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Christopher J. Langdon

A new method for encapsulating low-molecular weight, water-soluble substances in lipid-walled microcapsules (LWMs) for delivery to marine bivalves was developed, characterized, and tested. LWMs produced by a spray technique (spray microcapsules, SMs) were demonstrated to encapsulate both aqueous (polymeric dye or oxytetracycline hydrochloride) and particulate (riboflavin) core materials within a capsule wall composed of triacylglycerides. Core materials were most effectively delivered in a capsule wall composed of tripalmitin. Addition of lower melting point lipids to soften the capsule wall significantly reduced (up to a nine-fold reduction) delivery of aqueous core materials, but did not significantly affect particulate riboflavin delivery.

The composition of the capsule wall was demonstrated to affect the digestibility of LWMs. Addition of 40% w/w fish oil to the tripalmitin wall was required for Manila clam spat (*Tapes philippinarum*) enzymatically digest LWMs (convert triacylglycerides to free fatty acids). Delivery of aqueous and particulate core materials by microcapsules was assessed by feeding clams LWMs containing an aqueous core of polymeric dye or a particulate core of oxytetracycline hemicalcium salt (OTC.HEM). The physical

appearance and absence of core material in capsules observed in clam fecal strands suggested release and delivery of core materials in the clam's digestive system.

Optimal methods for encapsulating and storing oxytetracycline were assessed. Oxytetracycline hydrochloride was most efficiently encapsulated (3.2 mg core/ 100 mg lipid) and retained (30%) as an aqueous core in LWMs produced by a double-emulsion process (double-emulsion microcapsules, DEMs). OTC.HEM was most efficiently encapsulated (7.4 mg core/100 mg lipid) and retained (66% of initial encapsulated core material remained after 24 hours suspension in seawater) as a particulate in SMs. SMs containing OTC.HEM were most stable in storage, retaining 86% of their core after 5 weeks of storage as a wet paste. Freeze-drying of SMs containing particulate OTC.HEM increased initial leakage losses of core material, but did not the affect the stability of capsules during long-term storage. Freeze-drying of DEMs containing aqueous OTC.HCl also increased initial leakage loses of core material, but improved the stability of DEMs during long-term storage.

Microencapsulation of Water-Soluble Substances for Delivery to Marine Bivalves

by

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF APPENDIX TABLES

Microencapsulation of Water-Soluble Substances for Delivery to Marine Bivalves

Chapter 1
General Introduction

Microencapsulation and aquaculture

Microencapsulation is a process by which an active ingredient (solid, liquid or gas) is sealed within a coating or shell to form particles ranging in size from less than a micron to several millimeters in diameter. The encapsulated material is frequently referred to as the "core", whilst the shell or coating material is termed the "wall." The techniques for creating microcapsules are most often employed by the pharmaceutical and food industries to both facilitate the delivery and improve the performance (e.g., storage, transport, release) of food additives and drugs (Arshady, 1993; Dziezak, 1988).

Techniques of microencapsulation have been employed in aquaculture to reduce losses of water-soluble nutrients from artificial feeds. "Complete" diets and dietary supplements (containing microencapsulated ingredients) for the larval and adult stages of finfish as well as crustacea are currently available from several manufacturers (e.g., Frippak Feeds, Basingstoke, Great Britain). These diets typically utilize methods of encapsulating ingredients within an alginate bead and/or a cross-linked protein wall capsule. While these methods prove suitable for containing dietary macromolecules, they do not eliminate leaching of low-molecular weight, water-soluble substances, such as water-soluble vitamins, antibiotics, and amino acids. As a result, these capsules may deliver nutritionally inadequate diets or, in the case of medicated feeds, doses below the level necessary for therapeutic effect. High concentrations of unencapsulated nutrients in

the culture water may result in the proliferation of bacteria, introducing or exacerbating disease problems. The discharge of nutrient or antibiotic laden waste-water from aquaculture facilities may also negatively impact the environment.

Progress in the development of encapsulation methods for containing low-molecular weight, water-soluble materials for cultured aquatic organisms has progressed slowly. Development of methods for effectively delivering these materials to bivalves has been further complicated by several factors associated with bivalve physiology. Bivalves are filter-feeding organisms that require potential food items to remain in suspension until they are ingested. Capsules containing water-soluble material must be either neutrally buoyant or somehow effectively maintained in suspension. Capsules must also be micron-sized to be ingested. Larval oysters begin to feed approximately 24 hours (at 21°C) after reaching the D-stage of development (personal communication, Dr. Christopher Langdon, College of Agricultural Sciences, Oregon State University) and the mouth of early veligers is estimated to be no more than 10 μm wide (Langdon and Newell, in press). For oyster spat less than 1 cm in shell length, a recommended median capsule diameter is less than 25 μm (Gabott *et al.*, 1975).

The very small particle size required for bivalve suspension feeders combined with the method of presentation (suspension in an aqueous environment) make it very difficult to effectively encapsulate and deliver a water-soluble material. As the size of the particle is reduced, the surface area to volume ratio increases having a two-fold effect: encapsulation efficiency may be reduced (less core can be encapsulated per quantity of wall material) and leakage of core materials from capsules may increase.

While an effective capsule should retain its core, the encapsulating wall material must be easily ruptured or digested for the core to be delivered to and assimilated by the target organism. Bivalves, unlike crustacea which masticate their food or grind food particles in a gastric mill, consume their food whole and digestion of particles and release

of ingredients must be achieved by the action of digestive enzymes, pH change, and the grinding action of the style (Langdon and Newell, in press).

Review of capsule types developed for bivalves

Attempts to produce artificial diets for a variety of aquaculture species have resulted in several capsule types which have been refined for delivery of dietary materials to bivalves. Currently, no completely satisfactory method of encapsulating low-molecular weight ingredients has been described.

Cross-linked, protein-walled capsules

Capsules prepared with walls consisting of protein and protein cross-linked with nylon, have been frequently used for the delivery of dietary materials to crustacea (*Artemia*, Jones, 1974 ; penaeid shrimp, Jones *et al.*, 1979; Jones *et al.*, 1987; Kanazawa *et al.*, 1982; brachyuran crab larvae, Levine *et al.*, 1983). Juvenile mussels (*Mytilus edulis* , Gabott *et al.*, 1975; Jones, 1978; *Mytilus trossulus* , Kreeger and Langdon, 1993; Kreeger and Langdon, 1994), juvenile oysters (*Crassostrea gigas*, Gabott *et al.*, 1975; Jones, 1978; Langdon, 1989; Langdon and DeBevoise, 1990), and oyster larvae (*Crassostrea virginica*, Chu *et al.*, 1982; *Saccostrea commercialis*, Southgate *et al.*, 1992) will ingest and digest protein-walled capsules. These capsules have shown promise in feeding trials as both replacements and supplements for algal diets (Chu *et al.*, 1982; Gabott *et al.*, 1975; Kreeger and Langdon, 1993; Laing, 1987; Langdon, 1977; Langdon, 1989; Langdon and DeBevoise, 1990; Southgate *et al.*, 1992). While protein-walled capsules (with and without nylon as a cross-linking agent) may be of some use in bivalve diets, this capsule type does not retain low-molecular weight, water-soluble materials (Chu *et al.*, 1982; Gabott *et al.*, 1978; Lopez *et al.*, 1994) and the

use of organic solvents in capsule preparation limits their potential for the delivery of dietary lipid (personal communication, Dr. Christopher Langdon, College of Agricultural Sciences, Oregon State University).

Gelatin-acacia capsules

Gelatin-acacia capsules formed by a coacervation reaction between gelatin and acacia have been used successfully for the delivery of lipids and lipid-soluble materials to bivalves. Langdon and Waldock (1981) used gelatin-acacia capsules to deliver dietary lipid to oyster spat (*Crassostrea gigas*). Growth of oyster spat fed algal diets supplemented with capsules containing the polyunsaturated fatty acid, docosahexaenoic acid (22:6ω3), was improved compared with that of algal-fed controls, and concentrations of this fatty acid in tissues of oysters fed on the lipid supplements were elevated. Chu and coworkers (1982; 1987) delivered fish oil and lipid-soluble vitamins to 2-day old oyster larvae (*Crassostrea virginica*) using gelatin-acacia capsules. These capsules were more easily digested than protein-walled (with nylon as a cross-linking agent) capsules (Chu *et al.*,1982), and were claimed to support development of larvae to metamorphosis (Chu *et al.*, 1987). Although useful for the delivery of dietary lipid and lipid-soluble vitamins, Chu and coworkers (1987) noted that the walls of gelatin-acacia capsules were permeable and only water-insoluble or macromolecular dietary components were effectively contained without loss.

Alginate particles

Delivery of combinations of dietary materials (lipids, solids, and high molecular weight water-soluble nutrients) to bivalves has been achieved by use of microgel particles. Microgel particles are gels of calcium carboxymethyl cellulose, calcium

alginate, gelatin, agar, carrageenan, or chitosan in which lipids or high molecular weight water-soluble substances, such as protein or starch, are entrapped (Langdon *et al.*, 1985). Microgel particles composed of calcium carboxymethyl cellulose (Langdon and Siegfried, 1984) and calcium alginate (Langdon and Bolton, 1984) have been used to deliver protein and carbohydrates to juvenile oysters (*Crassostrea virginica*). While microgel particles are extremely versatile and those composed of calcium carboxymethyl cellulose were digested by juvenile oysters (Langdon and Siegfried, 1984), they are not impermeable and are of little use for encapsulating low-molecular weight, water-soluble substances (Lopez *et al.*, 1994).

Lipid-walled microcapsules

Langdon (1983) described a process of double-emulsion by which an aqueous phase is encapsulated within lipid droplets (menhaden oil + 10%w/v ethyl cellulose) and demonstrated the digestibility of these capsules by oyster spat (*Crassostrea virginica*). Lipid-walled microcapsules (LWMs) prepared by the double-emulsion process [double-emulsion microcapsules (DEMs)] were shown to have very good retention of low-molecular weight , water-soluble materials, retaining 81% of the encapsulated amino acid glycine after 24 hours of suspension in seawater (Langdon *et al.*, 1985). Langdon and Siegfried (1984) delivered dietary materials with some success to oyster spat (*C. virginica*) using a combination of microgel particles (calcium carboxymethyl cellulose) containing protein and starch together with lipid walled microcapsules (primarily composed of menhaden oil) containing a mixture of water-soluble vitamins. Chu and coworkers (1987) reared oyster larvae (*C. virginica*) to metamorphosis on lipid-walled microcapsules (primarily composed of cod liver oil) containing protein, dextrose and vitamins. More recently lipid-walled microcapsules composed of triacylglycerides and

prepared by the double-emulsion process have been used to deliver nutrients to penaeid shrimp larvae (Villamar and Langdon, 1993) and marine fish larvae (Lopez *et al.*, 1994).

Liposomes

Another development in microencapsulation of materials for bivalves has been the use of phospholipid vesicles (liposomes). Liposomes administered to the blood stream of mammals accumulate in cells that exhibit phagocytotic behavior. Studies have shown that phagocytosis may play a role in bivalve digestion and that much of the proteolytic digestion in oysters may occur within the cells lining the digestive diverticula (Owen, 1974). Parker and Selivonchick (1986) demonstrated that liposomes (containing entrapped fluorescent materials) fed to juvenile *Crassostrea gigas* may be phagocytized intact and that liposome-encapsulated, radiolabeled glucose and amino acids are assimilated and used in the synthesis of protein and glycogen. The described liposomes represent a valuable tool for delivering simple and complex materials for intracellular digestion, but are unstable and do not retain encapsulated water-soluble materials well when they are suspended in seawater (personal communication, Dr. Christopher Langdon, College of Agricultural Sciences, Oregon State University).

Limitations of lipid-walled microcapsules

Of the capsule types discussed, only lipid-walled microcapsules prepared by the double-emulsion process described by Langdon (1983) appear to show promise as efficient delivery devices of low-molecular weight, water-soluble materials to aquatic and marine suspension-feeders. While lipid-walled capsules composed of menhaden oil and ethyl-cellulose are digestible by larvae and show retention of water-soluble materials, Langdon reports that the capsules themselves are fragile (the lipid being liquid at room

temperature) and difficult to store (personal communication, Dr. Christopher Langdon, College of Agricultural Sciences, Oregon State University).

Villamar and Langdon (1993) developed a technique for creating lipid-walled microcapsules (LWMs) by a double emulsion technique using the triacylglyceride tripalmitin (melting point 66°C) as the wall material and have shown successful encapsulation of several water-soluble vitamins. These capsules are more robust than those made from menhaden oil and have exhibited excellent storage and retention characteristics. While designed to withstand the rigors of incorporation into the complex gel particles used as diets for penaeid shrimp larvae (Villamar and Langdon, 1993), these capsules may be suitable, provided that they are digestible, as a means for delivering materials to bivalve larvae and post-metamorphic stages.

While LWMs composed of triacylglycerides are relatively stable and show promise as effective delivery devices for low-molecular weight water-soluble materials, the double-emulsion process for encapsulating materials is limited. The most obvious restriction of the double-emulsion process used to form LWMs is that it is most efficient with aqueous core materials (Langdon and Singleton, unpublished data; Langdon and Buchal, unpublished data). As a result, the core material's aqueous solubility limits the quantity of core which may be encapsulated. Materials such as the water-soluble vitamin, riboflavin, have limited solubility (1 gram to 3 to 5 liters of water), and as a result are poorly encapsulated (0.7 mg riboflavin/100 mg lipid) as an aqueous core in DEMs (Langdon and Singleton, unpublished data) While encapsulation of slightly soluble materials, such as riboflavin, may be improved (up to five-fold) by encapsulating core materials as a particulate, the efficiency of the double-emulsion process (the percentage of added core material successfully encapsulated in DEMs) is reduced (from 60-100% for aqueous core solutions to 0-20% for particulate core materials; Langdon and Buchal, unpublished data). Another short-coming of the double-emulsion process is that the process is composed of several steps during which core material may be lost or diluted

(these steps seem to especially restrict the encapsulation of particulate core materials). Finally, the double-emulsion process is complex. While useful for laboratory-scale production of LWMs, the double-emulsion encapsulation process is poorly suited for commercial production methods.

A spray technique for encapsulating either aqueous or solid core materials in lipid-walled microcapsules should be less complex than the double-emulsion process, improve encapsulation and delivery of low-molecular weight, water-soluble materials, and be better suited to commercial production processes. Indeed, particulate riboflavin could be encapsulated in spray microcapsules (SMs) in greater quantities (12.03 mg riboflavin/100 mg lipid) and with greater efficiency (83% of added riboflavin successfully encapsulated) than in DEMs (2.16-3.79 mg riboflavin/100 mg lipid, 20-22% of added riboflavin encapsulated; Langdon and Buchal, unpublished data).

The general goals of this research were to describe the encapsulation and retention of a range of core materials within SMs and to demonstrate that SMs are ingested and digested by marine bivalves.

Chapter 2
Production of Lipid-Walled Microcapsules by a Spray Technique

Introduction

Villamar and Langdon (1993) developed a technique for creating lipid-walled microcapsules (LWMs) by a double-emulsion technique using tripalmitin (melting point, 66°C) as the wall material and have shown successful encapsulation of several water-soluble materials (e.g., thiamin and riboflavin). These double-emulsion lipid-walled capsules (DEMs) are more robust than those made from menhaden oil (Langdon, 1983) and exhibit excellent storage and retention characteristics (Langdon, unpublished data). DEMs composed of triacylglycerides and containing aqueous polymeric dye are broken down and their contents released in the gut of penaeid shrimp larvae (Villamar and Langdon, 1993). These capsules may be suitable, provided that they are digestible, as a means for delivering dietary material to bivalve larvae and post-metamorphic stages.

The double emulsion process for producing LWMs is restricted by the fact that it is most efficient with aqueous core materials (Langdon and Singleton, unpublished data; Langdon and Buchal, unpublished data). As a result, the core material's aqueous solubility limits the quantity of core which may be encapsulated. In order to improve the encapsulation of low-molecular weight, water-soluble materials having a limited solubility (e.g., riboflavin), an efficient technique for encapsulating solid core materials is needed. The food industry employs a technique termed spray cooling to encapsulate core materials within a lipid coating (Blenford, 1986; Jackson and Lee, 1991; Lamb, 1987; Taylor, 1983). The process itself is simple and may be summarized as follows: capsule coating materials are heated above their melting point and combined with a core material. The wall/core emulsion or suspension is sprayed into a chamber containing cooled or ambient air. As the temperature of the atomized lipid decreases below its melting point , the lipid

solidifies trapping the core material within a lipid-walled capsule. Since lipid is used as the coating for these capsules, the core contents are well protected against aqueous environments.

Preliminary experiments were conducted to modify this spray-cooling technique to produce spray microcapsules (SMs) containing particulate vitamins and of a suitable size for ingestion by penaeid shrimp and bivalves. SMs composed of the triacylglyceride tripalmitin (TP, Sigma Chemical Co.) softened with 2% w/w EC-25 (a mixture of mono- and di-glycerides and phospholipid, Durkee Foods) encapsulated a core of powdered riboflavin with seventeen-fold greater efficiency and exhibited equal retention of encapsulated core as DEMs containing droplets of aqueous riboflavin (Langdon and Buchal, unpublished data). Preliminary feeding experiments with mussel (*Mytilus edulis*) larvae (200-240 μm in shell length) demonstrated that microcapsules as large as 8 μm in diameter were ingested, but not digested. SMs composed of mixtures of tripalmitin and lower melting-point lipids (liquid at room temperature, 21°C), such as triolein (a triacylglyceride) or fish oil (a mixture of triacylglycerides), appeared to be partially digested by the larvae. As a result of these preliminary experiments, the effect of several lipid wall compositions on the encapsulation efficiencies (amount of core material encapsulated) and retention of SMs for aqueous core materials (either a polymeric dye or an antibiotic) and particulate core materials (riboflavin) was investigated.

Materials and methods

Spray microcapsule production

Core materials

The polymeric dye, poly(vinylamine) sulfonate (Poly-R, Sigma Chemical Co., Poly-R 478) and the antibiotic, oxytetracycline hydrochloride (OTC.HCl, Sigma Chemical Co.) were chosen for encapsulation as aqueous cores in SMs. Poly-R is a high-molecular weight, water-soluble dye which has been encapsulated in DEMs and used in feeding studies with penaeid shrimp larvae (Villamar and Langdon, 1993). OTC.HCl is a low-molecular weight antibiotic which has been encapsulated in DEMs. DEMs containing OTC.HCl have been incorporated into complex feed particles for use with penaeid shrimp larvae (Langdon and Villamar, unpublished data). These materials were chosen for encapsulation in SMs based upon their previous successful encapsulation in DEMs. The difference in the molecular weights of the two core materials was expected to result in different encapsulation and leakage characteristics of capsules having similar lipid wall compositions. The vitamin, riboflavin was chosen for encapsulation as a particulate core in SMs based upon previous difficulty experienced in effectively encapsulating this material in DEMs. Successful encapsulation of both aqueous and particulate core materials in SMs was expected to demonstrate the flexibility of producing lipid-walled microcapsules by a spray process.

Preliminary experiments were conducted to determine the maximum solubility of the Poly-R and OTC.HCl. Poly-R was determined to be soluble at concentrations up to 10% w/v in distilled water at room temperature (21°C). Solubility of OTC.HCl was improved by acidification; OTC.HCl was soluble at concentrations of up to 50% w/v in 0.2 N HCl. The ratio of aqueous core material to encapsulating lipid used in SM

preparations was set at 3.5 ml to 10 grams (core to lipid) based upon previous experiments to determine optimal core/lipid ratios for formation of DEMs (Langdon and Singleton, unpublished data).

Riboflavin was micronized to a particle size of less than 25 μm using a ball mill (Microdismembrator, B Braun Instruments). The proportion of particulate riboflavin added to the encapsulating lipid for SM preparations was chosen such as to allow the formation of a free-flowing core/lipid mixture upon melting of the lipid. Up to 2 grams of ground riboflavin could be added to 10 grams of molten lipid, while retaining the desired consistency of the core/molten lipid mixture.

Emulsions/suspensions of core and wall materials

Core materials of 10% w/v aqueous Poly-R or 50% w/v OTC.HCl in 0.2 N HCl were added to a molten (90°C) lipid mixture of either 100% w/w tripalmitin (TP, Fluka Chemical Co., >95% pure by manufacturer's GC analysis), or 60% w/w TP /40% w/w triolein (TO, Fluka Chemical Co., >65% pure by manufacturer's GC analysis), or 60% w/w TP/40%w/w fish oil (FO, Dale Alexander's Fish Oil Concentrate) at a ratio of 3.5 ml core to 10 grams of encapsulating lipid. The core/wall mixture was homogenized for 40 seconds (Ultra-Turrex, Jankel-Kunkel) at maximum power to form an emulsion.

Ground riboflavin was added to the above molten (90°C) wall materials at a ratio of 2 grams core to 10 grams wall material. Riboflavin was evenly dispersed into the molten wall material by sonication (Braun-Sonic 2000, B Braun Instruments).

Formation of spray microcapsules

Emulsions and suspensions of core/wall materials were added to a preheated (90°C) thin-layer chromatography (TLC) flask (Kontes Glassware) and sprayed

(compressed air, 120 liters/minute) into a collection chamber containing cold (5°C) 0.2% w/v aqueous polyvinyl alcohol (PVA, Sigma Chemical Co., cold-water soluble) (Figure 2.1). SMs were collected and passed through a 40 μm mesh Nitex screen. SMs less than 40 μm in diameter were retained and filtered onto a glass fiber filter (Whatman 934-AH). SMs were rinsed with 500 ml of a cold (5°C) aqueous solvent (SMs containing Poly-R rinsed with distilled water; SMs containing riboflavin rinsed with 0.05 N NaOH; SMs containing OTC.HCl rinsed with 0.2 N HCl) to remove unencapsulated or exposed core material. SMs received a final rinse of cold (5°C) distilled water and were filtered to produce a wet paste. SMs were stored as a wet paste in sealed glass scintillation vials at 5°C in darkness. Triplicate batches of each core/wall material combination were prepared and stored separately.

