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FINAL REPORT

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MICROENCAPSULATION TECHNIQUES FOR PRAWN FEED FORMULATION

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SUMMARY

Three techniques were tested as methods to encapsulate soluble nutrients for inclusion in feed pellets for adult prawns - spray cooling, spray drying, and fluid bed coaintg. Powdered vitamin C was used as the model nutrient for most experiments. Spray drying and fluid bed cooling techniques gave capsules with poor leaching properties. However, spray cooled microcapsules using a hydrogenated vegetable oil (HVO) coating were suitable for inclusion in prawn feed pellets. The leaching rate of nutrient from the capsules was a strong function of coating to core ratio. For ratios of 4:1 or higher, approximately 25% of the vitamin C was leached in two hours. Much higher leaching rates were shown at lower ratios. The capsules varied in size from 80 to 250 μm, depending on the atomising air pressure to the nozzle. Leaching rates from methionine core capsules were as low as 5 to 7% for coating to core ratio of 2:1.

Digestibilty tests using dyes and radioactivelly labelled proline confirmed that the HVO capsules were digested and taken up into the prawn tissue. Feeding trials using different levels of free and encapsulated vitamin C added to a base diet showed that encapsulated vitamin C at a rate of 50 mg per 100g dry feed supplied the prawn's vitamin C requirements. This level of vitamin C is much lower than that needed (and currently used) for free vitamin C. Although growth rates in all experiments (including the control diet) were low, the mortality rate in prawns fed encapsulated vitamin C was lower than for other diets tested and no exoskelaton lesions were detected.

These results have demonstrated that microencapsulation with hydrogenated vegetable oil does provide an effective way of protecting micronutrients in aquaculture diets from oxidation and leaching. Suitable conditions for encapsulation have been identified which will allow scale up to a commercial process. The spray cooling encapulation process is cheap and easy. It is flexible and handles easily fine powders as core materials.

However the microcapsules have not been tested in a commercial pellet press or extruder and may be damaged at the temperatures and pressures involved in these feed manufacture processes. There is possibly less risk associated with their use in an extruder where they could be added towards the end of the extrusion process. It is strongly recommended that feeds containing microencapsulated nutrients are tested in a commercial pellet press or extruder as a final step before attempting to commercialise the process.

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1. OBJECTIVES

The objectives of this project, as stated in the original application were:

1. Develop a technique to encapsulate soluble nutrients for inclusion in feed pellets for adult prawns.

2. Develop new or improve existing techniques for encapsulating artificial whole diets for prawn larvae.

3. Test the effectiveness of the techniques by prawn feeding trials.

4. Encourage local feed manufacturers to use the techniques in the development of prawn feed for local industry and for export.

The main focus of the project has been on encapsulation of soluble nutrients to include in feed pellets for adult prawns (objectives 1, 3 and 4) which we perceived to have greater scope and importance than larval feed development. Results of this work are contained in this report. However, a parallel project on larval feed was undertaken by a PhD student at the University of Queensland (not funded by the FIRC). Results of the larval feed work are published in the student's PhD thesis (Komari, 1993).

2. BACKGROUND

2.1 The Need for Encapsulated Nutrients

Development of a long term aquaculture industry requires efficient production and process technologies. Development of expertise in both the biology and process engineering of aquaculture is essential. One area for the application of process engineering is the preparation of prawn feed. Provision of reliable cheap food which satisfies all the prawn nutrition requirements is a major problem in prawn farming and the cost of feed is a major operating expense (Maguire and Allen, 1988).

Adult prawns are fed artificial food in the form of extruded pellets. The main constituents of the pellet (proteins, fat, starches) have relatively low solubility in water. However, essential vitamins and other low molecular weight water soluble substances are also included in feed pellets (Dall and Smith, 1986, 1987, Dall and Moriaty, 1983). To achieve maximum growth rate, these nutrients must be included in the diet.

However, these nutrients are quickly leached from food pellets and are thus unavailable to the prawns (Goldblatt et al., 1980). Our proposed solution is to encapsulate the soluble nutrients in a polymer coating that remains intact until the pellet is digested. Thus, soluble nutrients can be successfully incorporated into the prawn feed pellets with resulting improvements in productivity.

2.2 Specifications for Encapsulated Nutrients

The desired properties of the encapsulation process and product capsules are:

- The coating must be strong enough to withstand extrusion of the prawn feed pellets.
- The coating must be water insoluble and impermeable to water soluble molecules.
- The coating must be physically broken down by the mandibles and gastric mill, or be digestible by the prawn.
- The product capsules should be less than 0.5mm is size.
- The encapsulation technique should be flexible enough to handle a range of different particles and powders. In particular, it must be able to handle fine powders in the range 5 to 50 μm.

A wide range of coating and encapsulation techniques are available and used in the pharmaceutical, chemical and mineral processing industries. At least three types of

processes could meet the criteria given above: spray cooling, spray drying and fluid bed coating. Each of these techniques was tested in this project.

2.3 Scope of the Work

The project was undertaken in two parts. First, equipment was developed to coat soluble nutrients, especially ascorbic acid, by three separate techniques. The physical properties of the capsules, particle size and leaching rate, were measured as a function of coating technique and operating variables. This work was conducted largely at the Department of Chemical Engineering at The University of Queensland and is described in section 3 of this report.

This first part of the study singled out spray cooled capsules using hydrogenated vegetable oil (HVO) as the coating to hold the most promise. In the second part of the study, extensive feeding trials were carried out using feeds incorporating encapsulated nutrients. This work was carried out at the CSIRO Marine Laboratories at Cleveland and is described in section 4 of this report.

3. DEVELOPMENT OF MICROENCAPSULATION TECHNIQUES

3.1 Experimental Equipment and Techniques

3.1.1 Materials

In the majority of experiments, powdered ascorbic acid was used as particles to be coated. Thsi material was very fine, typically less than 10 μm. In some experiments, porous silica powder (SIPERNAT 22) saturated with ascorbic acid solution was coated. This was done to increase the size of the particles to be coated to approximately 80 μm. In some experiments, crystalline ascorbic acid (David Craig and Co., mean size 160 μm) was used. Other model powders and particles were also used including radioactively labelled proline, water soluble dyes, and methionine.

Two types of coating were used. Waxy coatings (parrafin wax, hydrogenated vegetable oil (HVO) and hydrogentated beef tallow) were used in spray cooling experiments. Table 1 gives data on these materials. Two protein or cellulose based coatings were used in spray drying experiments - ethyl cellulose and zein. For these coatings, ethanol was used as a solvent.

Table 1. Properties of waxy coatings

Coating Material	Supplier	Melting Point	SFC* at 40°C
parrafin wax	Aldrich Chemicals	56 to 61°C	
hydrogenated palm stearine (HVO)	Meadow Lea Foods	51°C	45%
hydrogenated edible tallow	Meadow Lea Foods	51°C	38%

* SFC is the solid fat content at 40°C.

The leaching properties of three commercially available stabilised ascorbic acid products was also tested and compared to our microcapsules. These products were:

Takeda CVC Type A/B: powder coated with 1.2% ethyl cellulose

Takeda CVC Type F: powder coated with 10% HVO

Balchem AS-165 CR-70: 0.5mm crystals coated with 30% hydrogenated soybean oil

3.1.2 Spray cooling experiments

A schematic of the spray cooling equipment is shown in figure 1. Approximately 200g of the coating material was accurately weighed and then melted in a 500 ml beaker on a hot plate. The coating temperature was maintained at 65°C. The desired mass of core particles to be coated were mixed with the melted coating material in the beaker to form a slurry. This slurry was vigorously stirred to ensure complete suspension of the core material. The coating slurry was pumped via a peristaltic pump to a simple two fluid nozzle (see figure 2). Within the nozzle the slurry was atomised into small droplets by compressed air. These droplets quickly cooled and solidified and were collected as they settled into a 80 litre plastic lined container. A hot water jacket around the nozzle ensured that the wax did not solidify in the nozzle itself or in the slurry feed line.

