homogenate and three subsamples of beef tallow (mg/kg).

	Vitamin A	
	Lamb livers	Tallow
Sample 1	106	0.27
Sample 2	173	0.24
Sample 3	254	0.22
mean ± SD	139 ± 74	0.25 ± 0.02

3.1.2 Visual inspection

All three solvents used are non-polar and will extract the non-polar and lipidic constituents of liver to varying degrees. Compounds like vitamin A and other carotenoids tend to be yellow, orange and red, and upon extraction it was observed that each solvent differed in its accumulation of yellow-coloured pigments. For example, after hexane extraction at room temperature the colour of the supernatant had changed very little (**Figure 4)**. By contrast, the supernatant from the soy oil treatment at 55 °C appeared richly yellow and also cloudy, indicating a potential alteration in its physical properties (**Figure 5**). Further analysis and evaluation are recommended to ascertain the specific nature of these changes and their potential implications for the overall quality of the extracted product.

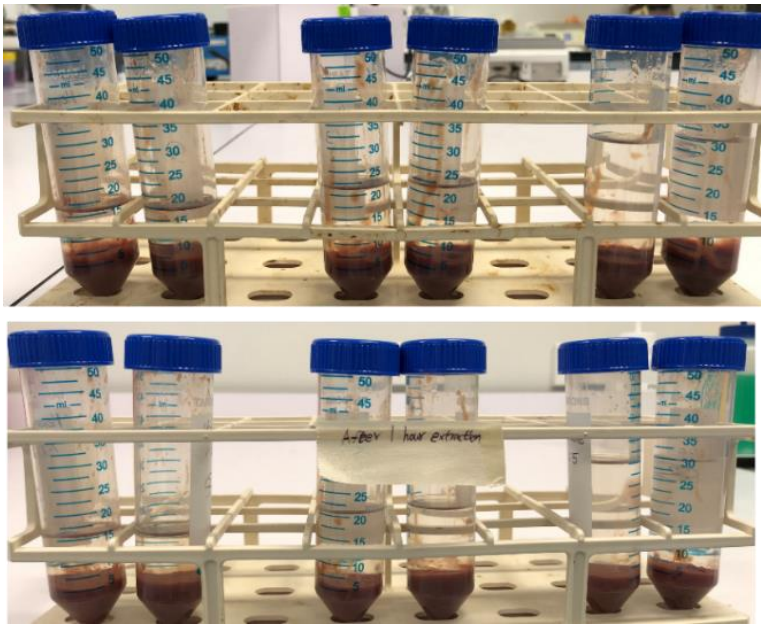

Figure 4. Hexane treatment: Photos of liver homogenate before (top) and after (bottom) extraction at liver:solvent (w/v) ratios of 1:2 (left), 1:3 (middle) and 1:5 (right).

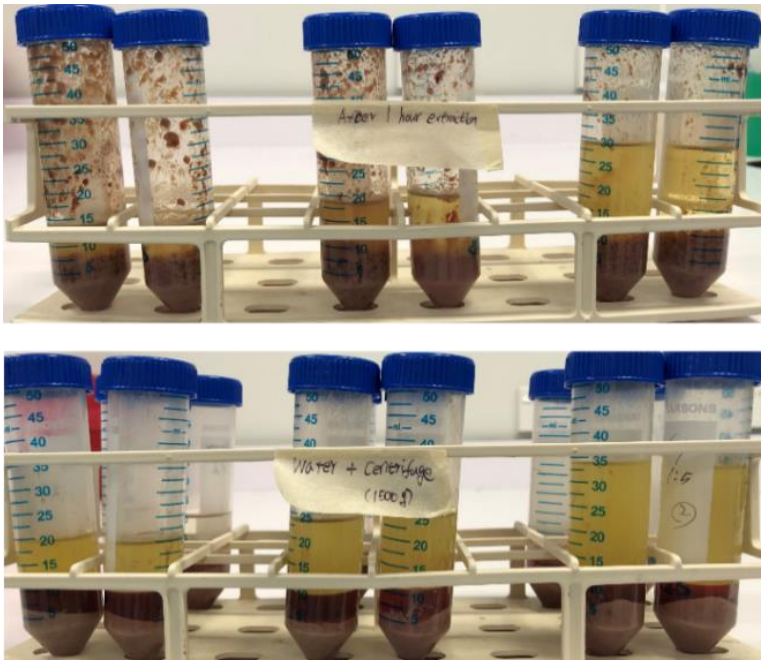

Figure 5. Soy oil treatment: Photos of liver homogenate before (top) and after (bottom) extraction at liver:solvent (w/v) ratios of 1:2 (left), 1:3 (middle) and 1:5 (right).