Measures of microcapsule performance

Encapsulation efficiency

Spray microcapsule preparations were characterized by the ratio of encapsulated core material to lipid. Triplicate samples from each batch of SMs were dissolved in chloroform and the core extracted with an aqueous solvent (the same solvents as used in the above rinses). Absorbance of aqueous extracts was measured against a solvent blank at the core material's absorbance peak (Poly-R: 251 nm, OTC.HCl: 354 nm, riboflavin: 266 nm). Absorbance was converted to core concentration using regression equations from standard curves prepared for each core material. A sample of the chloroform phase of the extract was added to a predried/tared crucible and dried for 24 hours in a 50°C oven. Lipid content of the extracted SMs was calculated based upon the amount of lipid recovered after evaporation of the chloroform solvent. Encapsulation efficiency was expressed as mg core/100 mg lipid.

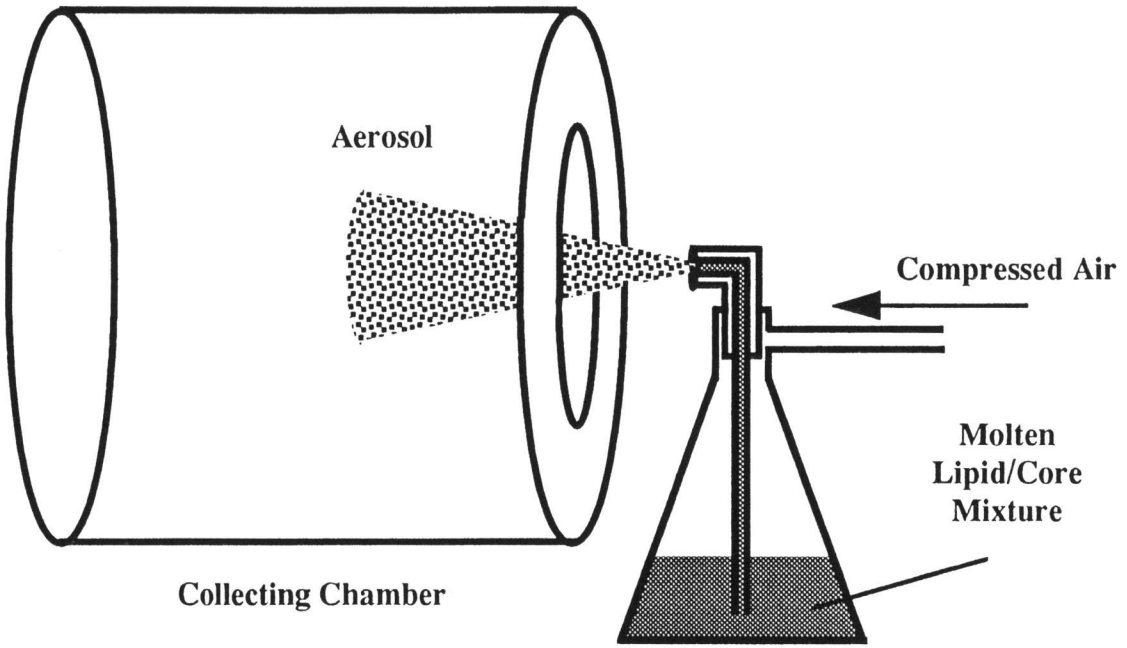

Figure 2.1: Spray microcapsule (SM) production. A mixture of molten lipid and core material was added to a thin-layer chromatography flask and atomized using compressed air. The resulting aerosol solidified forming SMs which dropped into a collecting chamber containing chilled 0.2% aqueous polyvinyl alcohol (PVA). SMs were drained from the collection chamber, passed through a 40 μm mesh Nitex screen, and filtered to produce a wet paste.

Retention efficiency

SM preparations were characterized by the percentage of initial core material retained by SMs after 24 hours suspension in seawater. SMs were suspended with low intensity (capsules not lysed by sonication) bursts of a sonicator (Braun -Sonic 2000, B Braun Instruments) into 0.5 ml of cold (5°C) 2% w/v aqueous PVA and taken to 5 ml in cold (5°C), filtered (Whatman GF/C) seawater (salinity 33 ppt, pH 8.0) containing 100 ppm of the preservative thimerosal (Sigma Chemical Co.). Six 0.5 ml aliquots of microcapsules were removed from the stirred suspension and added to glass scintillation vials containing 19.5 ml of filtered seawater. The final concentration of SMs added to the scintillation vials was adjusted such that the quantity of core material contained within the SMs was free to dissolve into the seawater. The contents of three vials were filtered onto glass fiber filters (Whatman GF/C) and rinsed with seawater to obtain zero hour (T=0 hr) measures of leakage. The remaining three vials were sealed and placed on a shaker table in darkness for 24 hours at 21°C. At 24 hours, suspensions of SMs were cooled on ice, filtered and rinsed with seawater to obtain 24 hour measures of leakage (T=24 hr). SMs retained on filters at T=0 hr and T=24 hr were extracted (as described above) and their encapsulation efficiencies calculated (T=0 hr and T=24 hr encapsulation efficiencies). The total amounts of core material recovered (in seawater filtrate and capsule extracts) at T=0 hr and T=24 hr were compared to test for degradation of core materials in seawater and errors in measurement of core materials. The 24 hour retention efficiency of microcapsules was expressed as the percentage of the initial core retained after 24 hours suspension in seawater and determined by dividing the encapsulation efficiency of microcapsules recovered after 24 hours by their initial encapsulation efficiency at zero hours [retention efficiency = (T=24 hr encapsulation efficiency/T=0 hr encapsulation efficiency) *100].

Delivery efficiency

The variable delivery efficiency was calculated to allow comparison of SM preparations in terms of the amount of core material which may be delivered after 24 hours suspension in seawater. "Theoretical" delivery efficiency (encapsulation efficiency * retention efficiency) assumes that no leakage (e.g., storage leakage) occurs between the measurement of a batch's encapsulation efficiency and the testing of a batch's 24 hour retention efficiency. Such a variable allows a comparison of the theoretical maximum delivery of core material by each batch of SMs. "True" delivery efficiency is simply the amount of material remaining in an aliquot of microcapsules after 24 hours in seawater (T=24 hr encapsulation efficiency from retention efficiency determinations described above). The true delivery efficiency reflects all leakage loses before (storage losses) and during the 24 hour retention efficiency test period. True delivery efficiency is less representative of the intrinsic characteristics of the SMs since it will vary with the amount of time elapsing prior to measurement of retention efficiency due to leakage over time in storage. Theoretical delivery efficiency based upon adjusting the encapsulation efficiency (amount of core material initially encapsulated on the day of microcapsule preparation) with the 24 hour retention efficiency, provides a value which describes the potential of the capsules to deliver their core material when suspended in seawater for 24 hours immediately after preparation (i.e., before storage leakage). This theoretical value allows more accurate comparison between capsule preparations held in storage for different amounts of time prior to measurement of retention efficiency.

Theoretical delivery efficiencies were calculated from the batch averages of encapsulation and retention efficiencies for each core type/lipid type combination (e.g., Poly-R/100% w/w TP), and expressed as mg core/100 mg lipid delivered after 24 hours suspension in seawater.

Statistical analysis

The effect of lipid wall composition (lipid type: 100% w/w TP, 60% w/w TP/40% w/w TO, or 60% w/w TP/40% w/w FO) on encapsulation efficiency was assessed separately for each core material (core type: Poly-R, OTC.HCl, or riboflavin) by one-way nested analyses of variance (ANOVAs) of the arcsine square-root transformed data (Sokall and Rolf, 1981). Replicate measures (n=3) of replicate batches (n=3) were nested within each lipid type to obtain estimates of measurement error and interbatch variability. The contribution of each nesting level to the overall variability of the data was calculated (Sokall and Rolf, 1981). Pairwise comparisons between lipid types within each core type were made using Tukey Honest Significant Difference (HSD) multiple range tests.

The effects of lipid type on retention efficiency and delivery efficiency were measured by separate one-way ANOVAs of the arcsine square-root transformed batch averages for each core type. Pairwise comparisons between lipid types within each core type were made using Tukey HSD multiple range tests.

The effect of core type on the retention efficiency of SMs was analyzed by grouping the core and lipid variables. A single one-way ANOVA was conducted on the arcsine square-root transformed, batch averaged retention efficiencies for each core type/lipid type combination (e.g., OTC.HCl/TP, Poly-R/TP, riboflavin/FO, etc.). Pairwise comparisons of core type /lipid type combinations (e.g., OTC.HCl/TP vs. Poly-R/TP) were made using Tukey HSD multiple range tests. In this way, the effect of the type of encapsulated core material on the retention efficiency of SMs composed of similar lipid types could be assessed.

The suitability of the data for each analysis by ANOVA was checked by viewing normal probability plots of residuals and by Bartlett's test for homogeneity of variance at the 5% level of significance.

Results

Statistical Analysis

The effect of lipid type, in those analyses where it was significant, accounted for approximately 97% of the variability. Although the effect of batch was significant ($p<0.05$ batch effect, one-way nested ANOVA) for each core type, the contribution of batch effect to the total variability was very small, accounting for 2.5 to 3% of total variability. The significance of batch effect was attributed to extremely low measurement error (0.05 to 0.08% of the total variability) and in terms of the overlying lipid effect considered insignificant. Therefore, the analysis of the effect of lipid type on encapsulation efficiency for each core type was carried out, by averaging the arcsine square-root transformed encapsulation efficiencies for each batch. Separate one-way ANOVAs for each core type were carried out using the resulting response variable of batch averages.

Spray microcapsules containing Poly-R

The mean encapsulation, retention and delivery efficiencies and associated standard deviations for each lipid type are listed in Table 2.1 and presented graphically in Figure 2.2.

Lipid type had a highly significant effect on encapsulation ($p<0.0001$, one-way ANOVA, $F_{2,6}=96.64$), retention ($p<0.0001$, one-way ANOVA, $F_{2,6}=33.82$), and delivery efficiency ($p<0.0001$, one-way ANOVA, $F_{2,6}=112.19$) of SMs containing Poly-R. The encapsulation, retention and delivery efficiencies of SMs composed of 100% w/w TP were significantly higher than those softened with either 40% w/w TO or FO ($p<<0.05$, Tukey HSD multiple range tests; Figure 2.2). There were no significant

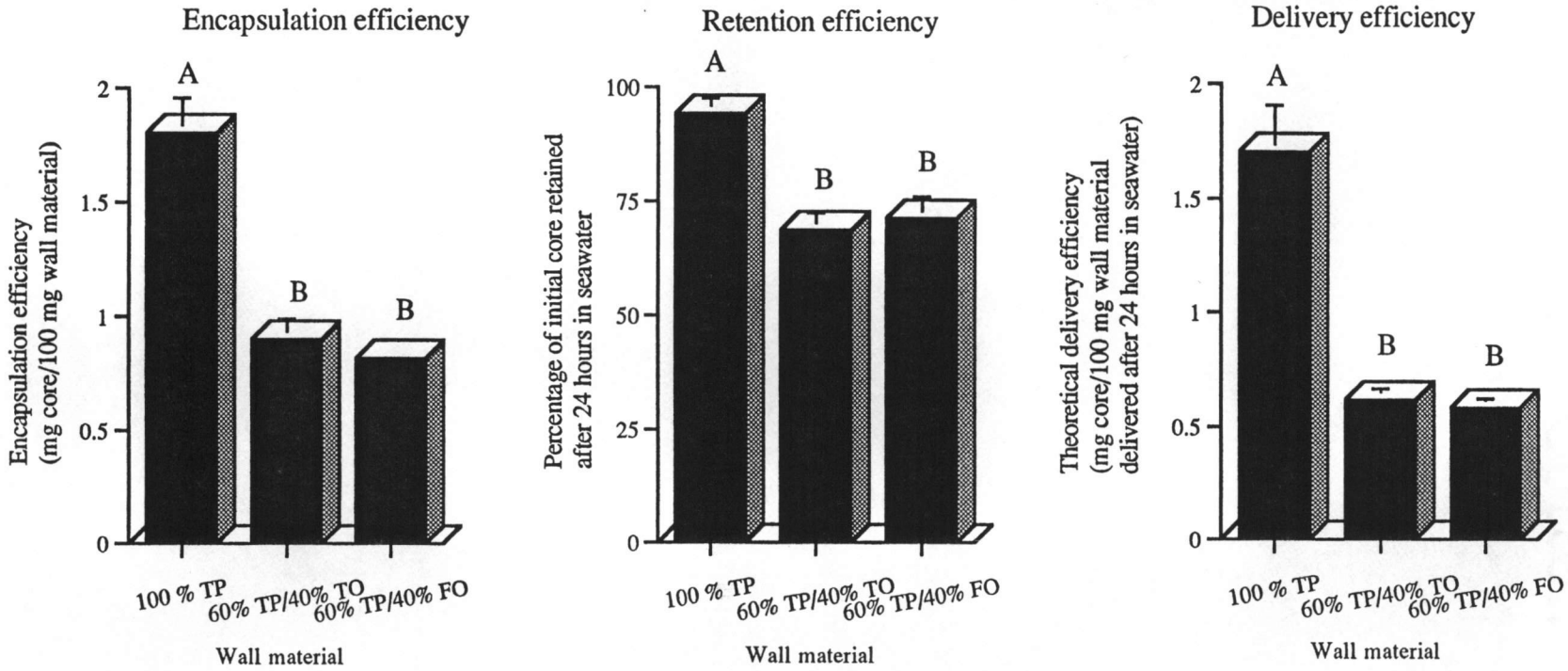

Figure 2.2: Encapsulation, retention, and delivery efficiencies of spray microcapsules (SMs) containing aqueous Poly-R 478 (Poly-R). 100% TP = 100% w/w tripalmitin, 60% TP/ 40% FO = 60% w/w triplamitin/40% w/w triolein, 60% TP/ 40% FO = 60% w/w tripalmitin/ 40% w/w fish oil. Letters within each graph denote significant differences ($p<0.05$, Tukey Honest Significant Difference (HSD) multiple range tests).

Table 2.1: Encapsulation efficiencies (mg core/100 mg lipid), retention efficiencies (percentage of initial core retained after 24 hours in seawater), and theoretical delivery efficiencies (mg core/100 mg lipid delivered after 24 hours in seawater) of spray microcapsules (SMs) containing 10% w/v aqueous Poly R-478 (Poly-R).

Lipid wall composition	Encapsulation efficiency (± standard deviation)	Retention efficiency (± standard deviation)	Delivery efficiency (± standard deviation)
100% w/w tripalmitin	1.81±0.15	94.23±3.24	1.71±0.20
60% w/w tripalmitin 40% w/w triolein	0.90±0.09	68.71±3.63	0.62±0.05
60% w/w tripalmitin 40% w/w fish oil	0.82±0.03	71.24±4.70	0.58±0.04

Note: Maximum possible encapsulation efficiency of SMs for Poly-R (based upon initial core concentration and ratio of core material added to lipid) = 3.5 mg core/100 mg lipid

differences between the encapsulation, retention or delivery efficiencies of SMs softened with 40% w/w TO or FO ($p>>0.05$, Tukey HSD multiple range tests; Figure 2.2).

Spray microcapsules containing OTC.HCl

The mean encapsulation, retention and delivery efficiencies and associated standard deviations for each lipid type are listed in Table 2.2 and presented graphically in Figure 2.3.

Lipid type had a highly significant effect on encapsulation ($p<0.0001$, one-way ANOVA, $F_{2,6}=114.38$), retention ($p<0.0001$, one-way ANOVA, $F_{2,6}=125.54$), and delivery efficiency ($p<<0.0001$, one-way ANOVA, $F_{2,6}=1237.11$) of SMs containing OTC.HCl. SMs composed of 100% w/w TP had significantly higher encapsulation, retention and delivery efficiencies than those softened with either 40% w/w TO or FO ($p<0.05$, Tukey HSD multiple range tests; Figure 2.3). SMs softened with 40% w/w FO encapsulated significantly more OTC.HCl than those softened with 40% w/w TO ($p<0.05$, Tukey HSD multiple range test), however, there were no significant differences

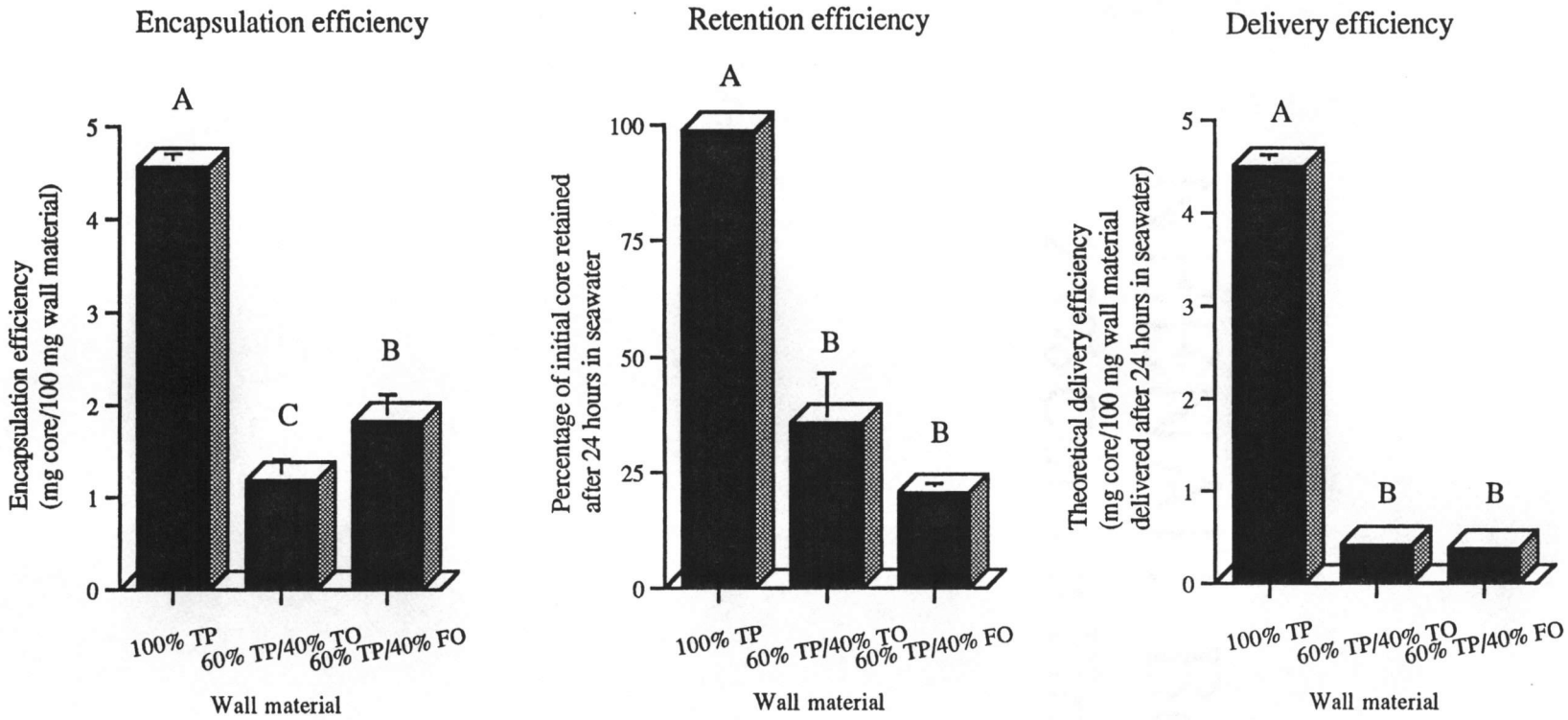

Figure 2.3: Encapsulation, retention, and delivery efficiencies of spray microcapsules (SMs) containing aqueous oxytetracycline hydrochloride (OTC.HCl). 100% TP = 100% w/w tripalmitin, 60% TP/ 40% FO = 60% w/w triplamitin/40% w/w triolein, 60% TP/ 40% FO = 60% w/w tripalmitin/ 40% w/w fish oil. Letters within each graph denote significant differences ($p<0.05$, Tukey Honest Significant Difference (HSD) multiple range tests).

Table 2.2: Encapsulation efficiencies (mg core/100 mg lipid), retention efficiencies (percentage of initial core retained after 24 hours in seawater), and theoretical delivery efficiencies (mg core/100 mg lipid delivered after 24 hours in seawater) of spray microcapsules (SMs) containing 300 mg/ml aqueous oxytetracycline hydrochloride (OTC.HCl) in 0.2 N HCl.

Lipid wall composition	Encapsulation efficiency (± standard deviation)	Retention efficiency (± standard deviation)	Delivery efficiency (± standard deviation)
100% w/w tripalmitin	4.56±0.14	98.65±1.66	4.50±0.13
60% w/w tripalmitin 40% w/w triolein	1.19±0.23	35.86±10.61	0.41±0.08
60% w/w tripalmitin 40% w/w fish oil	1.83±0.28	20.63±1.96	0.37±0.03

Note: Maximum possible encapsulation efficiency of SMs for OTC.HCl (based upon initial core concentration and ratio of core material added to lipid) = 10.5 mg core/100 mg lipid

between either the retention or delivery efficiencies of these SMs ($p<0.05$, Tukey HSD multiple range tests; Figure 2.3).

Spray microcapsules containing riboflavin

The mean encapsulation, retention and delivery efficiencies and associated standard deviations for each lipid type are listed in Table 2.3 and presented graphically in Figure 2.4. Lipid type had only a marginally significant effect on the encapsulation efficiency of SMs containing particulate riboflavin ($p=0.074$, one-way ANOVAs, $F_{2,6}=4.14$). The effect of lipid type on the retention efficiency of these SMs was highly significant ($p=0.0004$, one-way ANOVA, $F_{2,6}=38.39$). SMs composed of 100% w/w TP retained significantly more riboflavin than those softened with 40% w/w TO or FO ($p<<0.05$, Tukey HSD multiple range tests; Figure 2.4). There was no significant difference between the retention efficiencies of SMs softened with 40% w/w FO or TO ($p>>0.05$, Tukey HSD multiple range tests; Figure 2.4). Delivery efficiency of SMs was not significantly affected by lipid type ($p=0.16$, one-way ANOVA, $F_{2,6}=2.54$).