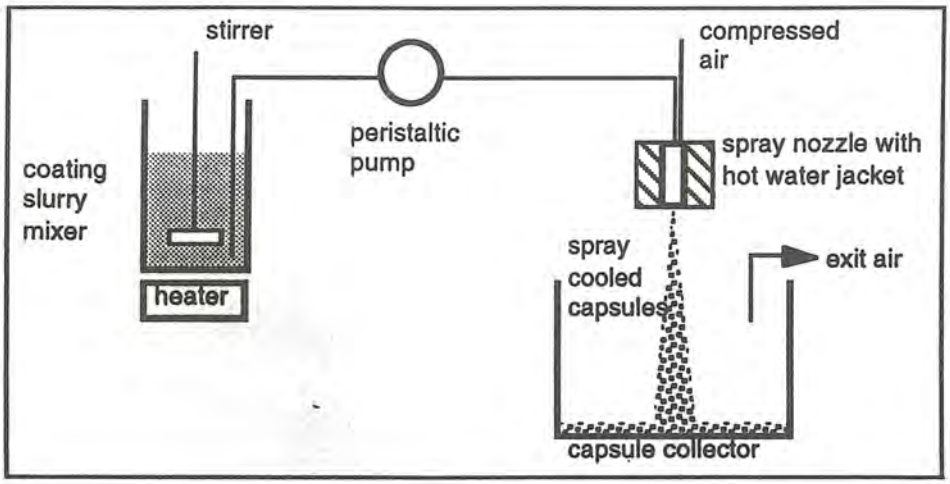

Figure 1. Schematic of Spray Cooling Experiment

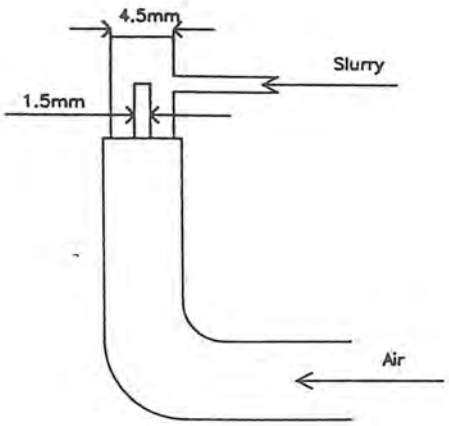

Figure 2. Details of Spray Cooling Nozzle

3.1.3 Spray drying experiments

A schematic diagram of the spray drying equipment is shown in figure 3. For spray drying experiments, the core particles were slurried in a solvent also containing the dissolved coating material. The slurry was pumped to a nozzle similar to that used for the spray cooling experiments. This nozzle was located at the top a 200mm I.D, 500mm long glass column. The air to the nozzle was preheated using a 2kW electric heater. The hot air evaporated the solvent from droplets formed in the nozzle as they traversed the column, leaving spray dried particles consisting of the core particles and coating materials. The column temperature was approximately 60°C. The spray dried particles were separated from the gas stream after leaving the column in a 100mm I.D. cyclone.

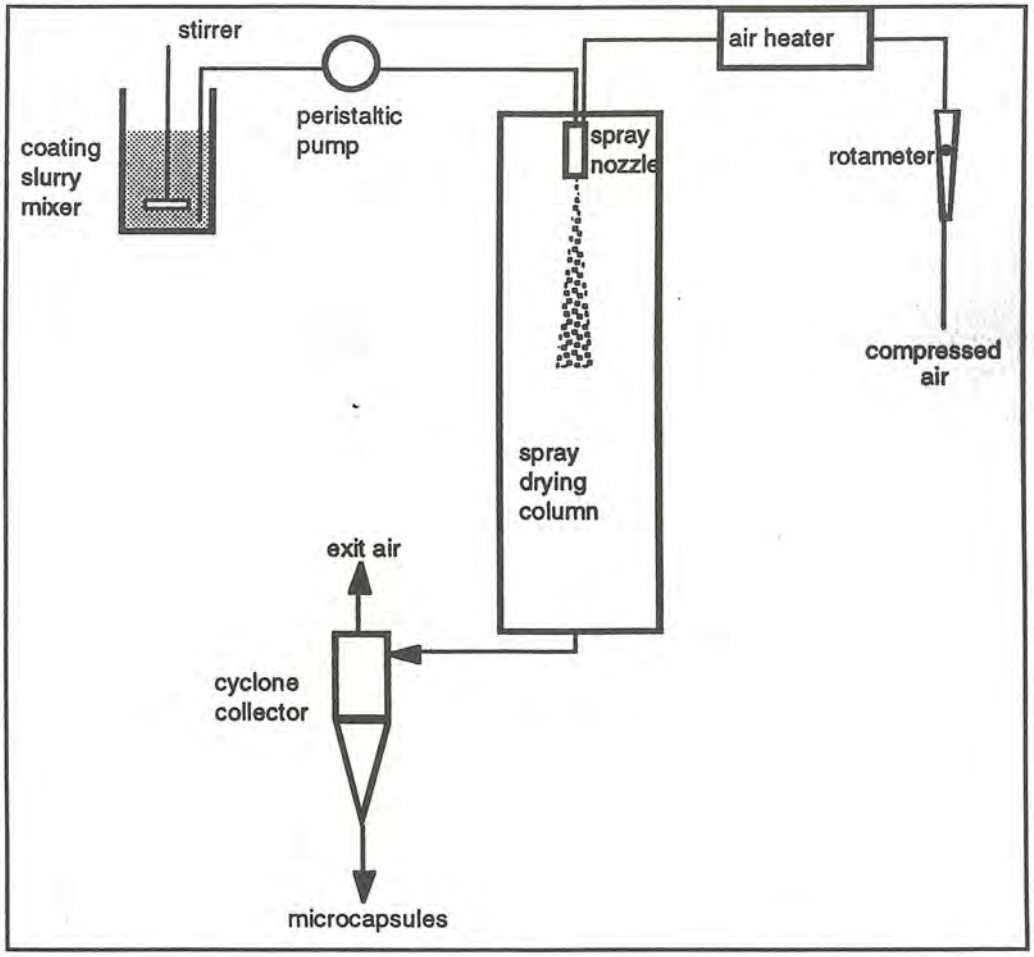

Figure 3. Schematic of Spray Drying Equipment

3.1.4 Fluid bed coating experiments

For fluid bed coating experiments, the desired material to be coated was first dissloved in water and absorbed onto porous silica particles (SIPERNAT 22) approximately 80μm in size. 1kg of the doped silica particles were then placed in a 0.2m I.D. glass

laboratory fluidised bed and fluidised with air (see figure 4). At the bottom of the bed was a distributor plate to support the bed and evenly distribute the incoming air. This plate was made of brass and was drilled with 1mm diameter holes on a 7mm square pitch pattern. The air flow rate was set using a rotameter and the air preheated, if necessary, using a 2 kW electrical heater. The coating (as a liquid or solution) was supplied through a two fluid nozzle as described above. The nozzle was placed in the bed from the top such that the end of the nozzle was fully submerged in the bed when it was fluidised. Droplets of coating collided with particles in the bed and the fluidising air acted to solidify the coating or evaporate the solvent to leave coated silica particles with the ascorbic acid, or other nutrient, trapped inside.