It was also observed in some of the incubations that four distinct phases were produced (e.g., for soy oil in Figure 5 bottom image and for hexane in **Figure 6**). The top layer of clear solvent likely containing extracted lipids and non-polar compounds, followed by a layer of unknown debris or emulsion or gelation, then a possibly aqueous layer that was strongly red-coloured, and finally a layer of liver residue at the bottom. The aqueous layer could have come from the sheared and lysed liver cells or from the added hot water. Its red pigments suggest substantial amounts of water-soluble haemoglobin (iron), which would be drawn into this water but not into the non-polar solvent. Further characterisation of the extracted pigments may reveal potential implications for the overall quality of the extracted product.

Figure 6. Hexane treatment: Photos of liver homogenate after centrifugation at ratio of 1:2 (left), 1:3 (middle) and 1:5 (right).

Tallow was challenging to implement as a solvent in these experiments. Although liquid at 55 °C during incubation in the water bath, it rapidly solidified in and around the liver homogenate matrix once removed (**Figure 7**). Considering the limitations associated with tallow's high melting point,

there is potential to explore processing techniques which fractionate it into lower melting points that will remain liquid at lower temperatures, allowing for easier handling and broader utilisation in final products or formulations.

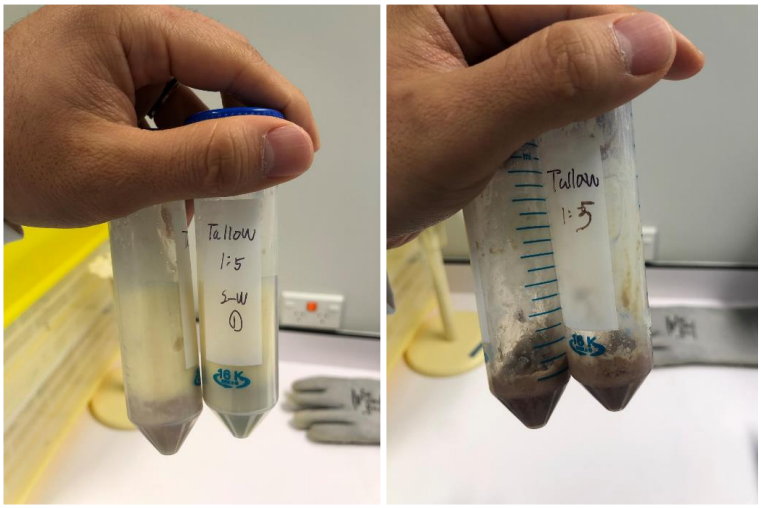

Figure 7. Tallow treatment: Photos of tubes after centrifugation when tallow supernatant fraction has solidified (left) and liver homogenate residue after removal of tallow by decanting and scraping (right).

3.1.3 Vitamin A Reduction

Extraction of liver homogenate by all three solvents at each ratio markedly reduced native liver vitamin A content from its expected starting value, calculated in **Table 2** as 139 mg/kg. Variability between ratios was modest compared to the extent of overall reduction (**Table 3**). There was also modest variability between replicates, such that all had very low vitamin A content. As a result, it is challenging to compare the efficiency of sample-solvents in reducing vitamin A. Based on ease of handling, a ratio of 1:3 was selected for subsequent experiments, during which individual livers (not combined homogenate) were evaluated.

Table 3. Vitamin A concentration in liver homogenate residue after extraction at each liver:solvent ratio, replicates 1 and 2 (mg/kg).