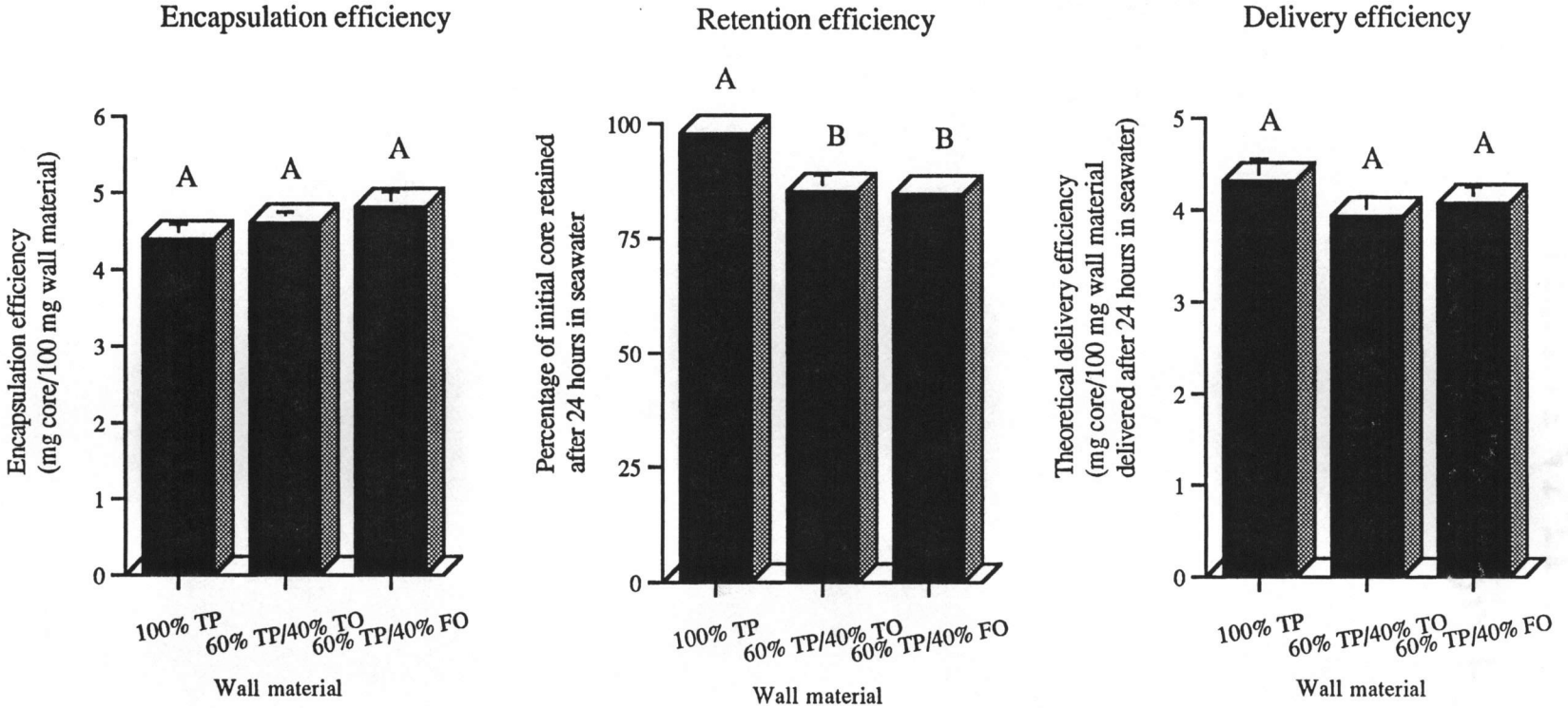

Figure 2.4: Encapsulation, retention, and delivery efficiencies of spray microcapsules (SMs) containing particulate riboflavin. 100% TP = 100% w/w tripalmitin, 60% TP/ 40% FO = 60% w/w triplamitin/40% w/w triolein, 60% TP/ 40% FO = 60% w/w tripalmitin/ 40% w/w fish oil. Letters within each graph denote significant differences ($p<0.05$, Tukey Honest Significant Difference (HSD) multiple range tests).

Table 2.3: Encapsulation efficiencies (mg core/100 mg lipid), retention efficiencies (percentage of initial core retained after 24 hours in seawater), and theoretical delivery efficiencies (mg core/100 mg lipid delivered after 24 hours in seawater) of spray microcapsules (SMs) containing particulate riboflavin.

Lipid wall composition	Encapsulation efficiency (± standard deviation)	Retention efficiency (± standard deviation)	Delivery efficiency (± standard deviation)
100% w/w tripalmitin	4.41±0.19	97.90±1.30	4.31±0.24
60% w/w tripalmitin 40% w/w triolein	4.62±0.14	85.14±3.60	3.94±0.21
60% w/w tripalmitin 40% w/w fish oil	4.82±0.20	84.58±1.52	4.07±0.18

Note: Maximum possible encapsulation efficiency of SMs for riboflavin (based upon initial core concentration and ratio of core material added to lipid) = 20 mg core/100 mg lipid

Effect of core material on retention efficiency

The combined variable of core type/lipid type had a highly significant effect on the retention efficiency of SMs ($p << 0.00001$, one-way ANOVA, $F_{8,18} = 87.03$). The average retention efficiencies and associated standard deviations for each core type/lipid type combination are displayed in Figure 2.5.

Retention of the different aqueous cores (Poly-R or OTC.HCl) by SMs composed of similar softer lipid mixtures was significantly different. A core of aqueous Poly-R was retained significantly better than a core of OTC.HCl in SMs composed of 40% w/w TO ($p<0.05$, Tukey HSD multiple range test of the core type /lipid type combination, OTC.HCl/TO vs. Poly-R/TO; Figure 2.5). Similarly, a core of aqueous Poly-R was retained significantly better than a core of OTC.HCl in SMs composed of 40% w/w FO ($p<0.05$, Tukey HSD multiple range test of the core type /lipid type combination, OTC.HCl/FO vs. Poly-R/FO; Figure 2.5). Retention of these aqueous cores encapsulated in 100% w/w TP did not differ significantly ($p>0.05$, Tukey HSD multiple range test of the core type/lipid type combination, OTC.HCl/TP vs. Poly-R/TP; Figure 2.5).

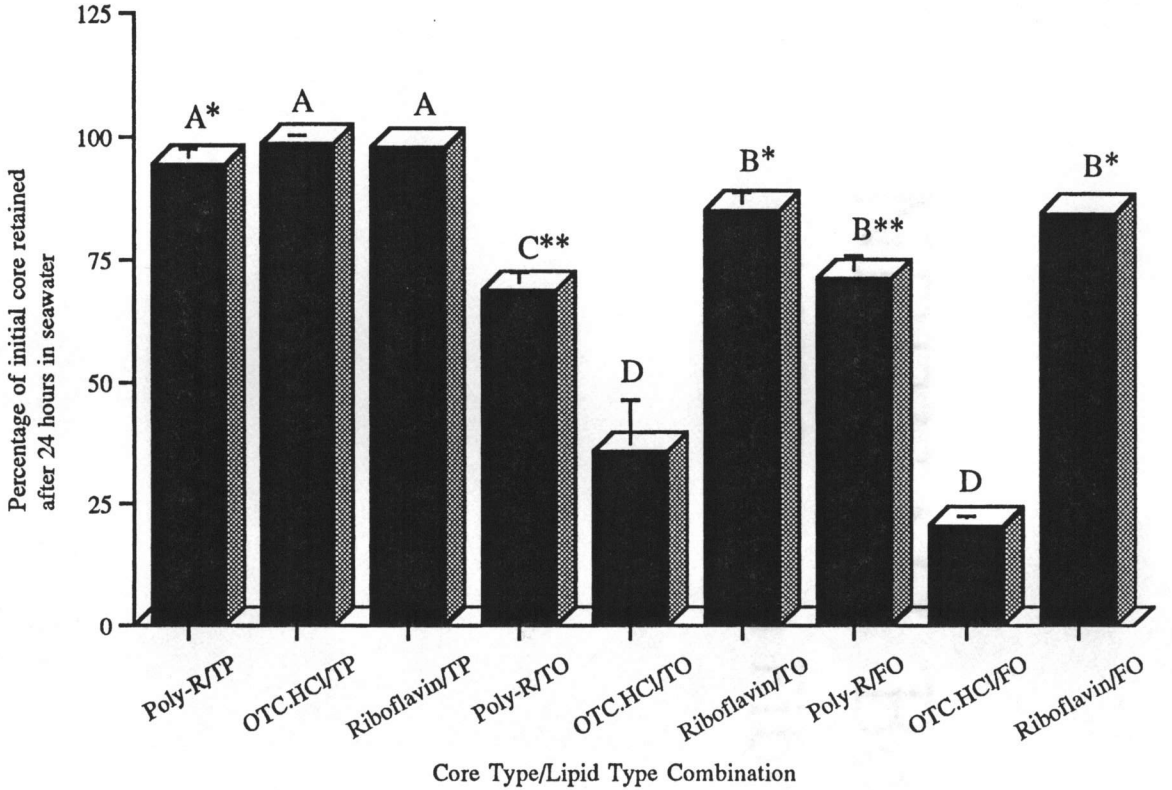

Figure 2.5: Retention efficiencies of spray microcapsules (SMs) containing aqueous Poly-R 478 (Poly-R), aqueous oxytetracycline hydrochloride (OTC.HCl), or particulate riboflavin. TP=100% w/w tripalmitin wall composition, TO=60% w/w tripalmitin/40% w/w triolein wall composition, FO=60% w/w tripalmitin/40% w/w fish oil wall composition. Letters denote significant differences ($p<0.05$ Tukey Honest Significant Difference (HSD)multiple range tests of core type/lipid type combinations) except as noted. Note: asterisk(s) denote a nonsignificant ($p>0.05$) difference between core type/lipid type combinations.

Riboflavin was retained better in SMs composed of the softer lipid mixtures (40% w/w TO or 40% w/w FO) than were either of the two aqueous cores (Poly-R or OTC.HCl). Retention of riboflavin by SMs softened with 40% w/w TO was significantly higher than retention of either aqueous core by SMs having a similar wall composition ($p<0.05$, Tukey HSD multiple range tests of core type/lipid type combinations: riboflavin/TO vs. Poly-R/TO, riboflavin/TO vs. OTC.HCl/TO; Figure 2.5). Retention of riboflavin by SMs softened with 40% w/w FO was significantly higher than retention of aqueous OTC.HCl ($p<0.05$, Tukey HSD multiple range test; Figure 2.5). Retention of riboflavin by SMs softened with 40% w/w FO was only marginally higher than retention of aqueous Poly-R by similar-walled capsules ($p=0.105$, Tukey HSD multiple range test ; Figure 2.5).

All core types (Poly-R, OTC.HCl, and riboflavin) were retained equally well within SMs composed of 100% w/w TP ($p>>0.05$, Tukey HSD multiple range tests of core type/lipid type combinations; Figure 2.5).

Discussion

General Overview

Preliminary experiments suggested that SMs could be used to effectively encapsulate a particulate material (riboflavin), but that the wall of the SMs required softening in order for digestion or release of core material by bivalves. The goal of the present experiments was to investigate the effect of softening the SM wall on several measures of SM performance (encapsulation, retention and delivery efficiency) and to determine whether there were any significant differences in these performance measures between SMs softened with either of two low melting point lipids (TO or FO).

The type of core material encapsulated may also affect SM performance. While aqueous and particulate cores were chosen for encapsulation to demonstrate the flexibility of the spray method for forming LWMs, the phase of the core material (aqueous or solid) may cause differences in the physical structure of the resulting capsules and their performance. Aqueous core materials were expected to be encapsulated as discrete droplets within a lipid bead, while particulate core materials were expected to be evenly distributed throughout the lipid bead (Figure 2.6). These differences in physical structure might affect SM performance (encapsulation, retention, and delivery efficiency).

Encapsulation efficiency may be largely affected by the ability of the lipid to surround the core material. In terms of a particulate core, this might be reflected by how well the core material is distributed and remains distributed in the lipid wall (a function of core and lipid characteristics) prior to solidification of the SM. In terms of an aqueous core, the encapsulation efficiency may be determined by the quality and stability of the emulsion formed with the aqueous core and lipid wall material (again, a function of undefined core and lipid characteristics) prior to solidification of the SM. Encapsulation efficiency might also affected by the rapid short term leakage of entrapped core through ruptures or pores in the SM wall prior to sampling and measurement of a batch of SM's encapsulation efficiency.

Retention efficiency may in large part be affected by diffusional losses of core material through the capsule wall. This diffusion may occur through pores or ruptures in the lipid wall which connect the core to the external capsule environment or by dissolution or transport of the core material into or through the lipid wall. The magnitude of pore formation, the rate of diffusion of core material through the pores, as well as the ability of the core to diffuse into or through the lipid wall itself, are dependent on characteristics of the core and lipid materials.

While the phase of the core material may affect the physical structure of the capsule, the characteristics of the core material itself may also affect capsule performance.

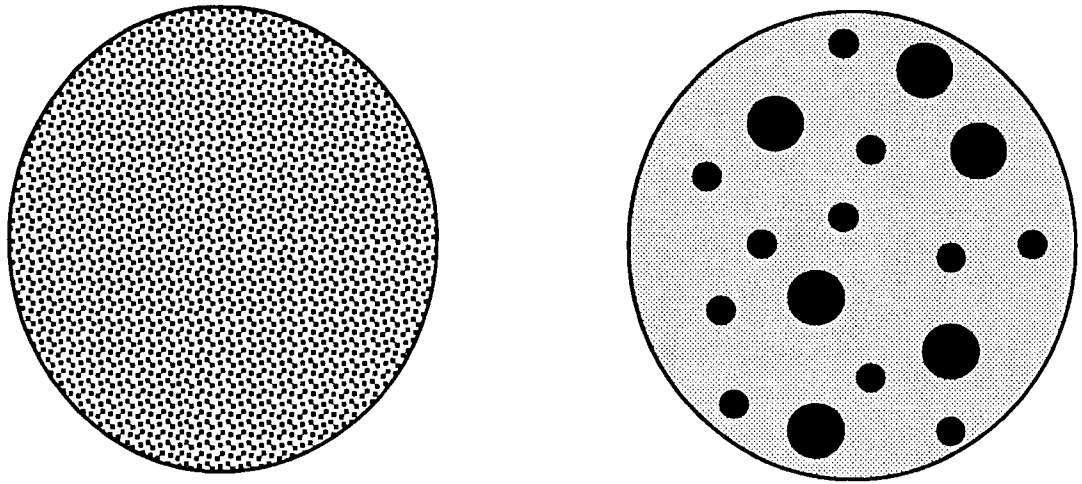

Figure 2.6: Spray microcapsules (SMs) containing particulate or aqueous core materials. Particulate core materials appeared distributed evenly throughout the lipid capsule. Aqueous core materials appeared as droplets entrapped in the lipid capsule.

Aqueous cores (Poly-R and OTC.HCl) having different molecular weights, aqueous solubilities, etc. were chosen to investigate whether differences in the characteristics of the core would impact the performance of SMs. Riboflavin was chosen for encapsulation in SMs since it had been encapsulated with only limited success by DEMs and was expected demonstrate the superiority the spray technique for encapsulating materials, having limited aqueous solubility, as a solid core.

Main effects

The effect of lipid type on SM performance varied with core type. Comparison of the encapsulation, retention, and delivery efficiencies of SMs for the aqueous core materials of Poly-R or OTC.HCl suggested that in each case a softening of the SM wall resulted in a significant reduction in core encapsulation, retention, and delivery. The effect of lipid wall composition on the performance of riboflavin SMs was less pronounced. While the effect of lipid type on encapsulation efficiency was only marginally significant ($p=0.074$, one-way ANOVA), encapsulation of riboflavin was greater for SMs with softer lipid walls (Table 2.3; Figure 2.4). As with SMs containing aqueous cores (Poly-R or OTC.HCl), retention efficiency of SMs containing riboflavin was significantly reduced as a result of softening the SM wall with either 40% w/w TO or 40% w/w FO. A slight increase in encapsulation efficiency in combination with a decrease in retention efficiency, interacted in such a way that SMs composed of the three different lipid mixtures were equally effective in delivering riboflavin ($p=0.16$, one-way ANOVA).

The observed reductions in encapsulation and retention efficiencies which accompany softening of the SM wall are most easily explained by changes in SM wall permeability caused by both the purity of the encapsulating lipids and the process of SM formation.

Effect of wall composition on retention efficiency

SMs are formed when a molten lipid/core mixture is atomized to produce lipid droplets. As the lipid droplets pass through ambient air in the collection chamber, they solidify and entrap the core material. Lipids entrapping core material may exist in either a "frozen" gel state or a liquid-crystalline state (Cullis and Hope, 1985). In the gel state, the hydrocarbon chains of the lipid adopt a highly ordered crystalline packing arrangement. The permeability of lipids in this gel state is generally very low. Above the lipid phase transition temperature (T_C), the lipid adopts a liquid-crystalline state in which the order of the hydrocarbon chains is disrupted and the rotation and molecular motion of the lipid increases. It is recognized that the stability of the gel-state and the fluidity of the lipid-crystalline state is influenced by the degree of saturation of the component lipids (Cullis and Hope, 1985); the presence (and degree) of unsaturated lipids interrupts the ordered packing of the hydrocarbon chains of the lipid in both states.

Addition of lipids of differing degrees of saturation or different classes of lipids may interrupt the packing arrangement of lipids in the gel-state. Braun and Olson (1986) observed that microcapsules formed from mixtures of lipids had pores or micro-canals which increased the permeability or the capsules. In general, increasing the proportion of dissimilar lipid in the wall of microcapsules will result in greater disruption of the capsule wall, exacerbate formation of pores in the capsule wall, and increase capsule wall permeability (personal communication, Dr. Mark Christensen, College of Pharmacy, Oregon State University). The tripalmitin used as a base material for these SM preparations exhibited an relatively high melting point (66°C) and consisted of 99.6% by weight palmitic acid (16:0 saturated fatty acid; Appendix A). Lipid in the wall of capsules prepared from 100% w/w tripalmitin would be expected to adopt an ordered crystalline packing arrangement and exist in a gel-state at room temperature (21°C). As a result of this wall structure, capsules formed from tripalmitin should have been fairly impermeable

to core materials. The added softening agents of TO and FO both contained a large proportion of longer chain unsaturated fatty acids (Appendix A) which might have been expected to disrupt the packing arrangement of the tripalmitin wall. This disruption may have caused the formation of pores or cracks in the capsule wall, increasing the permeability of the SM. A confounding issue is the fact that both the added triolein and fish oil exist in a liquid-crystalline state at the temperature in which the SMs were stored (5°C) and tested for 24 hour retention efficiency (21°C). Addition of these lipids, rather than causing the formation of pores, may have simply caused a change in the phase of the encapsulating lipid mixture, disrupting the order of the lipids and increasing the fluidity of the capsule wall. Regardless, the permeability of SMs softened with TO or FO increased significantly relative to SMs composed of 100% w/w TP (Figures 2.2, 2.3, and 2.4).

While addition of unsaturated lipids and softer lipids to spray microcapsules may increase wall permeability by either disrupting the gel-state of the TP capsule or causing a phase transition of the encapsulating lipid, previous experiments have indicated that specific "contaminants" in the bead wall can have profound effects on wall permeability. Addition of phospholipid (soy phosphatidylcholine, PC) to the wall of SMs containing riboflavin has been observed to cause substantial reductions in retention efficiency (Appendix B). Addition of 5 and 10% w/w PC to the lipid wall increased leakage of these SMs by 18 and 35%, respectively. Analysis of the lipids (TP, TO, and FO) used in the current experiments indicated that while the TP was composed solely of triacylglycerides and cholesterol, both the TO and FO were contaminated with similar quantities of phospholipid (7.4% and 7.8% by weight, respectively; Appendix C). Therefore, approximately 3% w/w phospholipid was added to the SM wall when the either 40% w/w TO or 40% w/w FO were added. While the reductions in retention efficiency observed for riboflavin SMs softened with TO or FO (approximately 12%; relative to SMs composed of 100% w/w TP ; Table 2.3) agree with the reductions reported in Appendix B (approximately 18% reduction in retention efficiency with

addition of 5% w/w phospholipid to a 100% w/w tripalmitin SM wall), the impact of lipid wall composition on the retention of aqueous core materials (OTC.HCl and Poly-R) by SMs was much more pronounced. SMs softened with TO or FO retained up to 78% less aqueous core material (OTC.HCl or Poly-R) than SMs composed of 100% w/w TP (Tables 2.1 and 2.2). The greater impact of wall composition on retention of aqueous cores is most likely the result of the core being in an aqueous phase and of greater solubility than the particulate riboflavin core used to test the effects of phospholipid on SM performance (Appendix B).