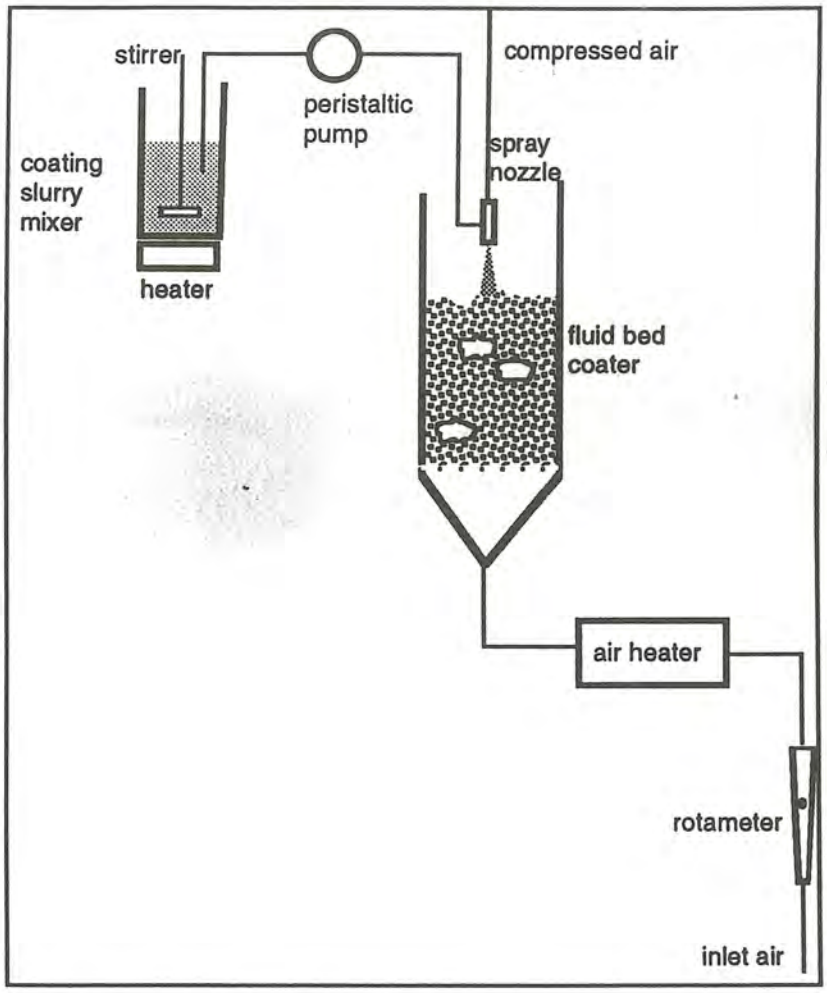

Figure 4. Schematic of Fluid Bed Coating Equipment

3.1.5 Analysis techniques

The two key measures of microcapsule quality were their size distribution and leaching properties in sea water. The capsule size distribution was measured using a Malvern Laser Sizer which uses a light diffraction sizing technique.

The leaching tests were conducted as follows. A carefully weighed sample (0.5 to 1 grams) of capsules were placed in 100 ml of sea water in a conical flask on a shaker table within a constant temperature bath at 32°C. At each desired time, a 1ml sample of sea water was taken from the flask and filtered into a phial for analysis. 0.5 ml of dichloroindophenol (DCIP) solution was then added to the sample and allowed to react for 45 sec. The sample was then placed in the UV spectrophotometer and the absorbance at 520 nm measured. Results were compared with a calibration curve produced by measuring the absorbance of known concentrations of ascorbic acid in sea water.

It was noted that for some experiments the measured amount of ascorbic acid in the sea water actually *decreased* at long times. The effect was due to the gradual oxidation of the leached ascorbic acid. To account for this effect, several solutions of ascorbic acid in sea water were carefully made up, placed on the shaker, and sampled as for the leaching experiments for 24 hours. The fraction of ascorbic acid oxidised at different times was measured and this data used to correct ascorbic acid readings from leaching tests. Only 7% of the ascorbic acid was oxidised in 2 hours, but 40% of oxidised over a 24 hour period.

3.2 Results and Discussion

3.2.1 Spray cooled microcapsules.

Spray cooled microcapsules were produced with three types of waxy coating (see Table 1). In most experiments, powdered ascorbic acid was used as the core material. However, crystalline ascorbic acid, vegetable dye, proline and methionine were also coated.

The choice of coatings was limited by their melting characteristics. To avoid degradation of the core material, it was necessary to keep the feed slurry at 65°C but the coating needed to be solid and hard at room temperature for reasonable handling. The initial experiments were performed using parrafin wax which meets these criteria admirably.

Using parrafin wax, the effect of wax flow rate, nozzle air pressure and wax to core ratio on the capsule properties were studied. All runs produced capsules of the desired size range, ie. with a median size in the range 50 to 200 μm. The median size decreased with increased nozzle pressure (and therefore air flow rate) as expected.

A typical leaching curve is shown in figure 5. Leaching tests showed nearly all the leachable ascorbic acid was removed from the capsules in the first hour, with little

additional loss after 2 hours. These leaching characteristics reflect the structure of the capsules. As the powder size is much smaller than the capsules size, the capsules are, in fact, a dispersion of the powder in a matrix of inpermeable solid wax. Ascorbic acid at or near the surface of the capsule is quickly leached away. The rest of the powder is protected by the impermeable wax matrix.

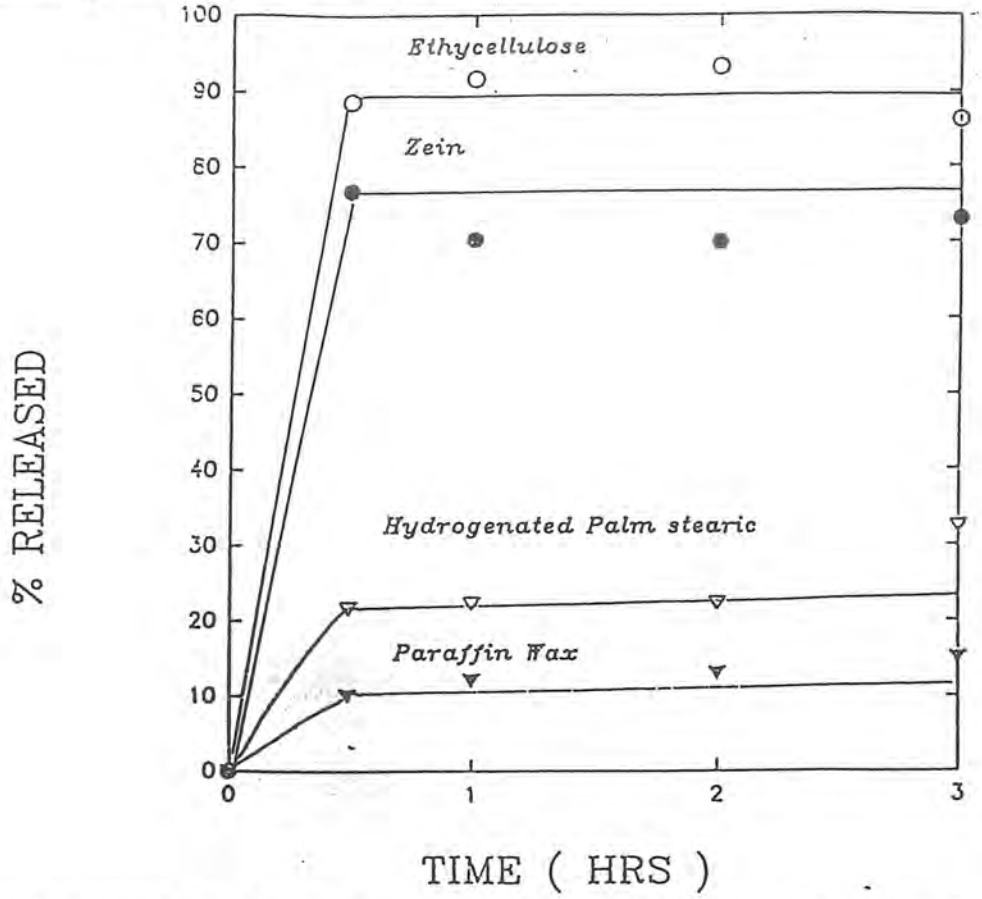

Figure 5. Typical Leaching Curves for Spray Cooled and Spray Dried Capsules

Between 10% and 70% of the ascorbic acid was leached in the first two hours, depending on experimental conditions. The leaching rate was a strong function of the coating to core ratio as was expected. Nozzle pressure also effected leaching rate because of its effect on capsule size. Smaller capsules have a proportionately higher fraction of powder exposed at the surface. A wax to core ratio of 4:1 and nozzle pressure of less than 150 kPa was required to reduce the leaching loss to 20% in 2 hours.

Thus, we were able to produce parrafin wax capsules with the desired physical characteristics. However, parrafin wax capsules were not easily broken down in the prawn's mandibles or gut (see section 4.2.1 below). Therefore, we looked for alternative edible waxy materials that would be digested by the prawn. Hydrogenated beef tallow and hydrogenated palm stearine (HVO) were both tried. The tallow capsules were too soft and sticky at room temperature, due to their high SFC (see Table

1). However, easy to handle capsules were produced using HVO as the coating material.