	1:2 ratio		1:3 ratio		1:5 ratio	
	rep1	rep2	rep1	rep2	rep1	rep2
Hexane	11	7	8	2	12	6
Soy oil	7	11	11	18	5	5
Tallow	18	11	4	11	10	13

3.2 Vitamin A extraction efficacy

3.2.1 Proximate composition

The average proximate composition of three additional lamb livers is shown in **Table 4**. Variability among them was low, and there were no significant differences from the livers used in Section 3.1 (see Table 1). Following the dehydration process, the moisture content of livers was reduced from 70.7% to 15.2%, with a corresponding increase in the weight percentage of ash, crude protein and

crude fat. Removing most of the moisture from liver tissue prior to extraction may improve the solvent's accessibility to the fat fraction and enhance the efficiency of vitamin removal.

The composition of liver residues after extraction (i.e., 'the pellet' separated by centrifugation) is also shown in Table 4. Comparing the unextracted and extracted materials reveals that not all of the solvent was expelled from the residue, i.e., the phase separation step was not rigorous. For instance, excess water was retained by the fresh liver after soy oil treatment and by the dehydrated liver after all treatments. The presence of residual soy oil and tallows increased the fat content of both fresh and dehydrated liver residues. These changes in moisture and fat led to commensurate dilution of ash and protein.

Table 4. Composition of unextracted liver and the residues remaining from fresh and dehydrated lamb livers after extraction by four solvent systems (wt%, mean ± SD, n=3)

	Moisture	Ash	Crude protein	Fat
Fresh				
unextracted	70.7 ± 0.6	1.7 ± 0.1	21.9 ± 0.6	0.6 ± 0.2
hexane	73.5 ± 0.3	1.30 ± 0.0	20.1 ± 0.1	1.30 ± 0.4
soy oil	79.8 ± 1.2	0.83 ± 0.1	14.4 ± 0.7	3.37 ± 0.3
tallow	71.5 ± 0.9	1.23 ± 0.1	20.0 ± 0.5	5.53 ± 2.8
low-tallow	71.4 ± 1.0	1.20 ± 0.0	20.4 ± 0.4	4.47 ± 1.3
Dehydrated				
unextracted	15.2 ± 0.9	4.3 ± 0.1	64.3 ± 1.5	13.4 ± 0.5
hexane	39.9 ± 0.5	2.83 ± 0.3	49.0 ± 0.6	6.3 ± 0.3
soy oil	55.4 ± 1.8	1.93 ± 0.1	31.2 ± 1.6	10.8 ± 0.5
tallow	39.3 ± 7.1	2.40 ± 0.1	36.6 ± 1.7	20.6 ± 5.4
low-tallow	34.9 ± 5.5	2.53 ± 0.1	38.2 ± 1.3	22.8 ± 4.4

Moisture content of the dehydrated liver residues suggests that they were partially rehydrated by water added to the oily treatments or contained entrapped solvent for the hexane (not watered) treatment. Dehydration of tissues can cause protein denaturation and increase hydrophobicity of the proteins. This can induce stronger hydrophobic-hydrophobic interactions with solvents. Interestingly, soy oil treatment produced residues with the highest moisture and lowest protein content. The liquid soy oil may have reacted with proteins and formed a protein-stabilised emulsion. When the supernatant was decanted after extraction, this emulsion layer of protein and fat was partially lost, resulting in relatively more moisture in the liver residue. Generally, emulsification is more effective at lower temperatures. Chen and Tao (2005) reported that emulsion stability was reduced when temperature increased from 30 to 70°C. Therefore, soy oil extraction performed at higher temperature may limit the formation of protein-oil emulsion.

Despite its 10°C lower melting point compared to whole tallow, the low-tallow was challenging to implement as a solvent and difficult to separate after extraction. If the tallow could be fractionated below ambient (25°C), it may be more suitable for food processing applications. Several attempts have been made to fractionate tallow fat at low temperatures. (Elhamirad & Zamanipoor, 2012; Ünsal & Aktaş, 2003).For example, Elhamirad & Zamanipoor (2012) conducted fractionation at temperatures of 25, 15 and 5 °C, while Ünsal & Aktaş (2003) peformed fractionation at 18.5 °C and below. Theses studies aimed to explore the possibilities of achieving better separation and utilisation of tallow through low-temperature fractionation methods.