Upon hydration phospholipids in a liquid-crystalline matrix may exhibit polymorphism (Cullis and Hope, 1985, Lindblom and Rilfors, 1992) forming any of a number of aggregates or structures. One structure is the familiar bilayer in which phospholipids orient tail to tail with their polar head groups exposed to the aqueous environment. A second and third structure are termed normal hexagonal and reversed hexagonal (H_{II}). Both hexagonal structures may be represented as cylinders. In the normal hexagonal structure, the cylinder is constructed of phospholipids oriented polar head groups outward (the center of the cylinder contains the hydrophobic tails of the phospholipids). In the reversed hexagonal structure, the cylinder contains an aqueous compartment into which the polar head groups of the phospholipids protrude. In triacylglyceride SMs containing aqueous core, phospholipids may exist in a combination of these states. In addition, in the presence of an aqueous core and an aqueous external environment, phospholipids may orient to expose their polar head groups at the surface of the SM (resembling their behavior in normal hexagonal structures) and into the aqueous cores (resembling their behavior in reversed hexagonal structures) (Figure 2.7). Aggregation or fusion of reversed hexagonal structures within the SM may result in the formation of pores and channels in the SM wall (Figure 2.7). These structures may increase wall permeability in several ways. The presence of phospholipid channels may

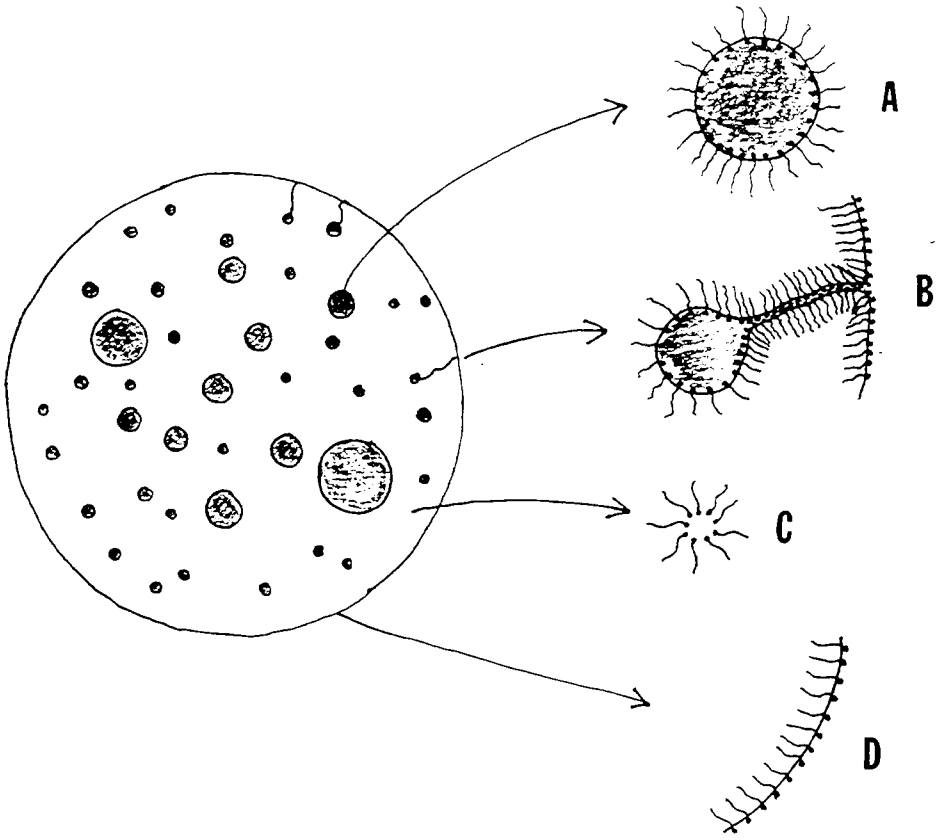

Figure 2.7: Possible behavior of phospholipids dissolved in a triacylglyceride (60%w/w tripalmitin/40% w/w fish oil) spray microcapsule (SM) containing aqueous core:

A) Phospholipid association with aqueous core. Polar head groups of phospholipids insert themselves into core. Hydrophobic tails extend out into the lipid bead.

B) Phospholipid channel connecting aqueous core material to surface of SM. Phospholipids line the channel inserting their polar head groups into the aqueous core and channel environment. Hydrophobic phospholipid tails extend into lipid bead.

C) Phospholipid micelle. Phospholipids aggregate within the lipid bead, orienting themselves with their polar head groups at the center of the micelle and hydrophobic tails extending into the lipid bead.

D) Phospholipid association with SM surface. Phospholipids aggregate at surface of SM exposing polar head groups to the external aqueous environment. Hydrophobic tails extend into lipid bead.

expose core materials to the external environment and facilitate their passage through the lipid wall. The association of phospholipids with the aqueous core may also improve mobility of the core within the lipid wall; the phospholipid may associate with the core making it appear more hydrophobic or the core material may simply be more soluble in the phospholipid than in the triacylglyceride. Phospholipids exposed at the surface of the capsule may make the surface of SMs more "wettable" and effectively increase contact with the aqueous environment. Over time phospholipid in the lipid wall, especially that exposed at the surface, will dissolve into the aqueous environment. This loss of phospholipid may destabilize the capsule wall, allowing additional pore formation and further loss of wall integrity.

On the basis of these leakage mechanisms, SM walls composed of 100% w/w TP should be relatively impermeable to fully encapsulated core contents. The homogeneous triacylglyceride composition (Appendix A) and high melting point (66°C) of TP-walled SMs should allow the lipids in the capsule wall to adopt an ordered crystalline packing arrangement. The absence of phospholipids contaminants in the TP-wall should preclude the formation of channels. Indeed, the retention efficiency for all three cores encapsulated within 100% w/w TP was very high, greater than 94% (Table 2.1, 2.2, and 2.3), and not significantly different between core materials ($p>>0.05$, Tukey HSD multiple range tests of core type/lipid type combinations; Figure 2.5). Addition of either TO or FO to these SMs should have caused a reduction in retention efficiency due to pore or channel formation caused by disruption of the packing order of the lipids in the capsule wall and/or the introduction of phospholipid impurities. Permeability of the capsule wall may also have been increased by altering the fluidity of lipid wall with the addition of these low melting point lipids. Indeed the retention efficiencies for the three core materials encapsulated in 100% w/w TP were significantly higher than like cores encapsulated within the two softer lipid mixtures ($p<0.05$, Tukey HSD multiple range tests; Figures 2.2, 2.3, and 2.4).

While it is difficult to identify the exact mechanism which caused the observed reductions in retention efficiency of tripalmitin SMs softened with either TO or FO, there is some evidence that the presence of phospholipid in the lipid wall is more detrimental to wall permeability than disruption of the packing arrangement of lipids in the capsule wall or alteration of the fluidity of the lipid wall.

Tripalmitin-walled DEMs containing OTC.HCl and softened with TO of two different purities retained significantly different amounts of core material while in storage (Appendix D). DEMs showed almost a 3 fold increase in retention (from 24 to 73%) when the purity of the TO was increased from 65% to 99% (manufacturer's reported purity; actual purity based upon fatty acid analysis, 51% and 96%, Appendix A). Analysis of the TOs used to soften the capsule wall indicated that one of the primary differences between the two TOs was the presence of phospholipid in the 65% (manufacturer's reported purity) pure preparation (Appendix C). If the increase in wall permeability observed in the current experiments was simply a result of the added lipids (TO or FO) fluidizing or disrupting the packing order of the lipids in the SM wall, then it could be argued that the retention of the described DEM preparations should have been more similar (i.e., both DEM preparations contained equivalent quantities of the lower melting point and unsaturated lipids). The fact that the retention efficiencies of these two DEM preparations (Appendix D) were so different suggests that the presence of phospholipid in the lipid wall may be a critical factor determining subsequent permeability of lipid-walled microcapsules.

Effect of core material on retention efficiency

While the differences observed in retention of core materials between SMs having different lipid wall compositions may be explained by the above mechanisms, SMs also exhibited differential rates of leakage between core types encapsulated in the same lipid

wall material. The retention efficiency of SMs for particulate riboflavin was expected to be quite high, because of the low solubility of the riboflavin in seawater (approximately 4.5 mg/100 ml). In contrast, the core materials of Poly-R and OTC.HCl were expected to leak from SMs at a more rapid rate than riboflavin, as a result of both their higher aqueous solubility and encapsulation in a dissolved form. Softer-walled SMs (40% w/w TO or 40% w/w FO) containing aqueous cores (OTC.HCl or Poly-R) did exhibit lower retention efficiencies than softer-walled SMs containing riboflavin (Figure 2.5). However, the retention efficiencies of softer-walled SMs containing OTC.HCl and Poly-R were also significantly different from one another. Softer-walled SMs containing OTC.HCl leaked significantly more core material than did comparable-walled SMs containing Poly-R ($p<0.05$, Tukey HSD multiple range tests; Figure 2.5). These differences suggest that retention of core materials by SMs is a function of not only of the encapsulating lipid, but also of the characteristics of the core material.

OTC.HCl and Poly-R, although both encapsulated as aqueous cores, differ in their molecular size and aqueous solubility. OTC.HCl is a much smaller molecule than Poly-R (molecular weights, 497 and 50,000 to 100,000 Daltons, respectively). OTC.HCl is also much more soluble than Poly-R (1.0 vs. 0.1 g/ml in water at 25°C). The differences in leakage between these two core types may be a reflection of these differences in size and solubility. OTC.HCl may simply diffuse faster through pores and channels in the lipid wall because of its smaller size and higher solubility. OTC.HCl may also interact differently with the phospholipid contaminants present in SMs softened with TO or FO, either being more soluble in the phospholipid or associating with the phospholipid such that diffusion of the OTC.HCl through the lipid wall is enhanced.

Effects of wall composition on encapsulation efficiency

SMs containing aqueous cores (Poly-R or OTC.HCl) exhibited significant reductions in encapsulation efficiency with the addition of TO or FO to the SM wall (Table 2.1 and 2.2; Figure 2.2 and 2.3). This may reflect either a true interaction between core and lipid or differential rates of core leakage from these capsules prior to measurement of encapsulation efficiency.

As noted, addition of TO or FO to SMs may interrupt the structure of the lipid wall, fluidize the lipid wall, and introduce a "contaminant" in the form of phospholipid. In contrast to present experimental results, past experiments have demonstrated that the presence of phospholipid may improve encapsulation of either particulate or aqueous core materials. Addition of 5% w/w phospholipid to tripalmitin SMs increased the amount of riboflavin encapsulated (Appendix B). Tripalmitin-walled DEMs softened with a low-purity (>65% pure by manufacturer's analysis) preparation of TO contaminated with phospholipid (Appendix C) showed a twenty-fold increase in encapsulation efficiency for OTC.HCl (Appendix D) compared to DEMs softened with pure (>99% pure by manufacturer's analysis) TO lacking phospholipid contaminants (Appendix C).

Phospholipid may improve encapsulation in DEMs by stabilizing (preventing the separation of the aqueous core and lipid) the primary emulsion created during formation of DEMs. Indeed, phospholipid emulsifiers are reported to increase the Hydrophile-Lipophile Balance (HLB) to favor oil/water emulsions (Magee and Olson, 1981). Maggee and Olson (1981) reported that encapsulation of glucose by milk-fat microcapsules was almost doubled by addition of 2.5% lecithin to the encapsulating fat mixture. Since preparation of aqueous core SMs relies upon atomizing a similar primary emulsion, it is logical that encapsulation efficiency would be similarly improved by the presence of phospholipid. Phospholipids present in a lipid mixture should associate with both aqueous and particulate cores, inserting or attaching their polar head groups to the

core material (Figure 2.7). Such behavior should aid in the dispersal of particulate core materials within encapsulating lipid and stabilize emulsions of aqueous core materials and lipid during the formation and solidification of SMs. These interactions should ultimately increase the amount of core encapsulated.

While encapsulation efficiency of SMs for the aqueous cores of Poly-R or OTC.HCl was significantly reduced (rather than improved) by addition of either TO or FO to the tripalmitin SM wall ($p<0.0001$, one-way ANOVAs for separate core types), it is impossible to separate the effects of the added phospholipid from the effect of softening the capsule wall. The results from the DEM preparations (Appendix D) do not compare the encapsulation efficiency of the DEMs softened with TO with those composed of pure TP. While the presence of phospholipids clearly improves encapsulation of aqueous OTC.HCl by DEMs softened with TO, this improvement may be negligible in relation to an overall negative effect of softening the capsule wall. It is worth noting however, that softening of the SM wall did improve with marginal significance ($P=0.074$, one-way ANOVA), the encapsulation of riboflavin. It was also noted (see "Effect of wall composition on retention efficiency") that the negative effects of softening the wall of these riboflavin SMs on retention efficiency was similar to those caused by addition of known quantities of phospholipid. This suggests that the effect of the softer lipids on the lipid wall structure and wall fluidity was minimal in comparison to the effect of phospholipid. Assuming that the two former effects (lipid wall structure and fluidity) of softer lipids on the wall integrity should be equal across core materials, there are two explanations for why addition of phospholipid contaminants along with the softening agents of TO and FO did not improve encapsulation of aqueous core materials.

The primary emulsion for DEMs is formed using sonication. Some core materials, such as OTC.HCl, form very good and repeatable emulsions. The quality of the emulsions with other core materials, such as Poly-R, can be highly variable and affected by the type of lipid (e.g., emulsions formed by sonication with 100% w/w TP

break down rapidly compared to those formed with 40% w/w TO or 40% w/w FO). In order to standardize the emulsions used in replicate SM preparations, primary emulsions of aqueous cores and encapsulating lipid were formed by a standardized period of homogenization (see Materials and methods). The emulsions formed by homogenization were not as robust as those formed by sonication and the stabilizing effect of the phospholipid observed in DEM preparations may not have been expressed. This may explain why the encapsulation efficiencies of SMs containing an aqueous core of OTC.HCl (Table 2.3) in the present experiments were lower than those of DEMs from past experiments (Appendix D).

A second explanation for why encapsulation efficiency was not improved by the presence of phospholipid in the capsule wall is that the aqueous core materials (OTC.HCl and Poly-R) were initially well encapsulated in the softer lipid SMs (40% w/w TO or 40% w/w FO), but that these core materials were rapidly lost during SM collection and filtration prior to storage. This second explanation is supported by the observation that across core materials, SMs composed of the softer lipid mixtures lost more core material during rinsing and filtration than SMs composed of 100% w/w TP. The loss of aqueous core material during filtration and rinsing, rather than in the collecting bath used in the spray process, suggests that core material was encapsulated in softer-walled SMs, but lost rapidly through fissures or channels in the capsule wall. This rapid loss of encapsulated material may be the result of a reduction in SM wall integrity due to softening of the wall or reflect the increased leakage associated with the presence of phospholipid in the SM wall (Appendix B and D). Regardless, these observations suggest that rather than reducing encapsulation of core, softer lipids increase short term leakage losses which occur prior to measurement of encapsulation efficiency.

A slightly significant difference ($p=0.074$) was observed between the encapsulation efficiencies of the three lipid types for a core of particulate riboflavin. Previous experiments (Appendix B) have suggested that the presence of phospholipids in

some concentration, may improve the encapsulation efficiency of particulate materials, although encapsulation is often so high as to preclude improvement (i.e., near 100% of available core is encapsulated based upon starting ratios of core/lipid). The presence of phospholipid should improve encapsulation of particulate material by improving dispersal of the particles within the encapsulating lipid. Indeed, during preparations of SMs containing riboflavin, particulate riboflavin tended to rapidly reaggregate after dispersal into 100% w/w TP. In contrast, riboflavin remained uniformly distributed in the softer lipid mixtures (40% w/w TO or 40% w/w FO) throughout the spray process. The interaction of riboflavin with phospholipid in the lipid wall seems to keep the material in suspension and prevent separation of the core and lipid during the spray process. As with softer-walled SMs containing aqueous core materials, loss of core material from softer SMs containing riboflavin during filtration and rinsing increased relative to SMs composed of 100% w/w TP. While it cannot be argued that unencapsulated core should have been lost immediately in the collecting bath (given the low solubility of riboflavin), it is likely that encapsulation efficiency of the core was improved by the presence of phospholipids, but that enough was lost during filtration such that the encapsulation efficiency of the softer SMs and those composed of 100% w/w TP were only slightly different.

Conclusions

The overall effect of softening the SM wall is reflected in the reduced delivery efficiencies of SMs for the various core materials. Softening of the lipid wall can result in as much as a 9 fold reduction in the amount of core delivered (OTC.HCl, Figure 2.3). While these reductions are significant, such a softening appears to be necessary in order for capsules ingested by bivalves to be digested or disrupted (See Introduction, this chapter, and Chapter 3, this thesis). SMs softened with either 40% w/w TO or 40% w/w

FO are equally effective for delivering any of the three core materials, suggesting that FO may be used in place of TO in further studies.

In addition to the above noted differences, SMs having walls composed of softer lipids were observed to be more "wettable" and entered suspension much more readily than those composed of 100% w/w TP. In terms of retention efficiency, an increase in "wettability" will enhance opportunities for leakage of core materials. In terms of encapsulation efficiency, the process of collecting SMs after preparation in an aqueous bath and the subsequent filtration and rinsing of SMs, present opportunities for core losses prior to encapsulation measurements. Manipulations of the spray process to eliminate exposure of the SMs to an aqueous environment, such as spraying the capsules into and collecting the capsules as a powder from a dry, chilled collecting chamber may reduce opportunities for leakage losses during preparation of SMs. In addition storing SMs as dry powders and freeze-drying SMs may reduce opportunities for leakage of core materials during storage. In addition, increasing the purity of lipids used in the SM wall, while cost-prohibitive on a commercial scale, should greatly improve retention characteristics of SMs. SMs composed of purified lipids may represent an effective vehicle for delivering water-soluble materials in a research setting. Further study to improve the stability of the primary emulsions of these purified lipid preparations need to be investigated to maximize encapsulation efficiency of these SMs.

The described lipid SMs represent a very efficient delivery device for particulate riboflavin. The ability of SMs to encapsulate other particulate materials needs to be investigated and the performance of these SMs assessed.

Chapter 3
Studies on the Digestibility of Lipid-Walled Microcapsules by the Manila Clam, *Tapes philippinarum*

Introduction

Fish oil triacylglycerols are reported to be a useful and inexpensive energy source for bivalve larvae (Heras *et al.*, 1993). These triacylglycerols are primarily composed of the highly unsaturated fatty acids eicopentaenoic acid (EPA) and docosahexanoic acid (DHA). The above fatty acids have been demonstrated to be important components of the diet and constituents of the tissues of bivalves and bivalve larvae (Langdon and Waldock, 1981; Napolitano *et al.*, 1988a,b; Trider and Castell, 1980).

Lipid-walled microcapsules (LWMs) composed of a mixture of triacylglycerides, 60% w/w tripalmitin (TP) and 40% w/w fish oil (FO), may be used to encapsulate particulate and aqueous core materials (Chapter 2). For core materials encapsulated within LWMs to be assimilated by target organisms, the core must be released either by physical rupture or enzymatic digestion of the capsule walls. In preliminary feeding studies, LWMs prepared using a wall material of 60% w/w TP/40% w/w FO were ingested by mussel larvae (*Mytilus edulis*). Digestion of the lipid wall and assimilation of capsule contents were not verified. However, changes in the morphology and the coloration of LWMs stained with Sudan Black suggested that larval digestive processes altered the structural integrity of the ingested capsules.

The lipid wall of LWMs may be digested by extracellular lipases which break down triacylglycerides into their component free fatty acids. The style of bivalves is reported to contain materials effective at emulsifying lipids (Kristenson, 1972) and it is thought to be a major source for extracellular lipases in the adult oyster *Crassostrea virginica* (George, 1952). Lipase isolated from the style of the surf clam, *Spisula*

solidissima, shows a specificity for the primary position of triacylglycerides (Patton and Quin, 1973). Lipase activity has also been reported in sections of the stomach and style sac of *Ostrea edulis* and *Crassostrea angulata.* (Mathers, 1973), the stomach, style, and digestive diverticula of the littoral bivalve, *Scrobicularia plana* (Payne *et al.*, 1978), and the style of the intertidal bivalves, *Crassostrea madrasensis* , *Meretrix meretrix* (Linnaeus), *Meretrix casta* (Chemnitz), *Katelysia opima* (Gmelin), and *Donax cuneatus* (Linnaeus)(Hameed, 1987).

Feeding experiments with Sudan III stained LWMs, LWMs containing particulate oxytetracycline hemicalcium salt, and LWMs containing polymeric dye were conducted to determine whether bivalves are capable of digesting and/or releasing core materials encapsulated in a capsule wall composed of 60% w/w TP/ 40% w/w FO.

Materials and methods

Lipid-walled microcapsules

Lipid-walled microcapsules (LWMs) were formed by two methods. LWMs containing particulate core materials are easily produced by a spray process (Chapter 2). A spray process was used to form spray microcapsules (SMs) either stained with the lipid-soluble stain, Sudan III (Sigma Chemical Co.) or containing the particulate antibiotic, oxytetracycline hemicalcium salt (OTC.HEM). Aqueous core materials are effectively encapsulated in LWMs formed by a double-emulsion process (Langdon, 1983). Double-emulsion microcapsules (DEMs) were used to encapsulate an aqueous polymeric dye, Poly-R 478 (Poly-R, Sigma Chemical Co.).

Sudan III-stained spray microcapsules

Wall materials of 100% w/w TP (Fluka Chemical Co., >95% pure by manufacturer's analysis) and 60% w/w TP/40% w/w FO (Dale Alexander's Fish Oil Concentrate) were heated to 90°C and stained to saturation with Sudan III (Sigma Chemical Co.). Stained, molten wall material was added to a preheated (90°C) thin-layer chromatography (TLC) flask (Kontes Glassware) and sprayed (compressed air, flow rate 120 liters/minute) into a collection chamber containing cold (5°C) 0.2% w/v aqueous polyvinyl alcohol (PVA, Sigma Chemical Co., cold-water soluble). Spray microcapsules (SMs) were collected and passed through a 40 μm mesh Nitex screen. SMs less than 40 μm in diameter were reserved and filtered onto a glass fiber filter (Whatman 934-AH). SMs were rinsed with cold (5°C) distilled water and filtered to produce a wet paste. SM preparations were stored as wet pastes in sealed glass scintillation vials at 5°C in darkness.

Spray microcapsules containing oxytetracycline hemicalcium salt

Oxytetracycline hemicalcium salt (OTC.HEM, Sigma Chemical Co.) was micronized to a particle size of less than 25 μm using a ball mill (Microdismembrator, B Braun Instruments). Ground OTC.HEM was added to molten (90°C) wall materials of 100% w/w TP or 60% w/w TP/40% w/w FO at a ratio allowing formation of a free-flowing slurry (2 grams core to 10 grams wall material). OTC.HEM was dispersed into the molten lipid by sonication (Braun-Sonic 2000, B Braun Instruments). The molten lipid/core mixture was added to a preheated (90°C) TLC flask (Kontes Glassware) and sprayed (compressed air, flow rate 120 liters/minute) into a collection chamber containing cold (5°C) 0.2% w/v aqueous PVA. SMs were collected and passed through a 40 μm mesh Nitex screen. SMs less than 40 μm in diameter were collected and filtered onto a

glass fiber filter (Whatman 934-AH). SMs were rinsed with 500 ml of cold (5°C) 0.05 N NaOH to remove unencapsulated antibiotic. SMs received a final rinse of cold (5°C) distilled water and were filtered to produce a wet paste. SM preparations were stored as wet pastes in sealed glass scintillation vials at 5°C in darkness.