A summary of ascorbic acid coating experiments with HVO are given in Table 2. The trends seen here are similar to those for parrafin wax coating. A coating to core ratio of at least 4:1 was necessary for leaching losses in the range 20 to 27%. Increasing the coating ratio above 4:1 had little effect on the ascorbic acid leaching, but the extent of leaching increases markedly when lower coating to core ratios are used (see figure 6). Ascorbic acid crystals of much larger mean size (180 μm) were also coated to see if the coating to core ratio could be reduced by increasing the particle size (and therefore

Table 2. Summary of Properties of HVO Capsules

Type of ascorbic acid	Coating to core ratio	Nozzle air pressure (kPag)	% leached after 2 hours	Median capsule size (μm)
powder	2:1	200	51	83
powder	3:1	60	83	240
powder	3:1	120	51	207
powder	3:1	240	72	140
powder	4:1	120	22	228
powder	4:1	120	24	
powder	5:1	180	42	128
powder	5:1	180	35	127
powder	10:1	120	28	150
powder	10:1	150	26	133
crystal	3:1	180	92	205
crystal	5:1	150	71	248
crystal	10:1	150	25	279

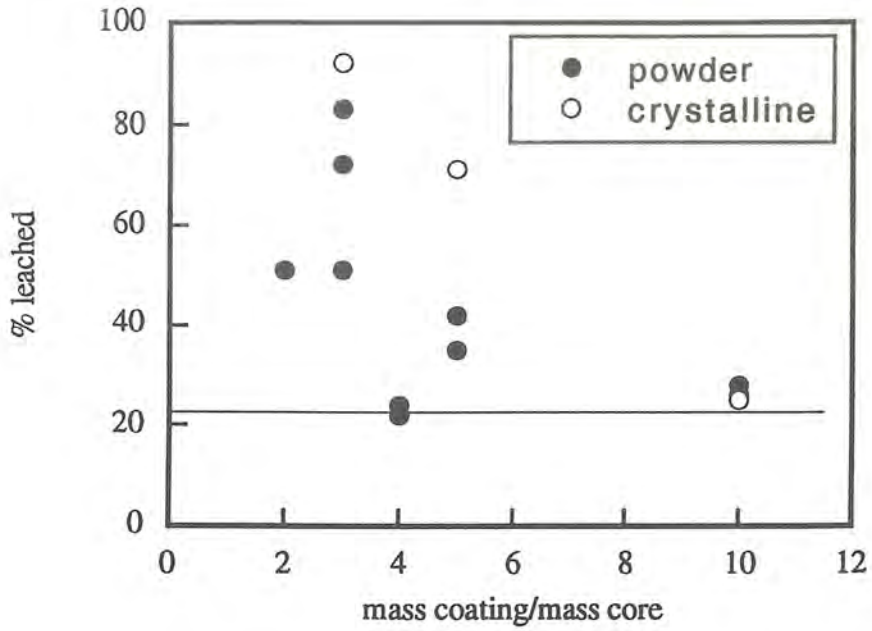

Figure 6. Effect of powder type and coating to core ratio on leaching

reducing the specific surface area). However, these experiments gave poorer leaching results than did those using powdered ascorbic acid. The high coating to core ratio requirement may possibly cause problems in balancing the saturated to unsaturated fatty acid balance in the prawn diet if HVO capsules are used.

In summary, the spray cooling method can provide capsules using both inert and digestible coatings which have the desired size and leaching characteristics. The technique is cheap, easy and very flexible. It will easily handle very fine powdered feeds. The only significant disadvantage of this method is the high coating to core ratio required.

3.2.2 Spray Dried Microcapsules

Spray drying has the potential advantages over spray cooling that a wider range of coating materials can be used and the required coating to core ratio may be smaller. Spray dried microcapsules were produced as described above in section 3.1.2. Results are summarised in Table 3.

Ascorbic acid was coated with ethylcellulose and zein, with coating to core ratios varying from 1:1 to 5:1. Both powdered and crystalline ascorbic acid were used. Fish oil was also tested as an additive to ethyl cellulose coatings.

Table 3. Summary of Properties of Spray Dried Capsules

Coating	Coating to core ratio	ascorbic acid type	% leached after two hours	median capsule size (μm)
ethyl cellulose	1:1	powder	62	96
ethly cellulose	5:1	powder	60	81
ethly cellulose	1:1	crystal	61	
ethyl cellulose	5:1	crystal	68	163
EC+20% fish oil	1:1	powder	72	78
EC+20% fish oil	5:1	powder	>100	85
EC+20% fish oil	1:1	crystal	70	87
EC+20% fish oil	5:1	crystal	>100	145
zein	5:1	crystal	80	120

In all experiments, small microcapsules ranging from 50 to 150 μm were produced. However, all capsules showed poor leaching properties with from 50 to 100% of the ascorbic acid leached from the capsules after two hours.

For the range of experiments performed, the particles formed were low density porous aggregates without a continuous film of coating or solid coating matrix. These particles did not protect the ascorbic acid to any extent. Some dissolution of ascorbic acid in the solvent, ethanol, may have contributed to the problem. After several months test work, spray drying was abandoned as a technique to produce the microcapsules.

3.3.3 Fluid bed coating

Powdered ascorbic acid is not suitable for fluidised bed coating because of its very small particle size. However, saturated ascorbic acid solution can be absorbed into porous silica particles (SIPERNAT 22) and then the silica particles coated with a HVO coating in the fluidised bed. Due to the larger particle size, it was hypothesised that lower coating to core ratios would be possible.

However, preliminary experiments using HVO to ascorbic acid ratios of between 6 and 12 to 1 showed virtually all the ascorbic acid was leached within 2 hours. The air in the fluid bed evaporates water from the doped silica particles, leaving substantial unfilled pore volume. It is likely that most of the HVO coating was filling these pores, rather than providing a coherent external coating. This explains the very poor leaching results.

Fluid bed coating was abandoned as a technique after these preliminary experiments.

3.3.4 Leaching tests on commercial ascorbic acid products

Several forms of stabilised ascorbic acid are available commercially. These are described in section 3.1.1 above. The leaching characteristics of these products were measured and compared with the results for spray cooled capsules we produced. The leaching results are shown in figure 7.

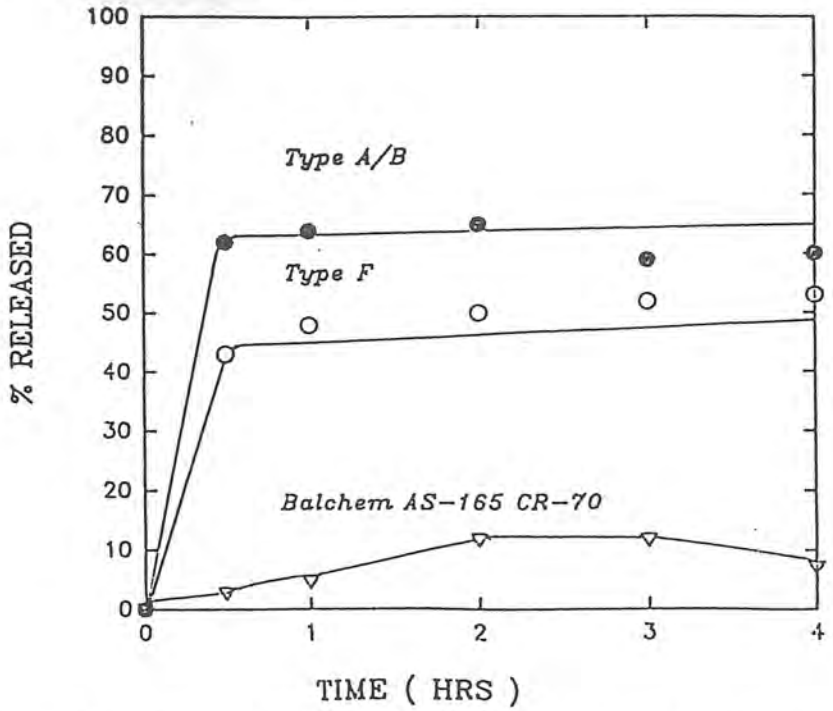

Figure 7. Leaching Characteristics of Commercial Ascorbic Acid Products

Both Takeda products (containing 1.2% ethlycellulose and 10% HVO respectively) had very poor leaching characteristics and would be unsuitable for aquaculture. The Balchem product showed good leaching performance with 10 to 15% of ascorbic acid leached after 2 hours. This product uses a HVO coating to core ratio of only 0.5 to 1. However, it is expensive as it uses 0.5mm sized crystalline ascorbic acid. The coating technique is unknown, but for particles of this size it was probably pan or fluid bed coating. These techniques are not adaptable to coating fine powders.