The composition of residue from fresh liver treated with hexane was similar to that of unextracted liver. An interesting phenomenon was observed in some of the hexane incubations, particularly with fresh liver; the solvent affected the physical properties of the sample and formed a soft gel-like texture, (**Figure 8**). It is worth noting that this change in texture occurred at ambient temperature. Gelation did not occur with the soy oil and tallow incubations. Further investigation will be required to identify composition of the debris.

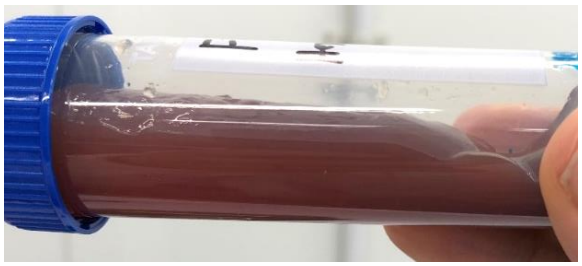

Figure 8. Photo of gel-like consistency formed when fresh liver was treated with hexane for 60 minutes incubation.

3.2.2 Percentage recovery

The recovery of residues and supernatants from each extraction treatment is shown in **Table 5**. There was little difference between solvent types. Theoretically the recovery of all residues should be 100%.

Table 5. Percentage recoveries of the residue and supernatant from fresh and dehydrated lamb liver after extraction by four solvent systems (as weight %).

	Residue (n=3)	SD	Supernatant (n=1*)	SD
Fresh				
hexane	97.3	12.4	96.7	<1.0
soy oil	96.5	11.1	79.4	<1.0
hi-tallow	96.6	8.5	82.4	2.9
lo-tallow	96.1	2.9	82.0	2.2
Dehydrated				
hexane	134.1	2.0	72.2	<1.0
soy oil	192.5	3.2	60.0	<1.0
hi-tallow	159.6	5.3	60.7	5.8
lo-tallow	154.2	21.3	61.7	2.7

*The recovery of supernatant was calculated from the average of duplicate results conducted in the third replication.

The excess residue recoveries for dehydrated liver were likely due to the observations about dynamics and proximate composition in Table 4. Their high relative protein content (i.e., undiluted by moisture) likely facilitated formation of protein:fat:water emulsions. As a result, there may be an emulsion remaining in the residue matrix that could not be removed by centrifugation and decanting steps of the experiment. Recall that hot water had been added to the soy oil and tallow extraction tubes to aid rinsing.

Over-recovery of the liver residue was unexpected for the hexane treatment. Those residues spent 1-2 hours of volatilisation time in the fume hood, which apparently was insufficient for complete

clearing of hexane from liver. The timing was a compromise against the risk of vitamin A degradation. This finding highlights the importance of taking additional steps to ensure food safety by thoroughly removing retained hexane. Industrial processes might involve volatilising under vacuum, or passing air through, like a fluidised bed.

3.2.3 Vitamin A reduction

In the fresh liver treatments, the extraction method using soy oil was the most efficient in reducing vitamin A, achieving approximately 70% reduction (**Table 6**). In the dehydrated liver treatments, tallow and low-tallow treatments showed similar efficiency in reducing vitamin A, with percentage reductions of 88% and 93%, respectively, while the soy oil treatment achieved a reduction of 89%.

Overall, the extraction process was found to be more effective and less variable for dehydrated liver compared to fresh liver. This difference may be attributed to the finely ground particles in dehydrated liver providing a large surface area, which may allow better interaction with the solvent (including by the removal of interfering polar water), whereas the fresh liver samples, with their non-particulate jellied puree consistency, may have hindered the efficiency of the solvent.

Table 6. Vitamin A concentration and percentage reduction in liver residues after extraction. Concentration is provided as mg/kg sample on a wet basis and mg/kg sample on a dry matter basis, calculated from moisture content in Table 4.