Double-emulsion microcapsules containing polymeric dye

Double-emulsion microcapsules (DEMs) containing an aqueous core of 10% w/v poly(vinylamine) sulfonate (Poly-R, Sigma Chemical Co., Poly-R 478) were formed by a modification of the technique of Langdon (1983). Aqueous core was added to a molten wall material (90°C) of 100% w/w TP or 60% w/w TP/40% w/w FO at a ratio of 3.5 ml core to 10 grams lipid (Langdon and Singleton, unpublished data). A primary emulsion was formed by sonicating the mixture (Braun-Sonic 2000, B Braun Instruments). Ten volumes of hot (90°C), 2% w/v aqueous PVA were immediately added to the primary emulsion and the mixture was homogenized (Ultra-Turrex, Jankel-Kunkel) to form a secondary emulsion. Capsules were solidified by adding the secondary emulsion to cold (5°C) distilled water. DEMs were passed through a 40 μm mesh Nitex screen. DEMs less than 40 μm in diameter were filtered onto a glass fiber filter (Whatman 934-AH). DEMs were rinsed with cold (5°C) distilled water and filtered to produce a wet paste. DEM preparations were stored as wet pastes in sealed glass scintillation vials at 5°C in darkness.

Clam feeding experiments

LWMs were fed to juvenile Manila clams, *Tapes philippinarum*, (7.5 to 16.8 mm in shell length, mean shell length 11.6 ± 2.1 mm) and resultant fecal strands collected and examined for evidence of digested or disrupted capsules and liberated core material.

Juvenile clams maintained in upwellers receiving ambient seawater (9-12°C) and effluent from red algal, *Palmaria mollis*, cultures were collected and rinsed with 12°C filtered (Whatman GF/C) seawater (salinity 33 ppt). Approximately 50 clams were added to a mesh basket (Vexar, 4 mm mesh) and suspended in a 3 liter glass beaker containing filtered (Whatman GF/C) seawater. LWMs were suspended in cold (5°C) 2% w/v aqueous PVA by brief sonication on ice. Enough concentrated LWM suspension was added to slightly cloud beakers containing clams. Beakers containing clams and capsules were placed in a dark, constant temperature room (12°C) and gently stirred. Control beakers containing capsules suspended in filtered seawater were treated similarly. An additional control (clams held in a solution of freely dissolved Poly-R equivalent to that delivered by DEMs) was used to control for uptake of dissolved Poly-R by clams..

At one, two and four hours, clams were agitated, rinsed with filtered seawater, and transferred to beakers containing a fresh suspension of LWMs. Fecal strands from one, two, and four hour cultures were examined to verify ingestion of capsules, digestion of the encapsulating lipid, and release of core material. At 6 hours, clams were agitated and transferred to beakers containing filtered seawater (LWMs not renewed). Fecal strands from 6 hour cultures were examined. At 6 hours, capsules in control beakers were filtered onto glass fiber filters (Whatman GF/C) and examined under a microscope for evidence of capsule rupture or core material leakage.

Fecal strand examination

Clams fed Sudan III-stained SMs

Fecal strands from animals fed 100% w/w TP or 60% w/w TP/40% w/w FO SMs were collected on a 25 μm mesh Nitex screen and gently rinsed with distilled water. Fecal strands were counter-stained with 0.1% Nile Blue Sulfate to determine whether the

lipid wall of the capsules had undergone enzymatic digestion (George, 1952). Free fatty acids resulting from the digestion of lipid (triacylglyceride, phospholipid, etc.) stained with Sudan III take up counter-stain changing the color of stained lipid from red to blue. Fecal strands from animals held in filtered seawater and starved for 6 hours were counter-stained to control for background staining of previously ingested material. SMs from control beakers were filtered onto glass fiber (Whatman GF/C) filters, rinsed with distilled water, and counter-stained as above. Counter-stained control SMs and feces were viewed by brightfield microscopy (Zeiss Instruments) and photographed with 160T Ektachrome slide film (Kodak).

Clams fed spray microcapsules containing OTC.HEM

OTC.HEM is a slightly soluble (12 mg/ml in water at 25°C) form of the antibiotic oxytetracycline. Particles of OTC.HEM are highly fluorescent when viewed by epifluorescent microscopy. It was expected that given its low solubility, OTC.HEM liberated from SMs would diffuse slowly from fecal strands. Liberated particles of OTC.HEM were expected to be easily viewed given their fluorescence. In addition, the fluorescence of this core material was expected to allow comparison of the relative amounts of particulate core material remaining in SMs excreted in fecal strands.

Fecal strands collected from clams fed OTC.HEM encapsulated in SMs composed of 100% w/w TP or 60% w/w TP/40% w/w FO were examined by epifluorescent microscopy (Zeiss instruments; green excitation filter set: green interference filter, BP 546/7; chromatic splitter, FT 580; red filter barrier, LP 590) and photographed with 160T Ektachrome slide film (Kodak). Control SMs were filtered out of suspension onto glass fiber filters (Whatman GF/C), examined and photographed as above.

Clams fed double-emulsion microcapsules containing Poly-R

Poly-R is a high molecular weight (50,000 to 100,000 Daltons) water-soluble dye. Given its high molecular weight, Poly-R liberated from DEMs was expected to diffuse slowly and become bound in the mucous coating of fecal strands. Comparison of the quantity and intensity of encapsulated red dye remaining in DEMs excreted in fecal strands, as well as the amount of liberated dye in fecal strands, was expected to reflect the relative success of LWMs of different lipid compositions at delivering aqueous core material.

Fecal strands collected from clams fed Poly-R encapsulated in DEMs composed of 100% w/w TP or 60% w/w TP/40% w/w FO were examined by brightfield microscopy and photographed. Feces from control clams held in seawater containing dissolved Poly R were examined similarly. Control DEMs were filtered out of suspension onto glass fiber filters (Whatman GF/C), examined and photographed as above.

Results

Clams fed on LWMs readily ingested capsules. Fecal strands collected and observed at one, two, four and six hours appeared to be composed almost exclusively of capsules.

Sudan III-stained spray microcapsules

Control SMs (composed of 100% w/w TP or 60% w/w TP/40% w/w FO) filtered out of suspension after 6 hours retained their integrity (spherical shape and smooth surface) and intensity of staining (Figure 3.1). SMs from both controls did not accept

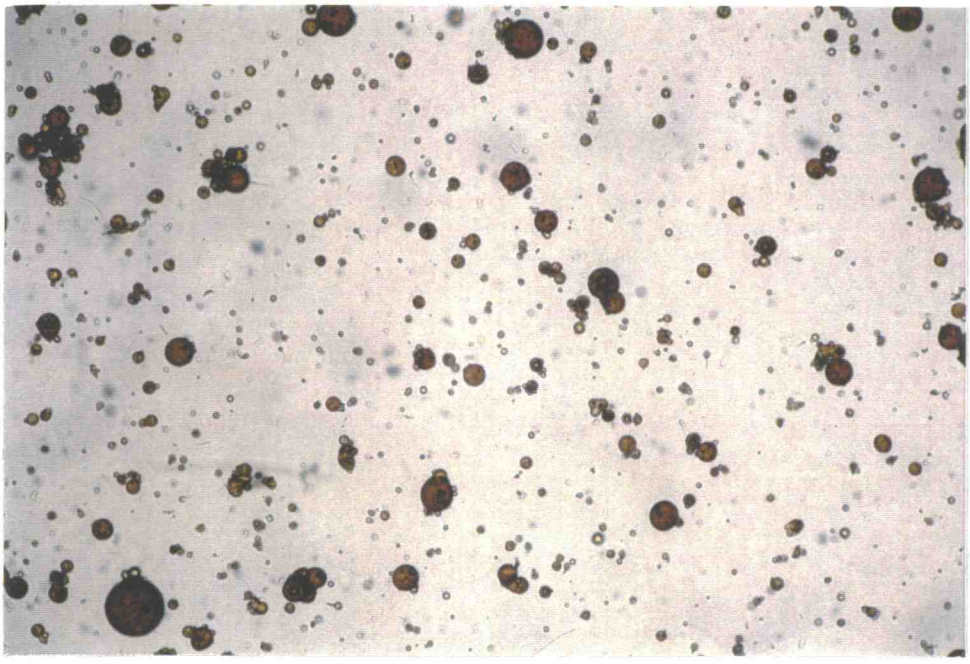

Figure 3.1: Sudan III-stained spray microcapsules (SMs) composed of 60% w/w tripalmitin/40% w/w fish oil. SMs recovered from control beakers after 6 hours suspension in seawater at 12°C. SMs were spherical and red in color. SMs did not accept blue counter-stain (Nile-blue sulfate). Magnification, 160X objective. Scale _____ = 50 μm.

counter-stain. Intact fecal strands were difficult to obtain from clams fed on SMs composed of 100% w/w TP. Fecal stands collected at 6 hours were fragile and contained intensely stained, smooth-surfaced, spherical SMs. SMs contained within these fecal strands did not accept counter-stain (Figure 3.2). Fecal strands from clams fed SMs composed of 60% w/w TP/40% w/w FO were robust and easily collected. These fecal strands appeared to be composed of a paste of partially digested SMs. SMs contained within these fecal strands were frequently irregular in shape and lighter in color than control SMs (both lipid wall compositions) or SMs composed of 100% w/w TP recovered from fecal strands. SMs (60% w/w TP/40% w/w FO) contained within fecal strands readily accepted counter-stain, changing from a pale red to pale blue (Figure 3.3). Fecal strands collected from starved animals did not accept counter-stain.

Spray microcapsules containing OTC.HEM

Control SMs (composed of 100% w/w TP or 60% w/w TP/40% w/w FO) filtered out of suspension after 6 hours were spherical and contained intensely fluorescent particles of OTC.HEM (Figure 3.4). Fecal strands from clams fed SMs composed of 100% w/w TP were fragile (Figure 3.5). SMs in these fecal stands retained their spherical shape and fluoresced brightly (Figures 3.6 and 3.7). No free particles of OTC.HEM were visible in these fecal strands. Fecal strands from clams fed on SMs composed of 60% w/w TP/40% w/w FO were more robust and easily collected (Figure 3.8). While no free OTC.HEM was visible in these fecal strands, SMs within these strands appeared to have lost most of their fluorescence (Figure 3.9). The SMs contained in these fecal strands were irregular in shape and contained less core material (Figure 3.10) than either control SMs or 100% w/w TP SMs observed in fecal strands.

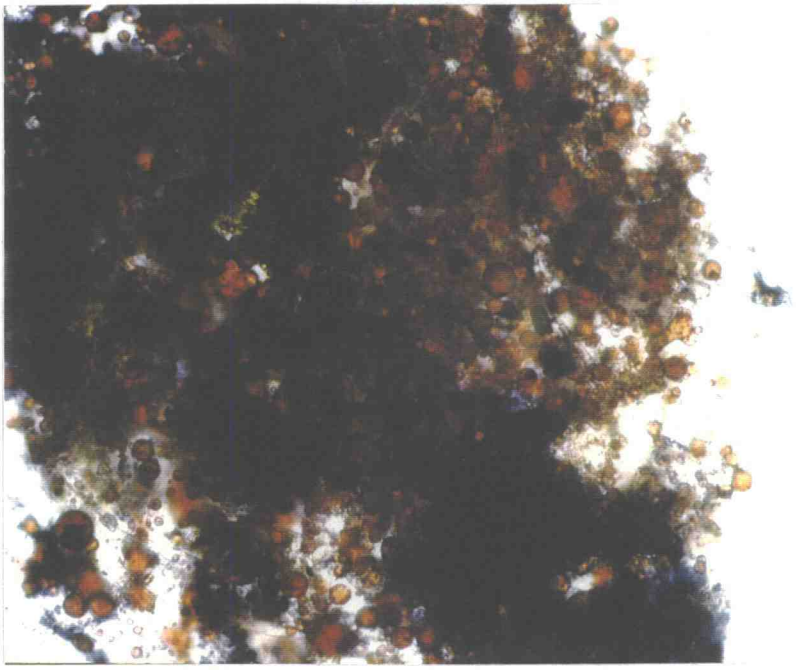

Figure 3.2: Fecal strand containing Sudan III-stained spray microcapsules (SMs) composed of 100% w/w tripalmitin. SMs were spherical, red in color and loosely bound in the fecal strand. SMs did not accept blue counter-stain (Nile-blue sulfate). Magnification, 160X objective. Scale _____ = 50 μm.

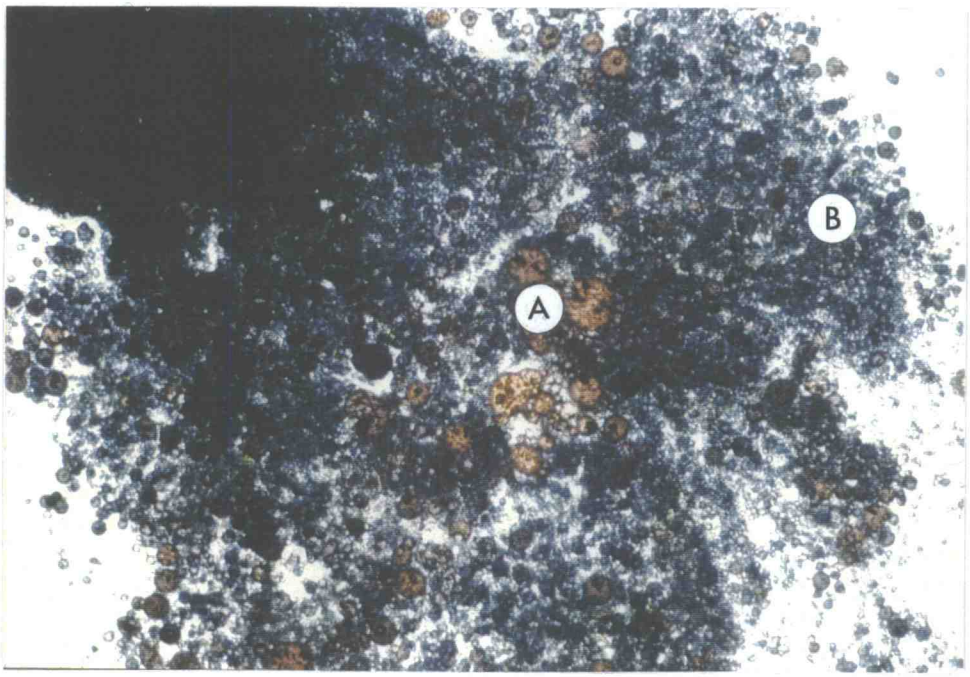

Figure 3.3: Fecal strand containing Sudan III-stained spray microcapsules (SMs) composed of 60% w/w tripalmitin/40% w/w fish oil. SMs were slightly irregular in shape and tightly bound in the fecal strand. Most SMs accepted blue counter-stain changing from red (A) to blue (B). Magnification, 160X objective.
Scale _____ = 50 μm.

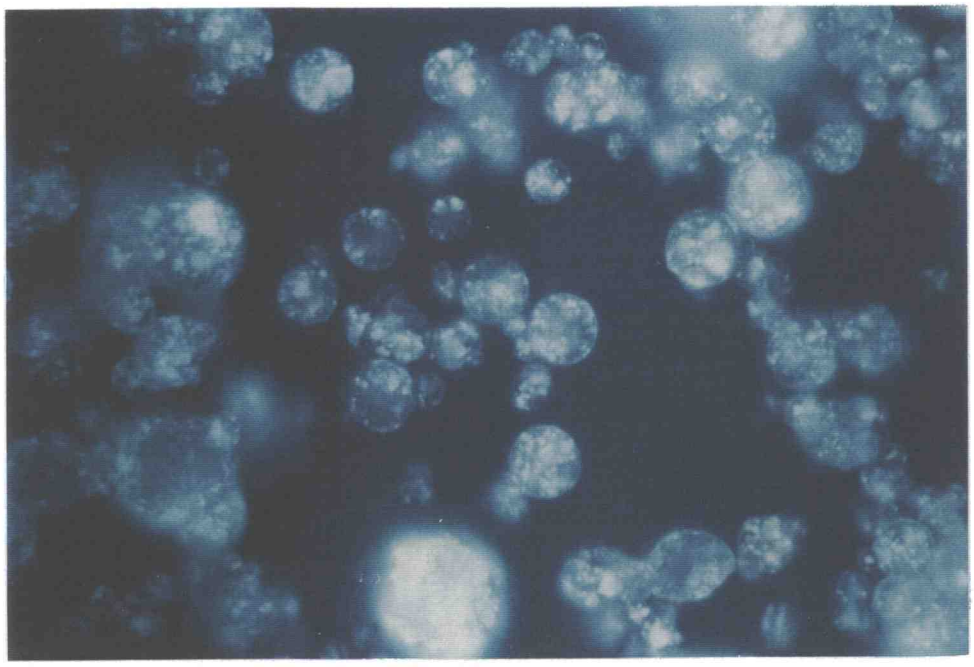

Figure 3.4: Spray microcapsules (SMs) composed of 60% w/w tripalmitin/40% w/w fish oil and containing particulate oxytetracycline hemicalcium salt (OTC.HEM). SMs recovered from control beakers after 6 hours suspension in seawater at 12°C. SMs were spherical and contain fluorescent (green bandpass filter set) particles of OTC.HEM. Magnification, 400X objective. Scale _____ = 20 μm.

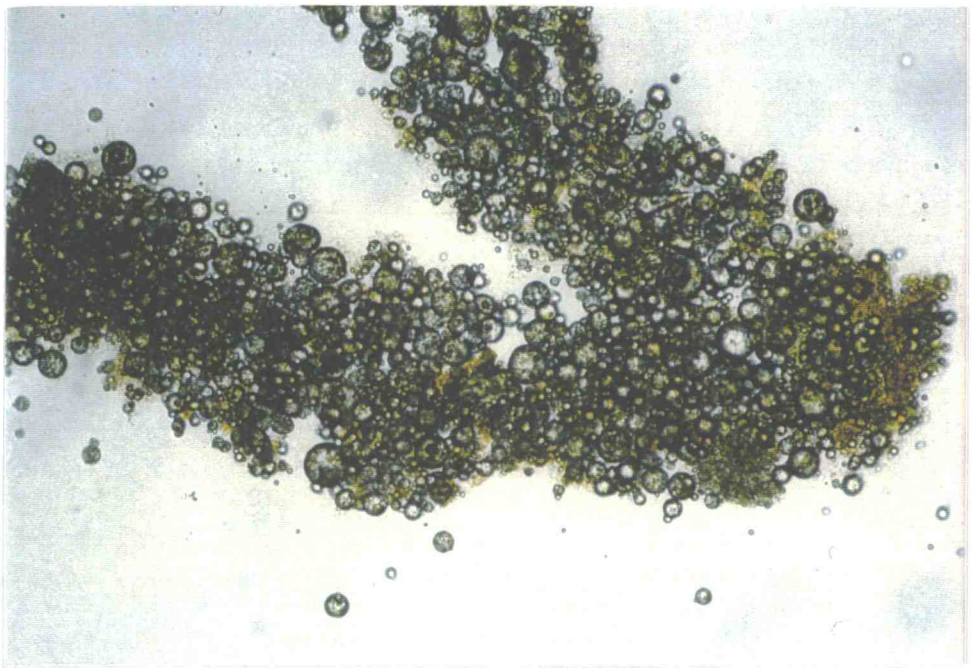

Figure 3.5: Fecal strand containing spray microcapsules (SMs) composed of 100% w/w tripalmitin and containing particulate oxytetracycline hemicalcium salt (OTC.HEM). SMs were spherical and loosely bound in the fecal strand. Magnification, 160X objective. Scale _____ = 50 μm.

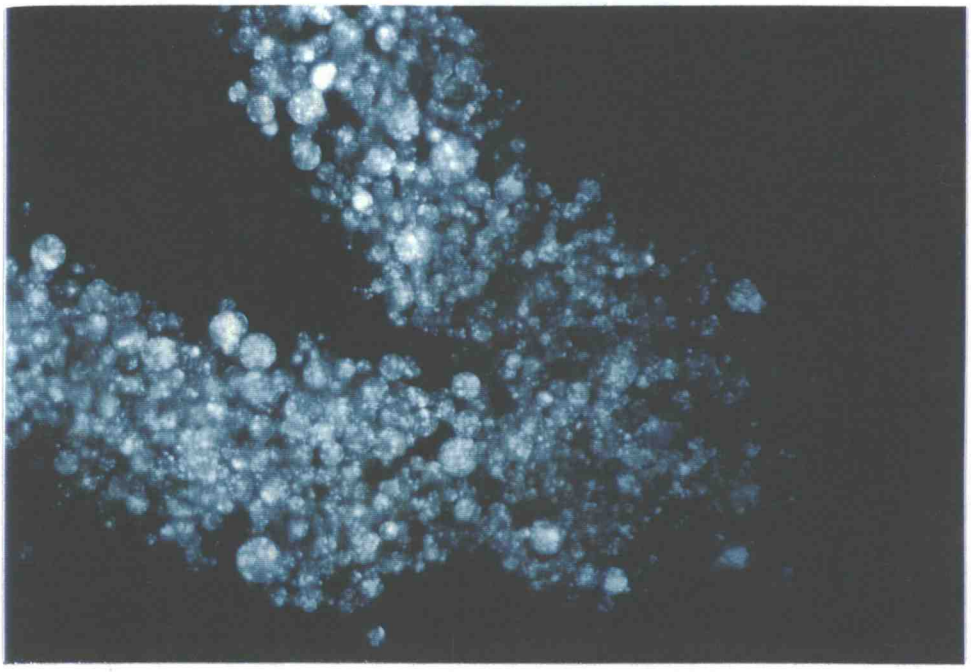

Figure 3.6: Fecal strand containing spray microcapsules (SMs) composed of 100% w/w tripalmitin and containing particulate oxytetracycline hemicalcium salt (OTC.HEM). SMs were spherical and loosely bound in the fecal strand. SMs contained intensely fluorescent (green bandpass filter set) particles of OTC.HEM. Magnification, 160X objective. Scale _____ = 50 μm.