3.3.5 Summary

Three potential encapsulation techniques were tested - spray cooling, spray drying and fluid bed coating. Only spray cooling provided capsules suitable to include in prawn feed pellets. Capsules produced by this technique using parrafin wax and HVO as coating material had good leaching and size characteristics and were suitable for further physical and biological testing. These tests are described in section 4 below.

4. PHYSICAL AND BIOLOGICAL TESTING OF FEED PELLETS CONTAINING ENCAPSULATED NUTRIENTS

A wide range of physical and biological tests were undertaken to test the suitability of encapsulated nutrients in prawn feed. These tests included leaching and degradation tests on pellets containing microcapsules, tests to confirm digestibility of capsules and uptake of nutrients, and feeding trials to measure prawn growth rate and mortality on feed including microcapsules.

Methods and materials for all the test are described in section 4.1 and results are discussed in section 4.2.

4.1 Materials and Methods

4.1.1 Leaching and degradation of core material in lipid microcapsules.

Preliminary investigations into the materials suitable for use as the microencapsulation medium involved the use of a dye Methylene Blue. With this dye it was easy to detect any leaching from the microcapsules into water. When the micrcapsules had been incorporated into a prawn feed and fed to prawns, the ability of the prawns to digest or rupture the microcapsules could be determined very readily by microscopic examination of the faeces.

Further trials were conducted using a radioactively labelled amino acid, ^{14}C-proline. The ^{14}C-proline was coated with HVO with a coating to core ratio of 4:1. These capsules were fed to prawns and the distribution of proline in the seawater and prawn tissue measured using a technique established by Smith and Dall (1991).

Leaching experiments were typically carried out with the microcapsules included in a prawn feed. A weighed amount of the feed pellets containing a known amont of ascorbic acid was placed in a beaker with 100 ml of seawater (later 0.1N HCl containing 0.2% dithiotheitol was used). The beaker was shaken at room temperature at 60 rpm and a time series of 1ml samples were drawn off for ascorbic acid analysis. At the conclusion of the experiment the feed pellets were broken up and extracted with hexane to remove the lipid coating of the microcapsules and the total ascorbic acid content was determined. The leaching rate was described as the percentage of total ascorbic acid in the feed pellets.

Ascorbic acid was used extensively as a core material in the development of the microencapsulation technique because of its importance in the diet of all aquaculture species and its high lability. The analysis of ascorbic acid is a complex process as

ascorbic acid is readily oxidised to a number of forms in some of which the vitamin activity is retained while in others it is lost. Samples of microcapsules, leachates and feeds prepared and stored for different lengths of time under various conditions were analysed to determine the integrety of the capsules and the level of protection given to the core material.

Initially ascorbic acid content of microcapsules and leachates were determined with a colorimetric method using the 2,6-dichloroindophenol derivative as described in section 3.1.5. However, this technique gave inconsistent results with the aquaeous solutions used and in feed extracts. The method then adopted was one in which the two active forms of vitamin C, ascorbic acid and dehydroascorbic acid were determined. The procedure involved dissolving the HVO coating of the microcapsules in hexane and extracting the vitamin C into a 0.2% solution of dithiotheitol (DTT) before making up to a specific volume. A sample of the extract was injected onto an HPLC and the quantity of ascorbic acid determined from the response at 254nm on a UV detector (Brokken, 1990). Even with this procedure consistent results were sometimes difficult to obtain, possibly due to interfering compounds in the prawn feed.

4.1.2 Microencapsulation of methionine.

The aim of this work was to determine the leaching characteristics of the essential amino acid methionine encapsulated in hydrogenated vegetable oil at various inclusion levels.

HVO microcapsules containing methionine were prepared as described in section 3.1.2 at the following coating to core ratios:-

Type 1	1.3 : 1
Type 2	2 : 1
Type 3	3 : 1
Type 4	4 : 1

The ingredients for the standard prawn diet were weighed out and 10 mg of microcapsules per 100g of dry food was included. The feed was mixed, extruded and then the pellets were steamed for 10 min. The steamed pellets were stored under refrigeration until used.

Leaching tests were then conducted as previously described for vitamin C capsules. At the end of the leaching experiment (24 hours) the remaining pellets were removed. 0.1to 0.2 g of the pellets were placed in a beaker and the lipid extracted into 4 ml of

hexane. 4 ml of water was added and the phases allowed to separate. 0.1 ml of the aqueous extract was pippetted into a volumetric flask and made up to 10 ml.

The methionine content of the seawater samples and the solutions prepared above was measured using an HPLC Amino Acid Analysis System. 10 µl samples were injected into the system where the ion exchange column had been removed. This resulted in the sample being converted to the o-phthalaldehyde derivative which passed directly to fluorescence detector. Peak areas from the samples were determined on a computer based integration system.

4.1.3 Feeding trials

Three feeding trials have been carried out with diets containing variations in the souce and content of vitamin C. The trials were carried out using juvenile tiger prawns, *Penaeus esculentus*. Thirty six prawns were held individually for each treatment and their growth rate and mortality monitored over for the duration of the trial: four, six and eight weeks.

These trials were conducted in a seawater aquaria system consisting of 10 x 360 litre black fibreglass tanks. The tanks were aerated with three airstones and supplied with preheated seawater exchanging at about 500% per day. Tank temperatures were maintained at 25 ± 0.5°C. Subdued lighting was provided in a 12h light /12h dark regime.

Eighteen *P. esculentus* juveniles (about 2 g, exact means varied between trials), caught by trawling on the seagrass beds at Oyster Point, Cleveland on Moreton Bay were placed in individual cages in each tank so that total weight and size range were as similar as possible across all tanks. The five treatments were duplicated and assigned to tanks in a completely randomised design. The prawns were acclimatised to the laboratory conditions and the control diet for seven days prior to the start of the trial. The animals were fed *ad lib.* twice a day at 0830h and 1630h. The feeding level was monitored and adjusted daily. Residual food and faeces were removed from the tanks before each feeding. Moults, mortalities and tank temperatures and salinities were recorded daily.

4.1.4 Diet formulation and preparation

The diets in the growth experiments were generally formulated to be identical except for the source and level of the vitamin C. The diets were based on a previously used purified diet that had given satisfactory performance (Table 4).

Table 4. Composition of base diet (% Dry matter)

Ingredients	% Dry matter
Casein	35.0
Albumen	5.0
Gelatin	5.4
Wheat gluten	7.0
Starch	20.0
Cholesterol	0.5
Lecithin	3.0
DL-Methionine	1.0
L-Tryptophan	0.5
Alginate	3.0
Mineral Mix	2.3
Choline chloride	0.6
Vitamin Mix	0.9
Cellulose	10.28
Cod liver oil	4.6

Vitamin C and microencapsulating material were added to the above diet. Cellulose content was varied to accommodate variations in the concentration of the different sources of vitamin C.

The dry ingredients were combined and mixed thoroughly. Water was then added (72 ml per 100g of ingredients) and mixed to a smooth dough. The cod liver oil was then added and mixed through before the dough was extruded through a 3mm diameter die. The spaghetti-like strands were cut into 15mm lengths before they were placed on a

gauze rack and steamed for 5 min. The pellets were stored in a sealed container at 4°C until used.

4.2 Results and Discussion.

4.2.1 Digestibility of microcapsules.

Following the initial assessment of leaching characteristics of spray cooled microcapsules (section 3.2.1) trials were carried out to determine the extent to which prawns could digest the various coating materials being considered for the microcapsules. Paraffin wax, and a range of vegetable and animal fats and oils were tested. Microcapsules were prepared containing a blue dye, Methylene blue, which were incorporated into prawn feed pellets. After the prawns had fed on the pellets, faeces were collected and examined under the microscope. With the paraffin coating none of the microcapsules had been ruptured and the blue core material could be clearly see inside all the microcapsules in the faeces (see figure 8). The presence of large numbers of complete microcapsules and the absence of any partially digested ones indicated that the prawns were unable to digest the paraffin wax and absorb the core material. Trials with hydrogenated vegetable oil showed no evidence of intact microcapsules in the entire fecal pellet (see figure 9). This indicated that the lipid coating was being digested and the dye was released into the digestive tract.