	Liver 1	Liver 2	Liver 3	Mean on wet basis	Reduction on wet basis (%)	Mean on DM basis	Reduction on DM basis (%)
Fresh							
unextracted	141	189	320	217 ± 93	--	741	--
hexane	79	62	152	98 ± 47	54 ± 11	370	50
soy oil	79	31	54	54 ± 24	70 ± 22	267	64
tallow	89	33	161	95 ± 64	56 ± 23	333	55
low-tallow	95	67	190	117 ± 64	46 ± 16	409	45
Dehydrated							
unextracted	389	295	593	426 ± 152	--	502	--
hexane	86	52	155	98 ± 52	78 ± 4	163	68
soy oil	45	17	86	49 ± 34	89 ± 4	110	78
tallow	47	29	71	49 ± 21	88 ± 1	81	84
low-tallow	41	5	38	28 ± 20	93 ± 4	43	91

As expected, the vitamin A concentration of the supernatants increased after the extraction process (**Table 7**). The supernatant from hexane treatment showed the highest concentration of vitamin A in both fresh and dehydrated liver samples. However, it is important to note that the highest concentration of vitamin A in the supernatant does not indicate the highest efficiency in reducing vitamin A in the liver. This could be due to hexane evaporation during storage or handling, leading to the high concentration of vitamin A in the supernatant.

Table 7. Vitamin A concentrations in supernatant after extraction (as mg/L).

	Liver 1	Liver 2	Liver 3	Mean
Fresh				
hexane	7.3	3.9	7.5	6.2 ± 2.9
soy oil	1.1	1.3	3.2	1.8 ± 1.1
tallow	2.9	2.4	2.3	2.5 ± 0.2
low-tallow	1.8	1.7	3.2	2.2 ± 0.8
Dehydrated				
hexane	37	75	102	71 ± 32
soy oil	28	19	21	22 ± 4
tallow	4	23	43	23 ± 19
low-tallow	17	9	40	21 ± 16

With the exception of the hexane treatment, no significant differences were observed between the soy oil, tallow, and low-tallow treatments in terms of vitamin A concentration. The vitamin A content ranged from 141-320 mg/kg in fresh liver and 295 to 593 mg/kg in dehydrated liver. Interestingly, the supernatant obtained from dehydrated liver samples showed a 10-fold higher concentration of vitamin A compared to fresh liver. Supernatants obtained in this way have a greater potential to be developed into a vitamin A-rich lipid ingredient.

While the project results show that liver vitamin A was substantially reduced by these simple extractions, it is not possible to perform a complete mass balance from the data, in which the initial quantity of vitamin A in liver is equated to its dispersal into post-extraction fractions of liver residue, solvent, aqueous emulsion and sometimes gel. First, laboratory analysis protocols designed for the modest vitamin A concentrations in red meat are not yet validated for the intensity of liver, nor for the changed matrix of dehydrated tissue. This adds uncertainty to the measurements of raw livers and liver residues. Second, vitamin A is labile to heat, oxygen and light, meaning that unpredictable and unaccounted-for losses will occur during all steps of handling. Finally, the aqueous emulsion fraction that separated from liver residue did not fully separate from the supernatant under moderate centrifugation. As only the 'clean' portion of recovered supernatant was tested for vitamin A, it is therefore unknown to what degree vitamin A may have been lost as part of emulsions.

In addition to vitamin A, other lipid and water-soluble compounds would have been extracted from liver into the supernatant during the process. Further research could evaluate the resulting composition and nutritional profiles, including the level of lipid oxidation, in order to fully understand the potential of these supernatants as a functional ingredient.

5. Conclusions

Vitamin A concentrations in livers from New Zealand lambs fell within the previously reported range, with up to two-fold variation between animals. However, our observations were from a very few samples collected from one North Island producer, and further work is required to understand how wider variation might impact vitamin A reduction.

It was found that simple, short-duration, warm temperature extractions using various non-polar solvents could substantially reduce vitamin A content in samples of fresh and dehydrated lamb liver.

The processed liver could be a safer ingredient for pet food, as it lowers the risk of vitamin A toxicity. Further work is required to understand quality, nutritional value and safety of such products.