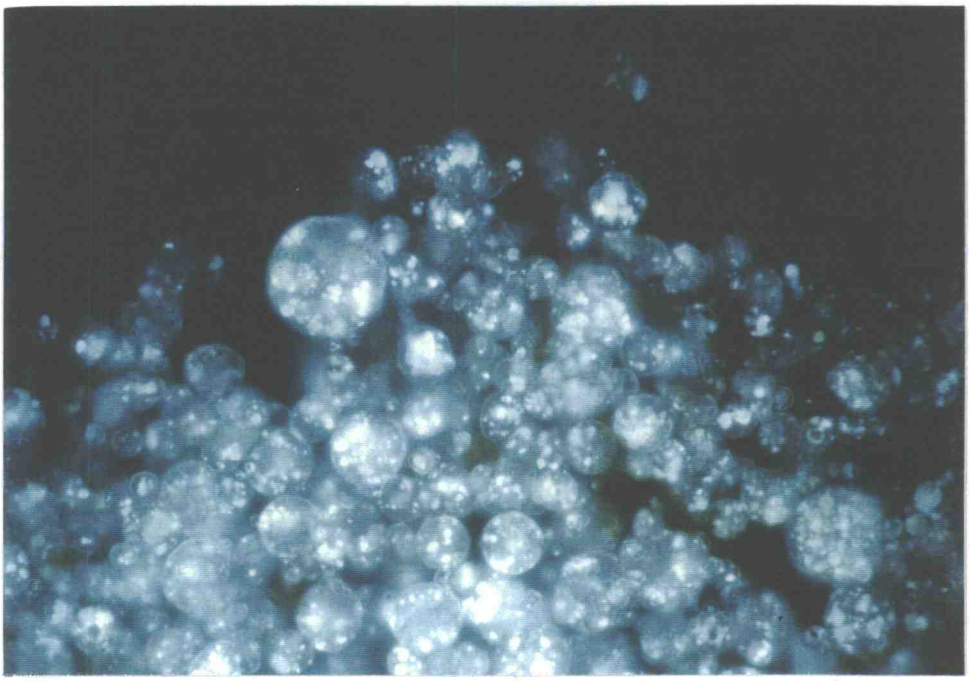

Figure 3.7: Fecal strand containing spray microcapsules (SMs) composed of 100% w/w tripalmitin and containing particulate oxytetracycline hemicalcium salt (OTC.HEM). SMs were spherical and loosely bound in the fecal strand. SBs contained intensely fluorescent (green bandpass filter set) particles of OTC.HEM. Magnification, 400X objective. Scale _____ = 20 μm.

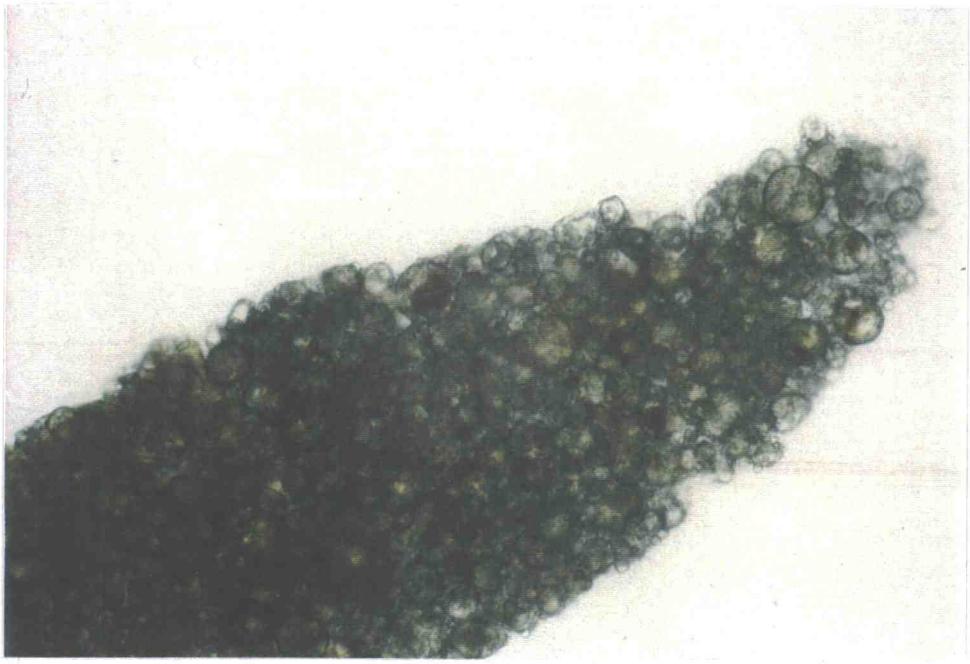

Figure 3.8: Fecal strand containing spray microcapsules (SMs) composed of 60% w/w tripalmitin/40% w/w fish oil and containing particulate oxytetracycline hemicalcium salt (OTC.HEM). SMs were irregular in shape and tightly bound in the fecal strand. Magnification, 160X objective. Scale _____ = 50 μm.

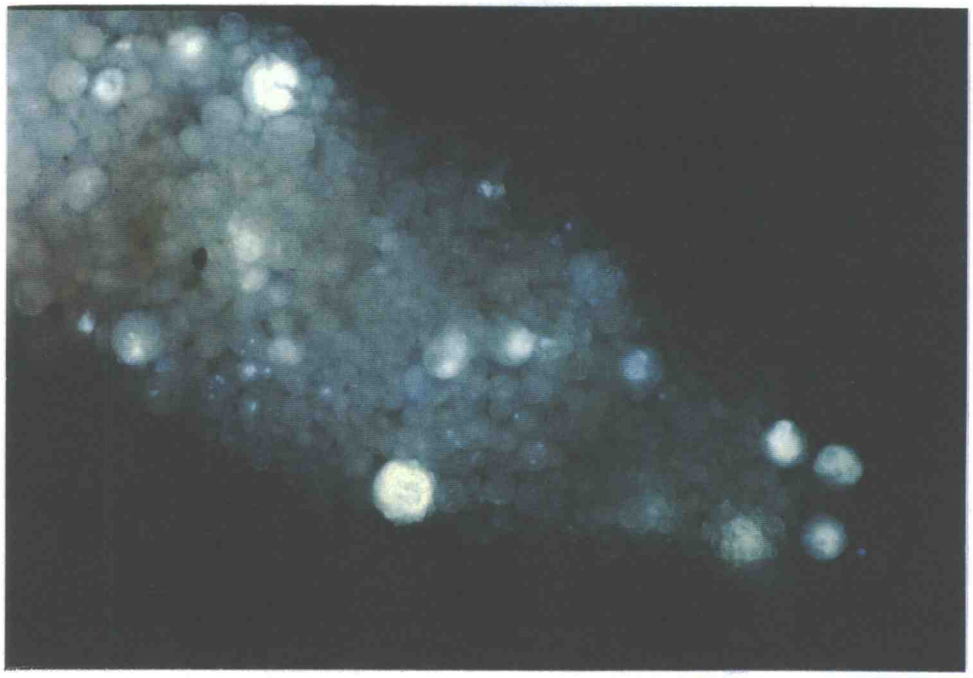

Figure 3.9: Fecal strand containing spray microcapsules (SMs) composed of 60% w/w tripalmitin/40% w/w fish oil and containing particulate oxytetracycline hemicalcium salt (OTC.HEM). SMs were irregular in shape and tightly bound in the fecal strand. SMs contained few fluorescent (green bandpass filter set) particles of OTC.HEM. Magnification, 160X objective. Scale _____ = 50 μm.

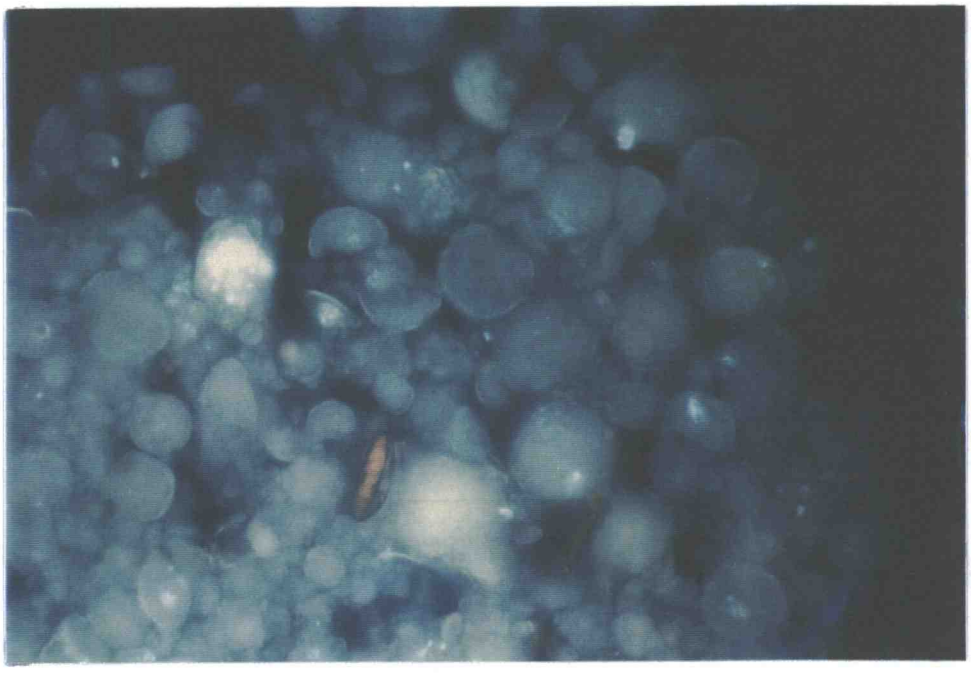

Figure 3.10: Fecal strand containing spray microcapsules (SMs) composed of 60% w/w tripalmitin/40% w/w fish oil and containing particulate oxytetracycline hemicalcium salt (OTC.HEM). SMs were irregular in shape and tightly bound in the fecal strand. Fecal strand appeared to be composed of a mash of capsule material. SMs contained few fluorescent (green bandpass filter set) particles of OTC.HEM. Magnification, 400X objective. Scale _____ = 20 μm.

Double-emulsion microcapsules containing Poly-R

Control DEMs (composed of 100% w/w TP or 60% w/w TP/40% w/w FO) filtered out of suspension after 6 hours retained their integrity and aqueous core of Poly-R (Figure 3.11). Fecal strands from clams fed DEMs composed of 100% w/w TP were fragile and easily disrupted. DEMs contained within these fecal strands retained their spherical shape and core of Poly-R (Figure 3.12). In contrast, fecal strands from clams fed DEMs composed of 60% w/w TP/40% w/w FO were more robust. These fecal strands often contained bands which appeared to be made up of partially digested capsule material. These bands often had a diffuse red coloration (Figure 3.13). DEMs within these bands were irregular in shape and rarely contained core (Figure 3.14). Some DEMs observed in these fecal strands appeared to be leaking core through fractures in their lipid wall (Figure 3.14). In some cases, the clear mucous coating of the fecal stand near such areas was lightly tinged pink. Feces collected from clams incubated for six hours in dissolved Poly-R did not exhibit any accumulation of dye.

Discussion

Juvenile clams can digest and release the contents of LWMs composed of 60% w/w TP/40% w/w FO. Digestion of capsules, defined as conversion of the triacylglycerides in the capsule wall to free fatty acids, seems to be fairly complete based upon the uptake of counter-stain by Sudan III-stained SMs composed of 60% w/w TP/40% w/w FO (Figure 3.3). By this same criterion, SMs composed of 100% w/w TP do not appear to be digested (Figure 3.2). Fecal strands from clams fed capsules composed of 100% w/w TP were also uniformly fragile across treatments, lacking a binding material, while fecal strands from animals fed on capsules softened with 40% w/w FO were more robust and appeared to be bound together by digested capsule

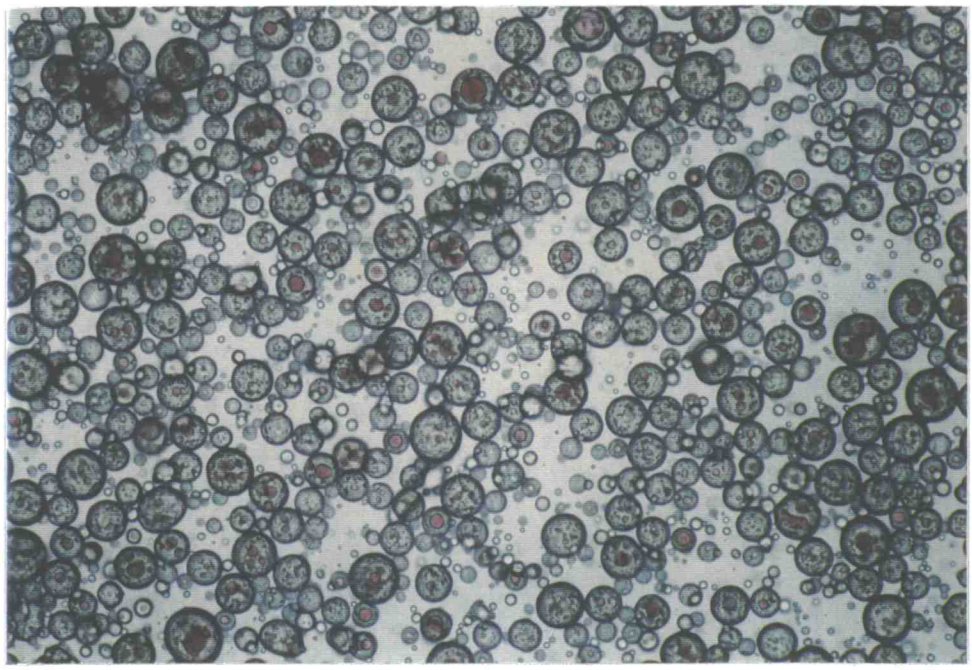

Figure 3.11: Double-emulsion microcapsules (DEMs) containing Poly-R 478 (Poly-R, Sigma Chemical Co.). DEMs recovered from control beakers after 6 hours suspension in seawater at 12°C. DEMs composed of 60% w/w tripalmitin/40% w/w fish oil. DEMs were spherical and contained a red core of aqueous dye. Magnification, 160X objective. Scale _____ = 50 μm.

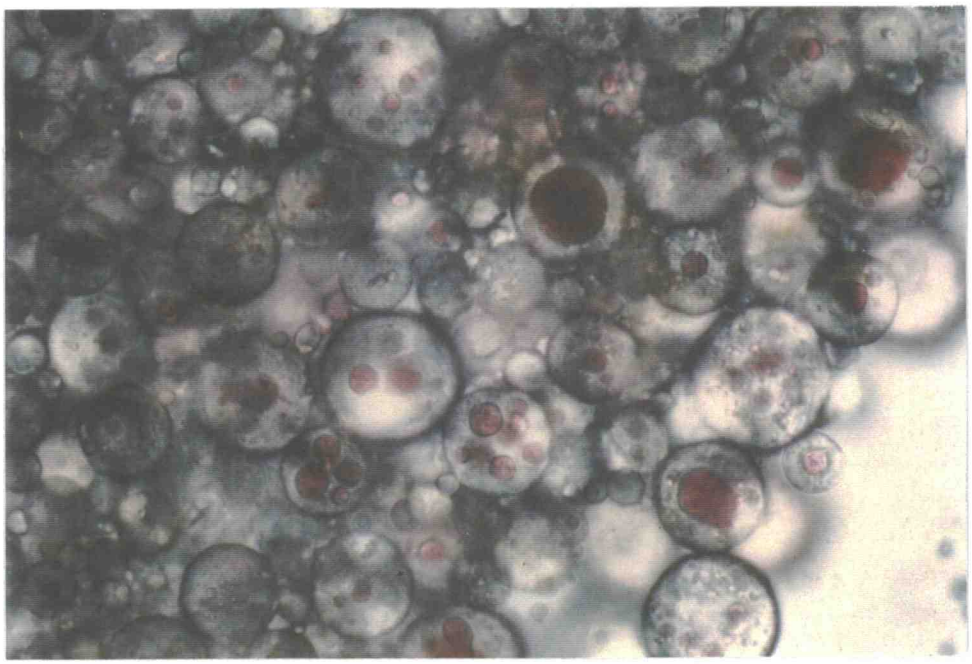

Figure 3.12: Fecal strand containing double-emulsion microcapsules (DEMs) composed of 100% w/w tripalmitin. DEMs contained an aqueous core of Poly-R 478 (Poly-R, Sigma Chemical Co.). DEMs were loosely bound in the fecal strand, spherical in shape and contained encapsulated core material. Magnification, 400X objective.
Scale _____ = 20 μm.

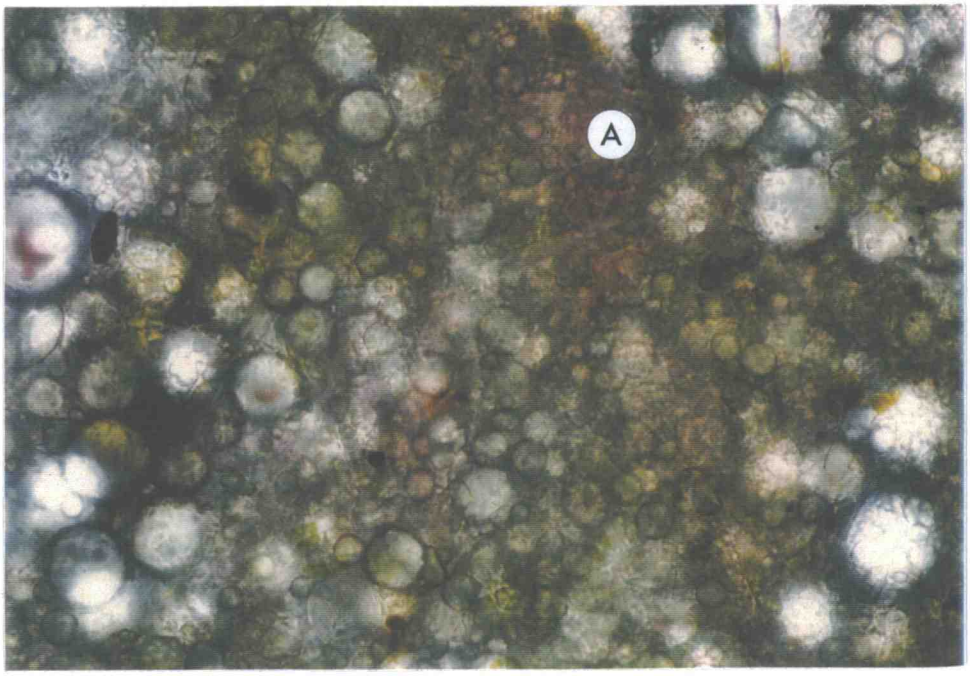

Figure 3.13: Fecal strand containing double-emulsion microcapsules (DEMs) composed of 60% w/w tripalmitin/40% w/w fish oil. DEMs contained an aqueous core of Poly-R 478 (Poly-R, Sigma Chemical Co.). DEMs were tightly bound in the fecal strand, irregular in shape and contained little encapsulated core material. Fecal strand contained a band of digested capsule materials having a diffuse red coloration (A).
Magnification, 400X objective. Scale _____ = 20 μm.

Figure 3.14: Fecal strand containing double-emulsion microcapsules (DEMs) composed of 60% w/w tripalmitin/40% w/w fish oil. DEMs contained an aqueous core of Poly-R 478 (Poly-R, Sigma Chemical Co.). DEMs appeared fractured and eroded. DEMs had lost most of the encapsulated Poly-R. Magnification, 400X objective.
Scale _____ = 20 μm.

material. The striking contrast in physical appearance between capsules composed of 100% w/w TP and 60% w/w TP/40% w/w FO further suggested some digestive processes to have occurred. 100% w/w TP capsules retained their integrity (spherical shape and smooth surface), while those softened with FO became irregularly shaped and pitted.

Softening LWMs by addition of FO to the capsule wall appears to be required to make core materials accessible. While capsules softened with FO appeared to be distorted by digestive processes, complete loss of capsule integrity or assimilation of wall material was not necessary for delivery or release of core. SMs delivering OTC lost their fluorescence, while still remaining recognizable as a SMs. This pattern was mimicked by DEMs delivering aqueous Poly R. Poly-R being a high molecular weight dye (50,000 to 100, 000 Daltons) was expected to be bound up in fecal strand material and show some accumulation in the binding mucus. While this was often not the case, DEMs (60% w/w TP/40% w/w FO) observed in fecal strands did not contain as much Poly-R as control DEMs (60% w/w TP/40% w/w FO) recovered from seawater or 100% w/w TP DEMs observed in fecal strands. This loss of core material without a loss of capsule integrity may be explained in terms of the permeability of a triacylglyceride wall in relation to one composed of a free fatty acid mixture. The results of feeding experiments with Sudan III-stained SMs demonstrated that the irregular though identifiable SMs are primarily composed of free fatty acids. While encapsulating lipid may not be completely assimilated, an increase in the free fatty acid composition of the capsule wall increases the permeability of the wall (personal communication Dr. Mark Christensen, College of Pharmacy, Oregon State University; Dr. Christopher Langdon, College of Agriculture, Oregon State University) and may allow effective delivery of encapsulated core materials.

Another mode of core release is suggested by the eroded or pitted (sometimes fractured) appearance of the LWMs composed of 60% w/w TP/40% w/w FO and observed in fecal strands. Loss of fluorescence in SMs containing OTC.HEM and loss

of encapsulated dye by DEMs containing Poly-R appeared to be related to the degree of distortion of the capsule wall. Erosion or fracture of the lipid wall, through enzymatic digestion or physical disruption, exposes encapsulated particulate and aqueous cores. The softening of the capsule wall by emulsification and enzymatic digestion may further increase the likelihood of fracture and release of core materials.