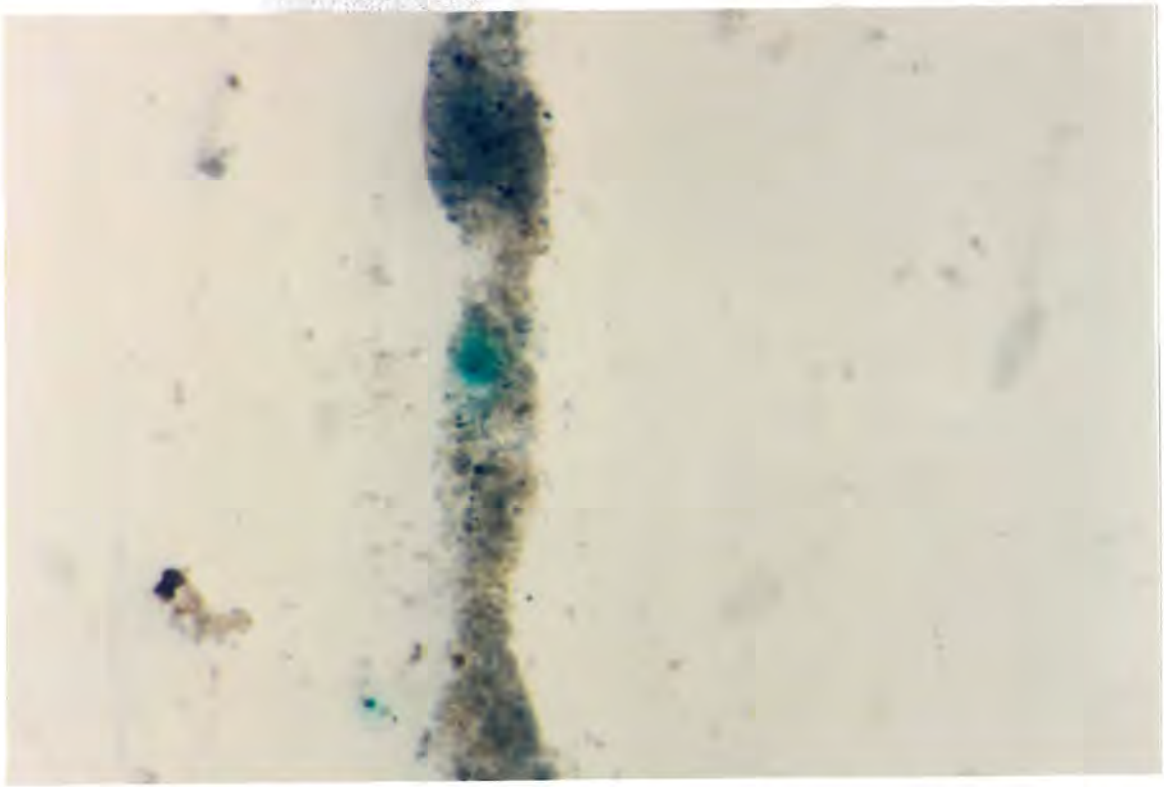

Figure 8. Photograph of prawn fecal pellet containing intact wax microcapsules (blue dots)

Figure 9. Photograph of fecal pellet from prawns feed with HVO microcapsules. No evidence of intact microcapsules.

Table 5. *Penaeus esculentus.* Distribution of radioactivity in seawater and in prawn tissues 3h after ingestion of food pellets containing ^{14}C-proline in HVO microcapsules and encased in zein. Results expressed as a percentage of recovered activity, are means of 7 replicates; standard errors in parentheses.

Compartment	HVO microcapsules	Zein encapsulated[1]
Seawater	41 (7.5)	10 (3.6)
Proventriculus	2 (0.3)	3 (1.3)
Digestive Gland	4 (0.5)	8 (1.3)
Hindgut and faeces	3 (1.2)	1 (2.0)
Muscle	28 (4.1)	52 (3.9)
Remainder	19 (3.5)	27 (1.2)

[1] Data from Smith and Dall, 1991.

These results encouraged us to investigate whether the nutrient in the microcapsule was capable of being absorbed and utilised by the prawn. Previous research by one of the collaborators had investigated the absorption and utilsation of the amino acid proline by *Penaeus esculentus* using radioactively labelled ^{14}C-proline bound in a protein matrix (Smith and Dall, 1991). The same labelled proline was then microencapsulated in a hydrogenated vegetable oil coating, fed to the prawns and the distribution of radioactivity in the various organs and tissues was measured (Table 5). The distribution pattern showed a close similarity to the distribution pattern obtained previously, indicating that the lipid coating was not preventing the absorption and utilisation of the core nutrients. The low levels of radioactivity in the hindgut and faeces indicate that a negligible amont of the core material was passing through the prawns. The results confirmed the suitability of HVO microcapsules as a carrier for micronutrients in prawns.

4.2.2 Leaching of core material from microcapsules incorporated in feed pellets.

Highly soluble components of aquaculture feeds leach very rapidly from the feed once it is placed into a pond. Most of the amino acid proline was found to leach out of diets bound with different binders within one hour (Figure 10). The leaching loss from commercially prepared feeds is likely to be similar to or slightly slower than these rates.

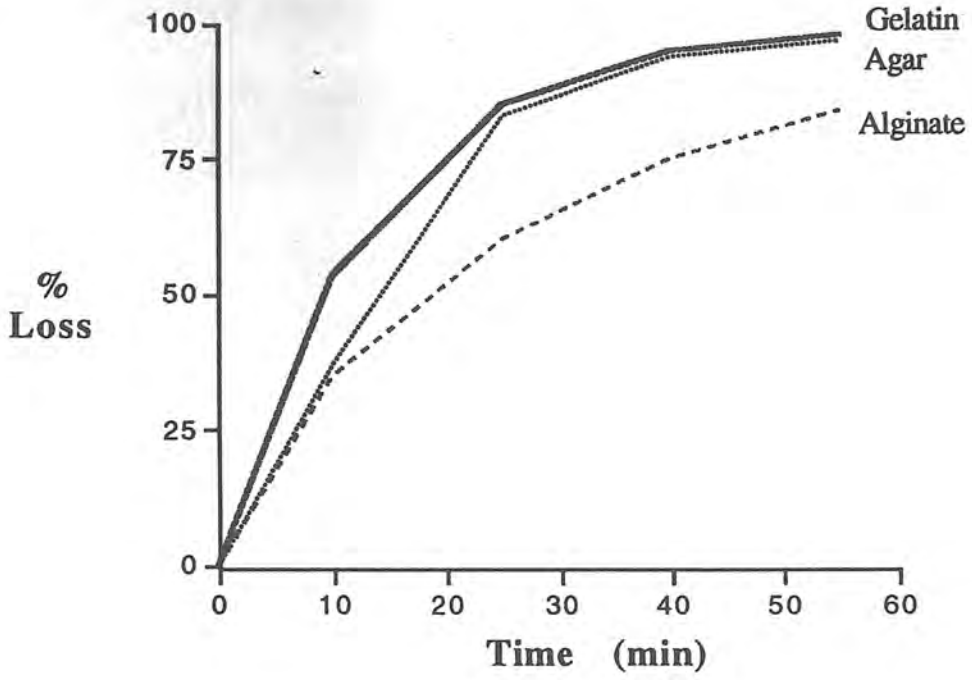

Figure 10. Leaching loss of proline (%) from prawn feed made using various binders.

A comparison was made of the leaching rate of vitamin C into acidic dithiothreitol from Takeda CVC-F90 coated crystalline vitamin C and HVO microcapsules made with a core to coating ratio of 4 : 1. The microcapsules were incorporated into feed pellets according to

the base formulation to give 50µg of vitamin C in 100g dry feed. Experiments were carried out in triplicate to provide a satisfactory level of replication.

The leaching rates of the Takeda coated vitamin C and the HVO microcapsules are very similar (see figure 11). Despite the steaming, which would cause the micrcapsules to melt and the on cooling resolidify, the loss of vitamin C was only about 30% over 2 hours. In commercial prawn farming prawns are fed four or five times per day and the bulk of the food provided is eaten within 2 hours of feeding. Under these circumstances the leaching rate of vitamin C from the feed would be acceptable.