Several permutations of liver preparation, incubation and extraction methods were trialled at laboratory scale. All these factors influenced the efficacy of vitamin A removal and product yield, and thus they have potential to be manipulated to create optimised conditions.

Implications of solvent choice

Three non-polar solvents were tried: hexane, soy oil and tallow. Hexane was included as a control due to its common use in the food industry for defatting processes. Soy oil was chosen because of its low melting point and affordability as an edible resource. It did not require heat to remain liquid, which would save energy, nor did it alter the texture of liver.

Tallow was a novel solvent choice. As a ruminant-derived by-product, it could be a 'clean label' option for processed liver ingredients. However, its high melting point of 55°C meant it was challenging to work with, required heat throughout processing, and was prone to re-solidification during tube handling and transfers. Further manipulations are needed to make it viable. A fraction of tallow with a melting point of 45 °C was tried here alongside unfractionated tallow used at 55 °C, however both temperatures caused thermal denaturing, the final products resembling cooked liver. This may affect nutritional characteristics and palatability of the processed product. Exploring methods to further lower the melting point of tallow and achieve desirable properties would be an interesting avenue for research. There have been successful attempts to chemically fractionate sheep tail fat into different melting points, resulting in a lipid fraction with melting point below 18.5°C (Ünsal et al., 2003).

While the current work focused on the effects of solvents on recoveries and vitamin A reduction, it is important to consider that these solvents may also affect the nutritional and physiological properties of the liver residue and its prospects as a value-added product.

Comparison of solvent ratios

Liver-to-solvent ratios tested across a narrow range did not substantively affect extraction efficiency, therefore we chose a 1:3 ratio for subsequent experiments. At this point it is not possible to identify an optimal ratio that might apply to scaled-up processes.

An additional brief bench test was tried with soy oil using an ultra-low solid-to-solvent ratio of 1:0.3. It appeared to have much poorer extraction efficiency than the 1:3 ratio, but a more thorough experiment is required to confirm the effects on emulsion formation, residue recovery and vitamin A.

Provisional extraction conditions

Fresh, slightly frozen liver was minced and used for extraction. When left at room temperature for approximately 10 minutes it lost structural integrity and became a liquid-like puree consistency. When combined with a warm solvent (i.e., tallow), the solvent was cooled and began to solidify. A straining filter was tried for separation, but the liver homogenate passed through along with the solvent. In large-scale trials, this effect of heat transfer (solvent cooling) could be an important consideration when determining incubation conditions. Note that these temperature and separation issues were not problematic when dealing with dehydrated liver.

The agitation method likely contributes to the efficiency of extractions. In these experiments we used only a simple and gentle approach: stirring or shaking for 60 minutes. Other conditions might

have produced different results. Agitation was probably responsible for the gelation that we observed occasionally in fresh liver treated with hexane (see Figure 8). We speculate that the stirring speed, combined with the effect of hexane on protein denaturation, altered the physical properties of the liver, even without heating. Gelled material could interfere with processing and handling at industrial scale.

Addition of hot water to the incubation was implemented to aid rinsing oily solvent from the liver residue. The water concurrently extracted water-soluble components, such as proteins and minerals, from the liver matrix. This separation might create another value stream from liver processing, however further investigation will be necessary to identify any changes in the nutritional quality of the liver residue when water is added versus when it is not.

Implications of liver format

The results for fresh liver can be compared across the different fatty solvents with some confidence, as they are in a similar physical format and have relatively similar water and crude fat contents. Similarly, the results for dehydrated liver can be compared across the fatty solvents, as they too are in a similar format (based on dehydrated liver, ground), with relatively similar crude fat contents. It is likely that the differences in moisture between fresh and dehydrated affected retention of the solvents and thus net efficacy. This might be further investigated by normalising on protein content of liver residue, which is independent of lipid contamination. However, comparing the vitamin A content across liver formats, from unextracted fresh liver to unextracted dehydrated liver, should be done with caution, as analytical recovery of vitamin A from dehydrated liver may be lower than that of wet liver due to the way the pre-chromatography sample extraction procedure works.