Conclusions

These experiments illustrate that LWMs are palatable and ingested by juvenile clams. The digestion of the encapsulating lipid and loss of core material suggest that this capsule type is potentially a useful delivery device for a wide variety of water-soluble core materials both in particulate and aqueous form. These experiments verify that a 100% w/w TP capsule wall composition must be softened in order for core materials to be released. Further research to verify the assimilation of core contents by target bivalves is necessary to truly claim efficient core delivery.

The benefits of such delivery devices has already been hinted at by Langdon (1983) i.e. if indeed water soluble vitamins increase growth of bivalve larvae on artificial diets then there is a defined application for capsules which can deliver such supplements in bivalve nursery and hatchery culture. The ability of juvenile clams to ingest and digest LWMs illustrates the suitability of these beads for bivalves, and possibly other aquaculture species possessing digestive systems capable of breaking down the triacylglyceride walls of LWMs.

Chapter 4
Studies on the Encapsulation and Storage of the Antibiotic Oxytetracycline in Lipid-Walled Microcapsules

Introduction

Vibriosis is a general term for the group of diseases caused by bacteria of the genus *Vibrio*. *Vibrio* species of bacteria are part of the normal microflora found in marine and brackish waters and are widely distributed throughout the world. Seven species of the genus (*Vibrio anguillarum* being the most common) have been described as pathogens in marine fish and shellfish (Austin and Austin, 1987).

Susceptibility of cultured organisms to disease is increased by stress and poor water quality. Typically, intensive aquaculture is characterized by high stocking densities and high feeding regimes which create these exact conditions. Vibriosis has become, economically, the most important disease in marine fish culture (Egidius, 1987). In a review of microbial diseases affecting mariculture in Japan, *Vibrio* species were cited as the primary causes of disease for red sea bream, horse mackerel, and kuruma shrimp and the fourth leading disease agent of the yellowtail tuna (Sano and Fukada, 1987). Japan alone attributes losses of approximately 19 million dollars annually to diseases caused by this bacteria (Austin and Austin, 1987). *Vibrio* species are cited as the most important disease causing microorganisms in Norwegian cod culture, salmonid culture in the Bergen region and have been widely observed in North America in cultured Pacific and Atlantic Salmon (Egidius, 1987).

Vibrio spp. are commonly observed in association with pond and raceway reared shrimp (Lightner, 1977). *Vibrio* spp. have been identified as causative agents for disease in cultured penaeid shrimp in the United States and Mexico (Lightner, 1977), China (Zheng *et al.*, 1990), Indonesia (Sunaryanto and Mariam, 1987; Mariam and Mintardjo,

1987), Malaysia (Anderson *et al.*, 1988), Thailand (Jiravanichpaisal *et al.*, 1994), Japan (De La Pena, *et al.* 1993; Takahashi, *et al.*, 1991), and Taiwan (Song *et al.*, 1993). Hatchery production of post-larval penaeid shrimp in Taiwan and the Philippines routinely requires addition of a variety of antibiotics within the culture water to reduce disease outbreaks, including vibriosis (Baticados *et al.*, 1990; Brown, 1989).

Vibrio species have been observed in several species of mollusc including: juvenile and larval oysters, scallops, hard shelled clam, and red abalone. Tubiash and coworkers (1970) identified two pathogenic marine *Vibrio* species (*V. anguillarum* and *V. alginolyticus*) infecting marine bivalve molluscs, and termed the disease caused by these strains bacillary necrosis. Hatchery production of oyster larvae is susceptible to periodic mass mortalities attributed to epizootics of *Vibrio* bacteria (Blogoslawski, 1981; Brown, 1981; Brown and Losee, 1978; Brown and Tettelbach, 1988; Elston *et al.*, 1981; Elston and Leibowitz, 1980; Garland *et al.*, 1983; Leibovitz, 1979; Lester, 1990; Lodeiro *et al.*, 1987). The impact of these mortalities can be quite severe. Elston and coworkers (1981) reported that one facility experienced a reduction of about a third in yearly production due to an extended epizootic in which up to 95% mortality was observed in oyster larvae.

Terramycin™ (oxytetracycline hydrochloride, OTC.HCl) and Romet™ (sulfadimethoxine and ormetoprim) are two FDA-approved antibiotic treatments for use with food fish in the United States (Williams and Lightner, 1988). Of the two, terramycin is preferred for treatment against *Vibrio* species. OTC.HCl may not be efficiently delivered in seawater due to its reaction with calcium and degradation under alkaline conditions. Thus, the preferred mode of administration of OTC.HCl is in medicated feed. Unfortunately, OTC.HCl medicated feed is often unpalatable (Marking *et al.*, 1988) to fish and can lead to significant reductions in feed ingestion (Hustvedt *et al.*, 1991). OTC.HCl is also very soluble in water (1 gram/ml at 25°C), and the rate of leaching from feed particles is probably quite high. One possible method for reducing

disintegration of feed and increasing retention of water-soluble materials is the encapsulation of materials within an impermeable, digestible shell or coating. Microencapsulation techniques have been used extensively in feed design for many aquaculture species, in particular penaeid shrimp, which tend to nibble food particles over an extended period of time. Effective encapsulation of water-soluble antibiotics (e.g., OTC.HCl) would reduce leaching of antibiotics into the culture water and increase feed palatability, thus reducing the amount of antibiotic needed in feed and the levels of antibiotic present in waste-water. In addition, encapsulation of antibiotics should protect antibiotics from environmental conditions which hasten their degradation.

Oxytetracycline may be encapsulated in lipid-walled microcapsules (LWMs) composed of digestible dietary lipid (see Chapter 2 and Chapter 3). LWMs composed of a mixture of 60% w/w tripalmitin /40% w/w fish oil are digested by juvenile Manila clams,*Tapes philippinarum*. (Chapter 3). The purpose of this research was to investigate the efficacy of encapsulating two forms of OTC, oxytetracycline hydrochloride (OTC.HCl) and oxytetracycline hemicalcium salt (OTC.HEM), having different aqueous solubilities (solubilities in water at 25°C, 1 gram/ml and 0.01 gram/ml, respectively) in LWMs. The encapsulation and retention efficiencies of aqueous core microcapsules, prepared by the method of double-emulsion (double-emulsion microcapsules, DEMs), and particulate core microcapsules, prepared by a spray technique (spray microcapsules, SMs), were compared for aqueous and particulate formulations of the two forms of OTC. The optimal method for encapsulating each OTC was determined. LWMs containing OTC encapsulated by the optimal method were examined over time to assess storage effects on capsule performance. The effect of freeze-drying on capsule performance was also assessed.

Materials and methods

Experiment 1: optimal method for encapsulating OTC

Preliminary experiments were conducted to determine the maximum solubility of each antibiotic in an aqueous phase. The maximum solubility of OTC.HCl was determined to be 300 mg/ml in 0.2N HCl. Solubility of OTC.HEM in aqueous phase improved in alkaline solution. While OTC.HEM exhibited maximum solubility in 0.5M (or higher) concentrations of NaOH, delivery of such a strongly basic solution to the digestive system of bivalves may adversely impact animal health. Solubility of OTC.HEM was improved by addition of weak sodium carbonate buffer. The maximum solubility of OTC.HEM was determined to be 12 mg/ml in 0.2 M sodium carbonate (pH 12). The maximum concentration of particulate core in molten lipid which formed of a free-flowing slurry of the core/molten lipid mixture was also determined for each antibiotic.

Double-emulsion microcapsules

DEMs were formed by the method of Langdon (1983). Three different aqueous solutions of OTC were prepared: 300 mg/ml OTC.HCl (Sigma Chemical Co.) in 0.2 N HCl; 12 mg/ml OTC.HCl in 0.2 N HCl; and 12 mg/ml OTC.HEM (Sigma Chemical Co.) in 0.2 M sodium carbonate (pH 12). Each solution was added to molten (90°C) lipid (60% w/w tripalmitin (TP; Fluka Chemical Co., >95% pure by manufacturer's analysis)/40% w/w fish oil (FO; Dale Alexander's Fish Oil Concentrate) at a ratio of 3.5 ml core to 10 grams lipid (Langdon and Singleton, unpublished data). The molten lipid/core mixture was emulsified by sonication (Braun-Sonic 2000, B Braun Instruments). This primary emulsion was diluted with 10 volumes of hot (90°C), 2%w/v

aqueous polyvinyl alcohol (PVA; Sigma Chemical Co., cold water-soluble). A secondary emulsion was formed by homogenization (Ultra-Turrex, Jankel and Kunkel) of the dilute primary emulsion. DEMs were solidified by addition of the secondary emulsion to cold (5°C) distilled water. DEMs were filtered through a 40 μm mesh Nitex screen. DEMs less than 40 μm in diameter were filtered onto a glass fiber filter (Whatman 934-AH) and rinsed with 500 ml of aqueous solvent [either 0.2 N HCl (OTC.HCl DEMs) or 0.05 N NaOH (OTC.HEM DEMs)] to remove unencapsulated or exposed core material. DEMs were rinsed with cold (5°C) distilled water and filtered to produce a wet paste. DEMs were stored as wet pastes in sealed glass scintillation vials at 5°C in darkness. Triplicate batches of each core (antibiotic/antibiotic concentration) material were prepared.

Spray microcapsules

OTC.HCl and OTC.HEM were micronized (Microdismembrator, B Braun Instruments) and sieved through 40 μm mesh Nitex screen. Ground particulate OTC.HEM or OTC.HCl (<40 μm in particle size) was added to molten (90°C) lipid (60% w/w TP/40% w/w FO) to form a final core concentration of 30% w/w OTC.HEM, 15% w/w OTC.HEM or 15% w/w OTC.HCl. A homogenous suspension was formed by sonication (Braun-Sonic 2000, B Braun Instruments) of the molten lipid/core mixture. Suspensions of the molten lipid/core materials were added to a preheated (90°C) thin layer chromatography (TLC) flask (Kontes Glassware) and sprayed (120 liters compressed air/minute) into a collection chamber containing cold (5°C) 0.2% w/v aqueous PVA (Figure 4.1). SMs were screened, filtered and stored in the same manner as DEMs above. Triplicate batches of each core (antibiotic/antibiotic concentration) were prepared.

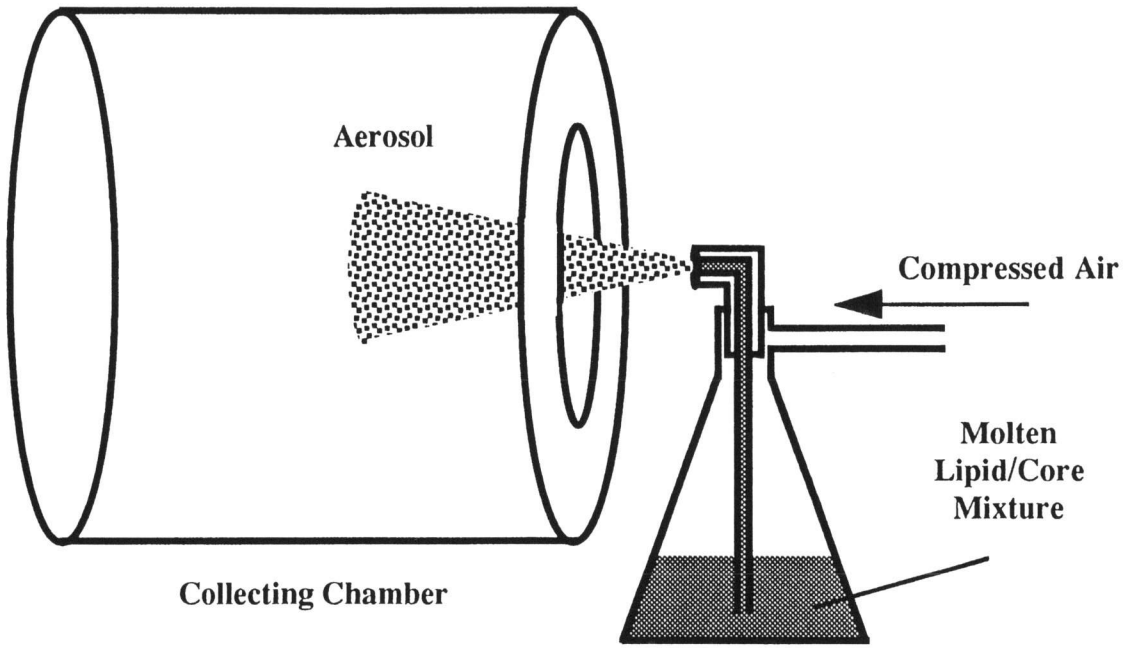

Figure 4.1: Spray microcapsule (SM) production. A mixture of molten lipid and core material was added to a thin-layer chromatography flask and atomized using compressed air. The resulting aerosol solidified forming SMs which dropped into a collecting chamber containing chilled 0.2% aqueous polyvinyl alcohol (PVA). SMs were drained from the collection chamber, passed through a 40 μm mesh Nitex screen, and filtered to produce a wet paste.

Encapsulation efficiency

Capsule preparations were characterized by the ratio of encapsulated core to lipid. Triplicate samples from each batch of capsules were dissolved in chloroform and the core extracted with an aqueous solvent (same solvents as used in above rinses; OTC.HCl core extracted with 0.2 N HCl; OTC.HEM extracted with 0.05 N NaOH). Absorbance of aqueous extracts was measured against a solvent blank at the core material's absorbance peak (OTC.HCl measured at 354 nm, OTC.HEM measured at 379 nm). Absorbance was converted to core concentration using regression equations from standard curves prepared for each core material. A sample of the chloroform phase of the extract was added to a pre-dried/tared crucible and dried for 24 hours in a 50°C oven. Lipid content of the extracted capsules was calculated based upon the amount of lipid recovered after evaporation of the chloroform solvent. Encapsulation efficiency was expressed as mg core/100 mg lipid.

Retention efficiency

Capsule preparations were characterized by the percentage of initial core material retained by capsules after 24 hours suspension in seawater. Capsules were suspended with low intensity (capsules not lysed by sonication) bursts of a sonicator (Braun-Sonic 2000, B Braun Instruments) into 0.5 ml of cold (5°C) 2% w/v aqueous PVA and taken to 5 ml in cold (5°C), filtered (Whatman GF/C) seawater (salinity 33 ppt, pH 8.0) containing 100 ppm of the preservative thimerosal (Sigma Chemical Co.). Three 1 ml aliquots of capsules were removed from the stirred suspension and added to three glass scintillation vials each containing 19 ml of filtered seawater. Ten milliliters of the contents of the three vials were filtered onto glass fiber filters (Whatman GF/C) and rinsed with cold, filtered seawater to obtain zero hour (T=0 hr) measures of core leakage.

The remaining contents of the three vials were taken to 20 ml with cold, filtered seawater. Vials were sealed and placed on a shaker table in darkness for 24 hours at 21°C. At 24 hours, suspensions of capsules were cooled on ice, filtered and rinsed to obtain 24 hour measures of core leakage (T=24 hr). Capsules retained on filters at T=0 hr and T=24 hr were extracted (as described above) and their encapsulation efficiencies calculated. Total concentrations of core material recovered (in seawater filtrate and capsule extracts) at T=0 hr and T=24 hr were compared to check for degradation of core materials in seawater. The 24 hour retention efficiency of capsules was expressed as the percentage of initial core retained after 24 hours of capsule suspension in seawater and determined by dividing the encapsulation efficiency of capsules recovered at 24 hours by their initial encapsulation efficiency at zero hours [retention efficiency = (T=24 hr encapsulation efficiency/T=0 hr encapsulation efficiency) *100].

Delivery efficiency

The variable delivery efficiency was calculated to facilitate comparison of capsule preparations in terms of the amount of core material which may be delivered after 24 hours suspension in seawater. "Theoretical" delivery efficiency (encapsulation efficiency * retention efficiency) assumes that no leakage (e.g., storage leakage) occurs between the measurement of a batch's encapsulation efficiency and the testing of a batch's 24 hour retention efficiency. Such a variable allows comparison of the theoretical maximum amount of core material which may be delivered by each batch of capsules. "True" delivery efficiency is simply the amount of material remaining per unit weight of lipid in capsules after 24 hours in seawater (T=24 hr encapsulation efficiency from retention efficiency measures above). The true delivery efficiency reflects all leakage loses before (storage losses) and during the 24 hour retention efficiency test period. True delivery efficiency is less representative of the intrinsic characteristics of the LWMs since it will

vary with the amount of time elapsing prior to measurement of retention efficiency due to leakage in storage. Theoretical delivery efficiencies were calculated from the batch averages of encapsulation and retention efficiencies, and expressed as mg core/100 mg lipid delivered after 24 hours suspension in seawater.

Statistical analysis

The effects of core (core type; core and core concentration) on encapsulation efficiency and retention efficiency were assessed within each capsule type (SMs or DEMs) by separate nested one-way analysis of variance (ANOVA) of the arcsine square-root transformed data (Sokall and Rolf, 1981). Replicate measures (n=3) of replicate batches (n=3) were nested within each core type to obtain estimates of measurement error and interbatch variability. The contribution of each nesting level to the overall variability of the data was calculated (Sokall and Rolf, 1981). Pairwise comparisons between core types within each capsule types were made using Tukey Honest Significant Difference (HSD) multiple range tests.

The effect of core on theoretical delivery efficiency within each capsule type (SMs or LWMs) was assessed by separate one-way ANOVAs of the arcsine square-root transformed batch averages for each core type. Pairwise comparisons between core types (within capsule types) were made using Tukey HSD multiple range tests.

Comparison of capsule types (SMs vs. DEMs) was made by combining the variables of core type and capsule type. Separate one-way ANOVAs were carried out on the arcsine square-root transformed batch averages of encapsulation, retention, and delivery efficiencies for each core type/capsule type combination (i.e., 30% w/w OTC.HEM/SM, 15% w/w OTC.HEM/SM, 15% w/w OTC.HCl/SM, 300 mg/ml OTC.HCl/LWM, 12 mg/ml OTC.HCl/LWM, and 12 mg/ml OTC.HEM/LWM).

Pairwise comparisons of core type/capsule type combinations were made using Tukey HSD multiple range tests.

Suitability of data for analysis by ANOVA was assessed by viewing normal probability plots of residuals and by Bartlett's test for homogeneity of variance at the 5% level of significance).

Experiment 2: Long-term storage of encapsulated oxytetracyclines

The best capsule type (SMs or DEMs) for encapsulation of each antibiotic (OTC.HEM or OTC.HCl) was chosen (on the basis of results from experiment 1) for use in long-term leakage experiments and to test the effect of freeze-drying on capsule performance during storage. Preliminary experiments conducted to investigate methods for resuspending freeze-dried SMs and DEMs suggested that freeze-drying capsules in a 2% w/v aqueous solution of PVA facilitated resuspension of capsules for testing after storage.

Spray microcapsule and double-emulsion microcapsule preparation

SMs and DEMs were prepared as in Experiment 1. Triplicate batches of SMs containing OTC.HEM (starting percentage, 20% w/w particulate OTC.HEM) were prepared and the batches pooled. SMs were passed through a 40 μm mesh Nitex screen and divided into two equal volumes for use in different storage regimes. "Wet-stored" SMs were filtered onto course glass fiber filters (Whatman 934-AH) and rinsed with 500 ml cold (5°C) 0.05 N NaOH to remove unencapsulated antibiotic. SMs received a final rinse of cold (5°C) distilled water and were filtered to produce a wet paste. The SM paste was stored in a sealed glass scintillation vial at 5°C in darkness. SMs prepared for freeze-drying were filtered and rinsed as described above, but received a final rinse of

cold (5°C) 2% w/v aqueous PVA. The resulting SM paste was diluted with cold (5°C) 2% w/v aqueous PVA to produce a thin slurry. The SM slurry was frozen in glass scintillation vials using a methanol bath (-50°C). Frozen samples were freeze-dried for 8 hours and stored in darkness at 5°C over silica dessicant.

Triplicate batches of DEMs were prepared using a starting ratio of 3.5 ml core material (starting core concentration 300 mg/ml OTC.HCl dissolved in 0.2 N HCl) to 10 grams lipid, as in experiment 1. Batches were pooled after preparation, passed though a 40 μm mesh Nitex screen and divided into two equal volumes. DEMs were processed and stored in the same manner as SMs except for a modification of the solvent rinse used to remove unencapsulated antibiotic (0.05 N NaOH rinse replaced with 0.2 N HCl).

Long-term storage effects

Capsule preparations (pooled batches) were sampled immediately after preparation (T=Day 0; prior to freeze-drying) and triplicate measures of encapsulation efficiency calculated (same method as Experiment 1). Twenty-four hours after capsule preparation, initial measures (in triplicate) of 24 hour retention efficiency (T=Day 1, retention efficiency) were calculated (same methods as Experiment 1). Further measures of 24 hour retention efficiencies were made on day seven, fourteen and twenty-eight of capsule storage.

After 5 weeks, a measure of long-term storage retention efficiency was made. Capsules were suspended in seawater, filtered onto glass fiber filters (Whatman GF/C), and rinsed with distilled water and an appropriate aqueous solvent (0.05 N NaOH or 0.02 N HCl) to remove free or exposed antibiotic. Rinsed capsules were sampled in triplicate and extracted to determine encapsulation efficiencies. Long-term storage retention efficiency was determined for each capsule type by dividing the capsule's encapsulation

efficiencies measured at 5 weeks by the capsule's original (T=Day 0) average encapsulation efficiency.

Two additional measures, the true delivery efficiency of capsules and a time course for the leakage of core materials from DEMs, were calculated for statistical comparison of capsule performance. The true delivery efficiency of capsules (defined as the percentage of originally encapsulated material remaining in capsules after storage leakage and 24 hour leakage in seawater) was calculated for each capsule type at one, seven, fourteen, and twenty-eight days of capsule storage. True delivery efficiency was calculated by dividing the encapsulation efficiency of capsules recovered after 24 hours suspension in seawater (T=24 hr encapsulation efficiency) by their average original T=Day 0 encapsulation efficiency [true delivery efficiency = (T=24 hr encapsulation efficiency day 1, 7, 14, or 28 of storage/average T=Day 0 encapsulation efficiency) * 100].