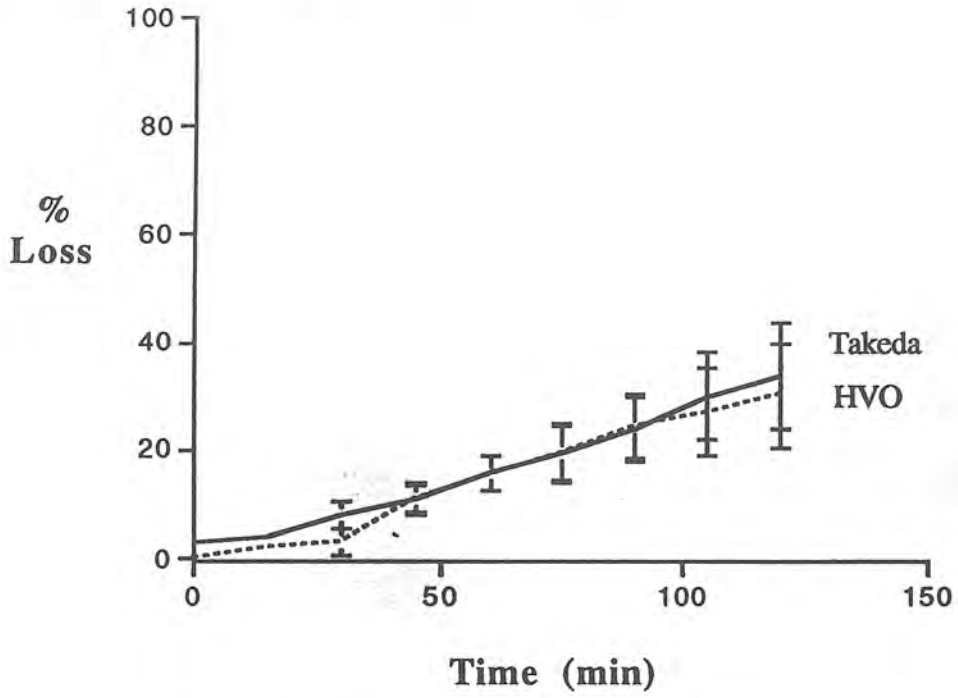

Figure 11. Leaching of encapsulated vitamin C from feed pellets into sea water.

4.2.3 Leaching of methionine

The leaching loss of methionine from the HVO microcapsules with a coating to core ratio of 3:1 and 4:1 was very low, reaching a maximum of less than 5% after 2 hours. At a coating to core ratio of 2:1 the loss was only slightly higher at about 7% but at 1.3:1 the results were more erratic (see figure 12).

It appears that the microencapsulation of amino acids in hydrogenated vegetable oil is an effective method for preventing leaching loss of these nutrients from laboratory prepared prawn feeds. These results are sufficently encouraging to warrant further experiments to determine whether at coating to core ratio of 2:1 is a practical level to use and to determine whether nutritional benefit can be gained from encapsulating crystalline amino acids in this manner.

There appears to be the potential to supplement diets with particular micrencapsulated crystalline amino acids to balance their amino acid profiles to meet the prawns' nutritional requirements. This application may be of greater practical value than the encapsulation of readilly oxidised compounds such as vitamin C.

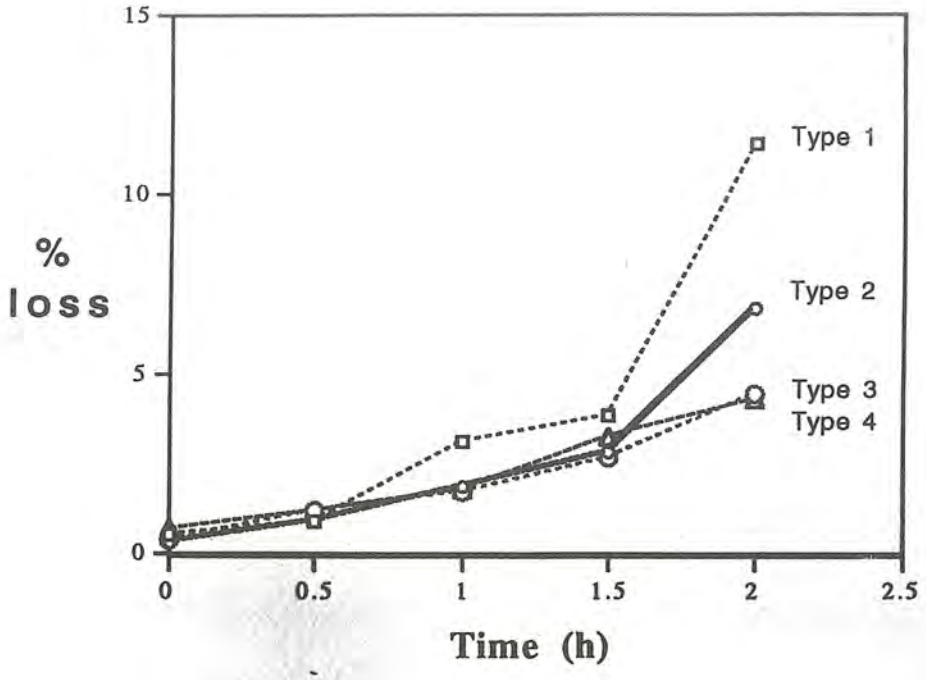

Figure 12. Leaching loss from prawn feed of methionine encapsulated in hydrogenated vegetable oil at various core to coating ratios.

4.2.4 Oxidation of vitamin C in steamed pellets

The laboratory pellets were steamed after extrusion to increase their strength. Commerial feed pellets are routinely made by steam pelletisation. This heating process could possibly cause degradation of the vitamin C. The loss of ascorbic acid activity from steamed laboratory pellets containing encapsulated vitamin C was measured and results are given in Table 6 below. All tests were done in triplicate.

To calculate loss of activity, the measured ascorbic acid activity was compared to that predicted from a mass balance of feed ingredients. Thus, these results show, firstly, that the encapsulation, mixing and low temperature extrusion processes do not degrade the vitamin C. Secondly, steaming does reduce the available vitamin C activity by 20 to 40%. Nevertheless, the majority of the vitamin C is available for the prawns in the steamed pellets.

Table 6. Loss of Activity of Vitamin C on Steaming

Time before testing	%loss of activity in unsteamed pellets	%loss of activity of steamed pellets
0.5 hr	0±10	39±5
72 hr	0±10	24±8

4.2.5 Feeding trials.

A feeding trial was carried out to determine the effect of providing vitamin C at various inclusion levels in HVO microencapsules. Also of interest was the effect of the microcapsule coating on growth and survival. The trial was run over six weeks using the base diet described in Table 4 with the combinations of vitamin C and HVO described in Table 7 below.

Table 7. Diet Formulations for Feeding Trial no. 1.

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Base Diet	88.60	88.60	88.60	88.60	88.60
Vitamin mix (w/o vit. C)	0.90	0.90	0.90	0.90	0.90
Vitamin C (free)	0.22	0.22	-	-	-
Vit. C in HVO microcaps.	-	-	0.25	0.05	-
HVO microcapsules	-	0.60	0.35	0.55	0.60
Cellulose	10.28	10.28	10.50	10.50	10.50

Figure 13 shows the mortality data for this feeding trial. The best survival rate in these experiments occured in prawns fed Diet 3, containing vitamin C microcapsules at a rate of 250mg.100g^{-1} dry feed (50mg Vitamin C). This feed performed better than the two diets containing 220mg free vitamin C per 100g feed. As expected the highest mortality occurred in the diet that contained no vitamin C.