In conclusion, this project was a proof of concept to assess whether simple solvent extraction methods can substantially reduce vitamin A or not. These were laboratory experiments and so the percentage recoveries (i.e., yields) and method efficacies could differ when scaled up. Factors such as loss during handling and reduced reproducibility may come into play. Additionally, there are various steps that can be improved, such as the separation method and agitation method, among others. Overall, we observed meaningful reductions in vitamin A that are likely sufficient to make lamb liver lower risk for inclusion in pet foods. This report provides recommendations for handling fresh and dehydrated livers, as well as selecting appropriate solvents. It is essential to further optimise and refine the extraction process to achieve higher efficiency and reproducibility.

5.1 Key findings

We found that all of the tested solvents were able to reduce the vitamin A content in both fresh and dehydrated liver. On a dry matter basis, 45 - 64% reduction in vitamin A was observed for fresh liver, and 68 - 91% reduction was observed for dehydrated liver.

5.2 Benefits to industry

The experimental project results show that vitamin A can be extracted from fresh or dehydrated liver using animal or vegetable lipids to produce a protein-rich liver residue with 45 - 68% less vitamin A (on a dry matter basis) and a solvent lipid-rich fraction containing 1.8 – 23 mg/L vitamin A.

Current maximum levels for vitamin A in dog and cat food formulations are 75 and 99 mg/kg DM respectively, and this has limited the usefulness of liver as an ingredient. Based on the average results observed here in this proof of concept, and if similar reductions can be obtained through a

commercial scale process, pet food formulations might be able to contain a post-extraction liver ingredient at quite high inclusion levels, assuming no other ingredients are contributing to vitamin A in the food formulation.

Based on the project results:

- A post-extraction liver product prepared from *fresh* liver could be incorporated in pet food formulations at 18 - 28% DM for dogs, and 24 - 37% DM for cats, before exceeding the maximum allowable value for vitamin A.
- A post-extraction liver product prepared from *dehydrated* liver could be incorporated in pet food formulations at at least 46% DM for dogs, and at least 61% DM for cats, before exceeding the maximum allowable value for vitamin A.

The ability to realise maximum inclusion rates will depend on industry users knowing the starting concentration of vitamin A in the liver and final values obtained. On-line quantitation of vitamin A is unlikely to be achievable in the near-term, therefore in practice a wide margin of error may still need to be applied to ensure pet safety. As liver becomes more frequently utilised and its variability measured, and the extracted products become optimised, the confidence in inclusions rates will improve.

6. Future research and recommendations

Further research is required to assess the quality and safety of the produced products, optimise the process unit operations and refine the business case for uptake of the innovation. This should include investigation of the following:

Product quality

- Characterisation of the nutritional profile of the recovered liver residue and lipid fraction
- Assessment of the palatability of both the liver protein and lipid fractions
- Assessment of microbial and residual components for safety
- Assessment of the quality and techno-functional properties of the liver protein fraction (e.g., gel strength, water/oil retention capacity and level of protein denaturation) and lipid fraction (e.g., peroxide value)
- Assessment of protein and lipid digestibility, or other health benefits of the products for specific applications

Process optimisation

- Optimisation of the time-temperature treatment for an appropriate set of edible oils to be used as non-polar solvents
- Establishment of a consistent extraction process that delivers known upper values of vitamin A in the final product irrespective of starting concentrations in the raw material, which may require a polishing stage to achieve
- Investigation of whether a continuous processing method can be employed, for example, an auger-based system which grinds, mixes and separates the solid and liquid fractions in a single production vessel
- Investigation of whether the solid and liquid fractions can be separated using simple sedimentation in a single vessel

- Investigation of whether solvent can be re-used multiple times to reduce cost and increase the vitamin A content of the solvent lipid-rich fraction

Business case

- Determination of recovery and extraction efficacy through pilot-scale trials
- Technoeconomic assessment to determine the viability of an industrial process at a range of commercial production capacities
- Determine “tipping point” for pet food brand owners for procuring modified Vitamin A liver product and time to deliver improved technical readiness level.

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