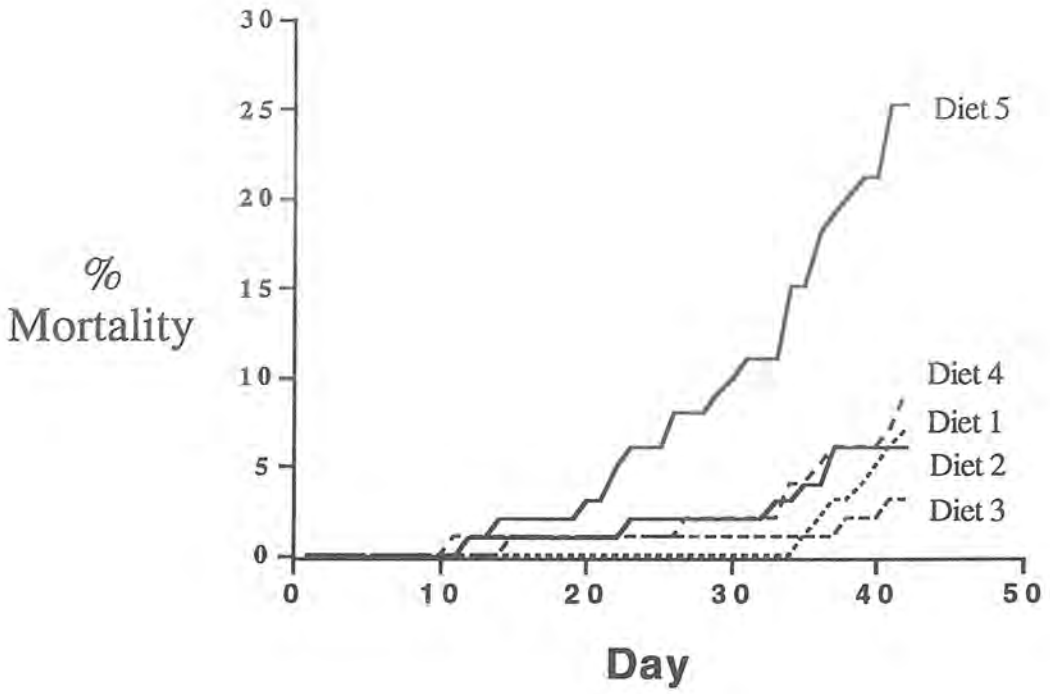

Figure 13. Mortality Data from Feeding Trail no.1. (Diets as specified in Table 7).

In these experiments the growth rates were well below the expected rate (see figure 14). The growth data is unreliable, showing inconsistencies that suggest factors other than those being tested have a dominant effect on the treatments. Diet 1 which was taken as the control diet, though containing the same ingredients as that of a diet used previously with success and considered as the control for this type of experiment, had been prepared somewhat differently to previous occassions. In these experiments the pellets were steamed after extrusion to improve their stability in water. This could have resulted in the degredation of some of the nutrients particularly the oxidation of some of the polyunsaturated fatty acids in the feed. The extent of oxidation could have varied between batches and diets leading to further difficulty in interpretation of the data.

Attempts were made to determine the extent of oxidation of the lipids by measuring the peroxide content of the lipid in the steamed diets using the method of Asakawa and Matsushita (1980). The method is a colorimetric micromethod using aluminium chloride as a catalyst. Despite repeated attempts to obtain consistent results, we were unable to determine the peroxide value of the lipid material with any level of confidence mainly because of the starch in the feed caused a cloudiness to the final solution. To counter the possible oxidation of nutrients in the steamed feeds, an antioxidant, butylated hydroxytoluene (BHT) was added to the feed at 3 parts per million.

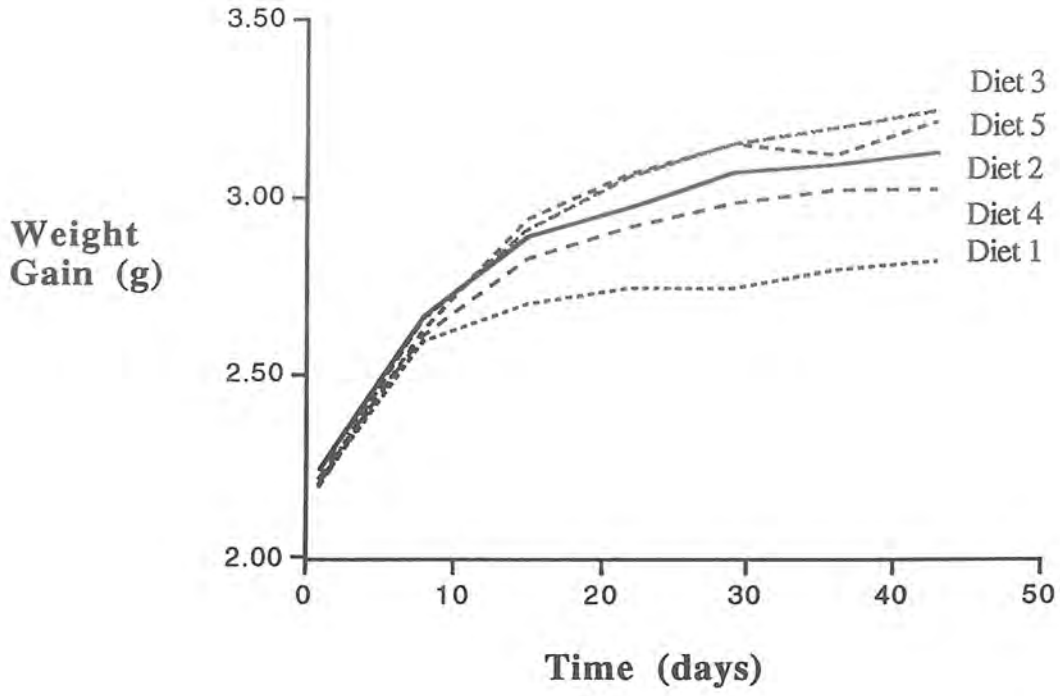

Figure 14. Growth Data from Feeding Trial no.1. (Diets as specified in Table 6.)

A second feeding trial was run to determine if the problem encountered in the first trial had been rectified by the addition of the BHT to the diets. This trial, with *Penaeus esculentus* was run over two weeks with three treatments. The base diet was that of Diet 1 in the previous experiment but (a) unsteamed (b) steamed (c) steamed but including 3ppm BHT. In this experiment the unsteamed diet did not perform as well as the steamed diets, presumably because of the lower pellet stability. The growth rate with the two steamed diets were not significantly different nor were they significantly different from the growth rate achieved in the first two weeks of the previous experiment with Diet 1.

In the third feeding trial *Penaeus monodon* was used as the test species with five treatments each with a different source or inclusion level of vitamin C (see Table 8).

The experiment extended over 8 weeks with the prawns being weighed every two weeks. Though the growth rate of prawns fed the diet containing $50mg.100g^{-1}$ microencapsulated vitamin C was higher than the growth rates of prawns fed the other diets, the difference was not significant. The mortality rate of prawns was also much lower in the prawns fed $50mg.100g^{-1}$ microencapsulated vitamin C (see Table 8). At the end of the experiment, none of these prawns had lesions on their exoskeleton whereas with the other treatments approximately 50% of the prawns had lesions.

Table 8. Mortality rates in prawns fed diets containing various sources of vitamin C.

Diet composition	Mortality (%)
Base diet with 220 mg free AA.100g^{-1}	31
Base diet with 50 mg micro encap. AA.100g^{-1}	25
Base diet with 10 mg microencap. AA.100g^{-1}	44
Base diet with 50 mg Takeda coated AA.100g^{-1}	44
Base diet with all vitamin mix microencapsulated	36

In many species of aquatic animal, vitamin C deficiency is characterised by reduced and sometimes deformed growth and high mortality (Tacon, 1991), Vitamin C deficiency in prawns is characterised by blackened lesions on the exoskeleton, reduced growth and high mortality (Guary *et al.*, 1976; Lightner *et al* 1979, Shigueno and Itoh, 1988). In our experiments the growth rates were low with all treatments and so cannot provide guide to the effectiveness of the microcapsules in protecting and carrying the vitamin C in the prawn feed. However, the mortality rate does provide a very good objective measurement of the effectiveness of the microcapsules as a carrier for the vitamin C. The difference in the incidence of lesions on the exoskeleton also indicates that the prawns were receiving sufficient vitamin C in their diets at an inclusion rate of 50mg. 100g^{-1} feed. This level of free vitamin C in prawn feeds has been demonstrated to be insufficient particularly as up to 75% of it could be destroyed during processing of the feed (Shiau and Hsu, 1994).

5. CONCLUDING REMARKS AND RECOMMENDATIONS

These results have demonstrated that microencapsulation with hydrogenated vegetable oil does provide an effective way of protecting micronutrients in aquaculture diets from oxidation and leaching. Suitable conditions for encapsulation have been identified which will allow scale up to a commercial process. The spray cooling encapulation process is cheap and easy. It is flexible and handles easily fine powders as core materials.

However the microcapsules have not been tested in a commercial pellet press or extruder and may be damaged at the temperatures and pressures involved in these feed manufacture processes. There is possibly less risk associated with their use in an extruder where they could be added towards the end of the extrusion process. It is strongly recommended that feeds containing microencapsulated nutrients are tested in a commercial pellet press or extruder as a final step before attempting to commercialise the process.

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