A time course for the storage retention efficiency of DEMs was calculated using data from 24 hour retention efficiency measures made after one, seven, fourteen, and twenty-eight days of storage. The amount of core retained in DEMs after storage at each time point was determined by dividing the capsules T=0 hr encapsulation efficiency by the capsules average original (T=Day 0) encapsulation efficiency [storage retention efficiency = (T=0 hr encapsulation efficiency at day 1, 7, 14, or 28 of storage/average T=Day 0 encapsulation efficiency)*100]. A time course for the storage retention efficiencies of SMs could not be calculated due to an error in experimental design. The solubility of OTC.HEM was not sufficiently high to allow all material lost during storage to readily dissolve during the capsule rinsing process prior to measures of T=0 hr encapsulation efficiency. As a result storage retention measures calculated for these capsules by the above method may appear high and constant over time.

Statistical analysis

Individual two-tailed t-tests were carried out within capsule types (SM or DEM) to measure the effect of storage method (wet-stored vs. freeze-dried) on 24 hour retention efficiency. Individual t-tests between wet-stored and freeze-dried capsules were carried out on the arcsine square-root transformed 24 hour retention efficiencies at each sample period (1, 7, 14, and 28 days of storage).

Long-term storage retention efficiency of capsules (wet-stored SMs, freeze-dried SMs, wet-stored DEMs, and freeze-dried DEMs) was compared by a one-way analysis of variance (ANOVA) using the response variable of long-term (5 week) storage retention efficiency. Long-term storage retention efficiency was calculated by dividing the arcsine transformed replicate measures of encapsulation efficiency after 5 weeks of storage by the arcsine square-root transformed average original encapsulation efficiency (T=Day 0).

Individual two-tailed t-tests were carried out within capsule types (SM or DEM) to measure the effect of storage method (wet-stored vs. freeze-dried) on true delivery efficiency. Individual t-tests between storage methods (within capsule types) were carried out for each sampling period using the arcsine square-root transformed true delivery efficiency of capsules calculated after 1, 7, 14, and 28 days of storage.

Individual two-tailed t-tests were carried out to measure the effect of storage method (wet-stored vs. freeze-dried) on the storage leakage of DEMs over time. Individual t-tests between storage methods were carried out for each sampling period (1, 7, 14, and 28 days) using the arcsine square-root transformed response variable, storage retention efficiency

Results: experiment 1

Statistical analysis

The effect of core type, in analyses where it was significant, accounted for approximately 97-99% of the variability. While the effect of batch was significant ($p<0.05$ batch effect, one-way nested ANOVA) for each core type, the contribution of batch to total variability was very small, accounting for 1 to 2% of total variability. The significance of batch effect was attributed to extremely low measurement error (≤1% of the total variability) and in terms of the overlying core effect considered insignificant. The analysis of the effect of core type on encapsulation and retention efficiencies was collapsed, by averaging the arcsine square-root transformed encapsulation and retention efficiency measures for each batch. Separate one-way ANOVAs for each capsule type were carried out using the transformed response variable of batch average (encapsulation efficiency or retention efficiency).

General

The encapsulation, retention and delivery efficiencies of SMs and DEMs for OTC.HEM and OTC.HCl are displayed in tables 4.1 and 4.2 and illustrated graphically in figures 4.2a and b, 4.3a and b, and 4.4a and b. Core type (core and core concentration) had a highly significant effect on encapsulation ($p<<0.0001$, one-way ANOVAs, $F_{2,6}$ = 378.67 and 265.77, SMs and DEMs, respectively), retention ($p<<0.0001$, one-way ANOVAs, $F_{2,6}$ = 442.15 and 126.82, SMs and DEMs, respectively), and delivery efficiencies ($p<<0.0001$, one-way ANOVAs, $F_{2,6}$ = 1826.90 and 493.88, SMs and DEMs, respectively) within each capsule type. Core type also had a highly significant

Table 4.1: Encapsulation efficiencies (mg core/100 mg lipid), retention efficiencies (percentage of core retained after 24 hours in seawater), and theoretical delivery efficiencies (mg core/100 mg lipid delivered after 24 hours in seawater) of double-emulsion microcapsules (DEMs) containing an aqueous core of either oxytetracycline hydrochloride (OTC.HCl) or oxytetracycline hemicalcium salt (OTC.HEM).

Initial concentration of aqueous core used in capsule preparation	Encapsulation efficiency (± standard deviation)	Retention efficiency (± standard deviation)	Delivery efficiency (± standard deviation)
300 mg/ml OTC.HCl	3.15±0.53	30.09±1.90	0.94±0.10
12 mg/ml OTC.HCl	0.16±0.01	43.92±2.34	0.07±0.01
12 mg/ml OTC.HEM	0.05±0.01	65.57±3.56	0.03±0.01

Note: Maximum encapsulation efficiency of DEMs for OTC (based upon initial core concentration and ratio of core material added to lipid) = 10.5 and 0.42 mg/100mg lipid for initial core concentrations of 300 mg/ml and 12 mg/ml, respectively.

Table 4.2: Encapsulation efficiencies (mg core/100 mg lipid), retention efficiencies (percentage of core retained after 24 hours in seawater), and theoretical delivery efficiencies (mg core/100 mg lipid delivered after 24 hours in seawater) of spray microcapsules (SMs) containing either particulate oxytetracycline hydrochloride (OTC.HCl) or particulate oxytetracycline hemicalcium salt (OTC.HEM).

Initial proportion of particulate core dispersed in lipid wall material	Encapsulation efficiency (± standard deviation)	Retention efficiency (± standard deviation)	Delivery efficiency (± standard deviation)
30% w/w OTC.HEM	7.42±0.53	65.57±4.46	4.85±0.11
15% w/w OTC.HEM	4.15±0.20	74.87±0.81	3.10±0.12
15% w/w OTC.HCl	1.00±0.15	12.98±1.36	0.13±0.02

Note: Maximum encapsulation efficiency of SMs for OTC (based upon the percentage of core material by weight of capsule materials) = 42.86 and 17.65 mg/100mg lipid for initial core concentrations of 30 and 15% w/w, respectively.

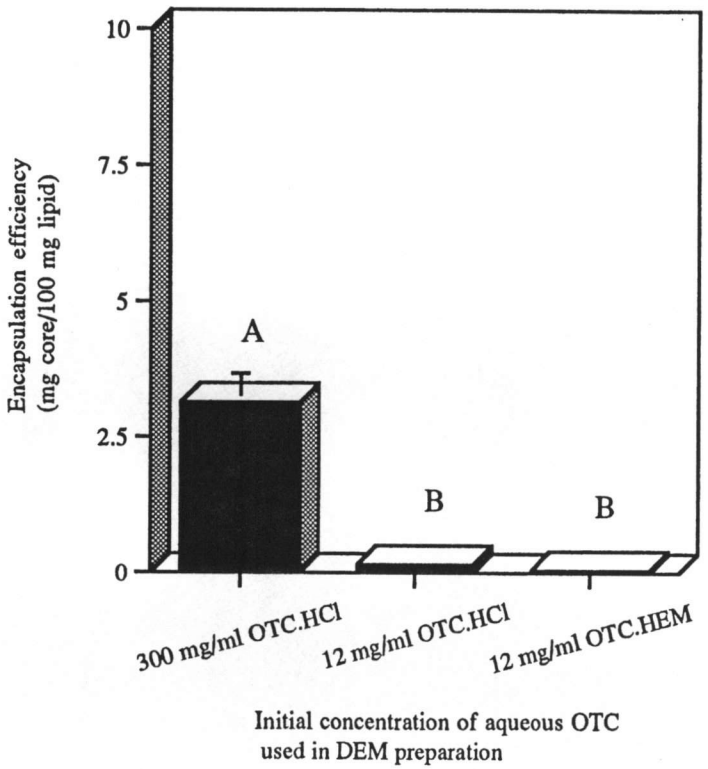

Figure 4.2a: Encapsulation efficiency of double-emulsion microcapsules (DEMs) containing aqueous oxytetracycline (OTC). OTC.HCl = OTC hydrochloride, OTC.HEM = OTC hemicalcium salt. Error bars = ±1 standard deviation. Letters denote a significant difference ($p<0.05$, Tukey Honest Significant Difference (HSD) multiple range tests).

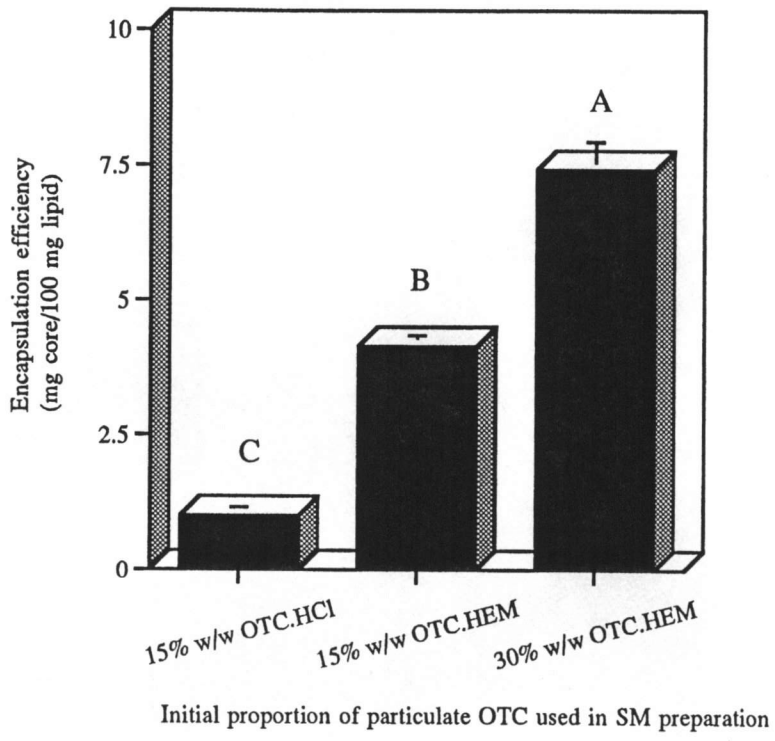

Figure 4.2b: Encapsulation efficiency of spray microcapsules (SMs) containing particulate oxytetracycline (OTC). OTC.HCl = OTC hydrochloride, OTC.HEM = OTC hemicalcium salt. Error bars = ±1 standard deviation. Letters denote a significant difference ($p<0.05$, Tukey Honest Significant Difference (HSD) multiple range tests).

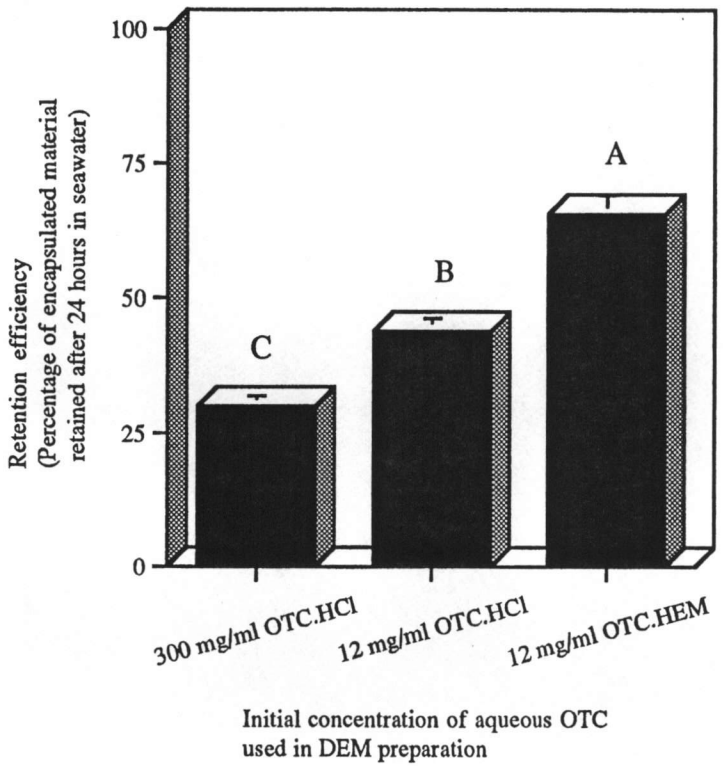

Figure 4.3a: Retention efficiency of double-emulsion microcapsules (DEMs) containing aqueous oxytetracycline (OTC). OTC.HCl = OTC hydrochloride, OTC.HEM = OTC hemicalcium salt. Error bars = ±1 standard deviation. Letters denote a significant difference ($p<0.05$, Tukey Honest Significant Difference (HSD) multiple range tests).

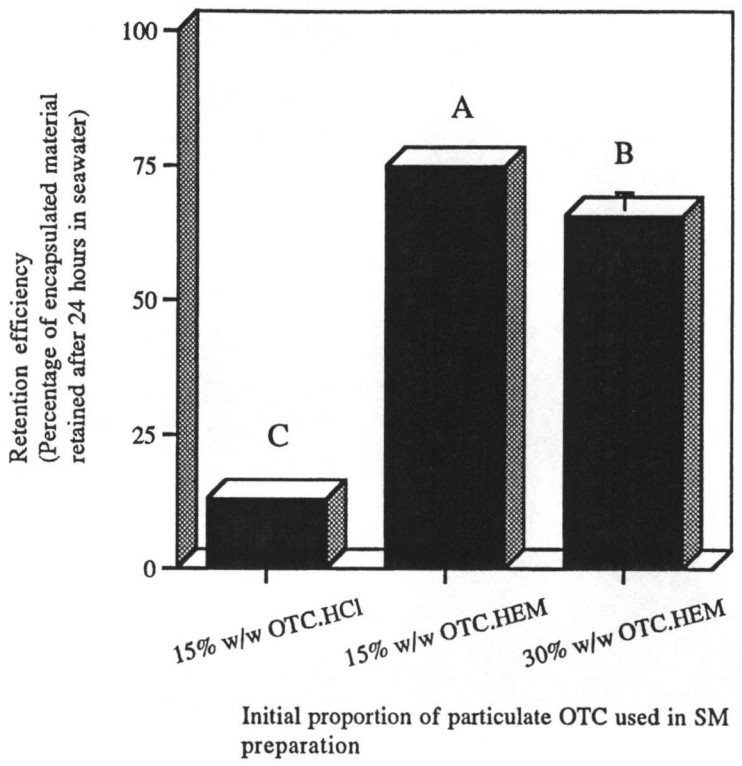

Figure 4.3b: Retention efficiency of spray microcapsules (SMs) containing particulate oxytetracycline (OTC). OTC.HCl = OTC hydrochloride, OTC.HEM = OTC hemicalcium salt. Error bars = ±1 standard deviation. Letters denote a significant difference ($p<0.05$, Tukey Honest Significant Difference (HSD) multiple range tests).

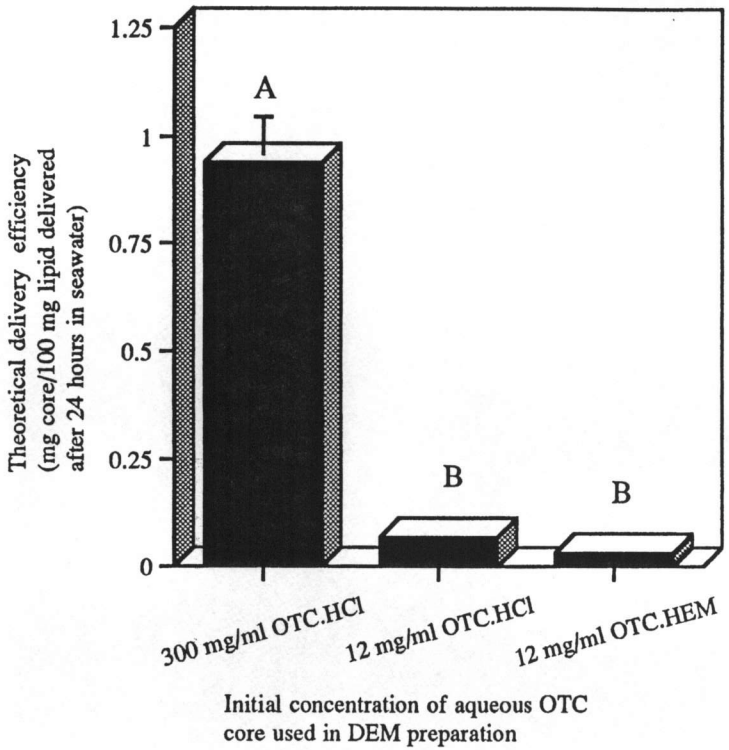

Figure 4.4a: Theoretical delivery efficiency of double-emulsion microcapsules (DEMs) containing aqueous oxytetracycline (OTC). OTC.HCl = OTC hydrochloride, OTC.HEM = OTC hemicalcium salt. Error bars = ±1 standard deviation. Letters denote a significant difference ($p<0.05$, Tukey Honest Significant Difference (HSD) multiple range tests).

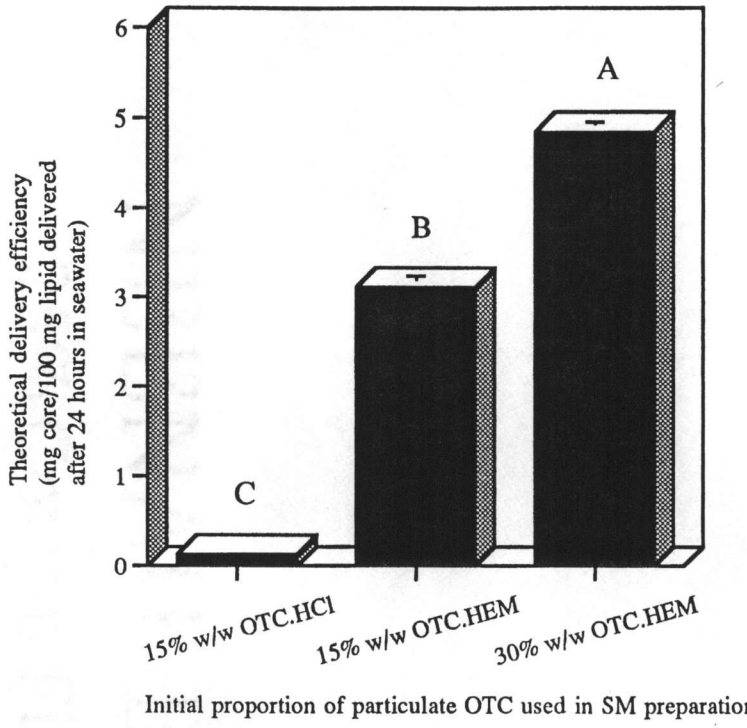

Figure 4.4b: Theoretical delivery efficiency of spray microcapsules (SMs) containing aqueous oxytetracycline (OTC). OTC.HCl = OTC hydrochloride, OTC.HEM = OTC hemicalcium salt. Error bars = ±1 standard deviation. Letters denote a significant difference ($p<0.05$, Tukey Honest Significant Difference (HSD) multiple range tests).

effect on encapsulation, retention, and delivery efficiency across capsule types ($p<<0.00001$, one-way ANOVAs of core type/capsule type combination, $F_{5,12}$ = 412.67, 237.27, and 3217.07, respectively).

Effect of core material across capsule types

OTC is most efficiently encapsulated, retained and delivered as a hemicalcium salt encapsulated in SMs ($p<0.05$, Tukey HSD multiple range tests of core type/capsule type combinations). As much as 7.42 mg OTC.HEM/100 mg lipid may be encapsulated in lipid SMs (Table 4.2). The retention efficiency of SMs containing OTC.HEM was more than twice that of DEMs containing OTC.HCl, such that five times more OTC.HEM may be delivered by SMs than OTC.HCl by DEMs (Tables 4.1 and 4.2).

OTC.HCl is most efficiently encapsulated (3.15 mg /100 mg lipid) in DEMs (Table 4.1). Significantly more OTC.HCl could be encapsulated, retained, and delivered by DEMs than by SMs ($p<0.05$, Tukey HSD multiple range tests of core type/capsule type combinations). The retention of both aqueous core concentrations of OTC.HCl (300 mg/ml and 12 mg/ml) by DEMs was significantly higher than the retention of particulate OTC.HCl by SMs ($p<0.05$ Tukey HSD multiple range tests of core type/capsule type combinations).

The retention efficiency of capsules (SMs and DEMs) containing OTC.HEM was significantly higher than those containing OTC.HCl, regardless of core concentration or capsule type ($p<0.05$, Tukey HSD multiple range tests of core type/capsule combinations).

Effect of core material within capsule types

Core type had a significant effect on the encapsulation, retention and delivery efficiency of SMs containing OTC ($p<<0.0001$, one-way ANOVAs). Significantly more OTC.HEM than OTC.HCl (4 times more) may be encapsulated in lipid SMs when starting with the same percentage by weight of particulate core in the prespray mix of capsule materials ($p<0.05$, Tukey HSD multiple range test; Figure 4.2b). OTC.HEM is also retained and delivered by SMs more efficiently than OTC.HCl ($p<0.05$, Tukey HSD multiple range tests; Figure 4.3b and Figure 4.4b).

Core type also had a significant effect on encapsulation, retention and delivery efficiency of DEMs containing OTC ($p<<0.0001$, one-way ANOVAs). Although more OTC.HCl (3 times more) than OTC.HEM was encapsulated in DEMs (Table 4.1) when capsules were prepared starting with the same aqueous core concentrations and ratios of core to lipid, this difference in encapsulation efficiency was not highly significant ($p=0.13$, Tukey HSD multiple range test; Figure 4.2a). At the higher core concentration of OTC.HCl (300 mg/ml), significantly more OTC.HCl was encapsulated in DEMs than OTC.HEM ($p<0.05$, Tukey HSD multiple range test; Figure 4.2a). OTC.HCl was retained significantly less well than OTC.HEM by DEMs ($p<0.05$, Tukey HSD multiple range tests; Figure 4.3a). However, due to its higher encapsulation efficiency, significantly more OTC.HCl than OTC.HEM was delivered by DEMs ($p<0.05$, Tukey HSD multiple range tests; Figure 4.4a).

Discussion: experiment 1

While DEMs and SMs may both be used to encapsulate either aqueous or particulate core materials, each method for producing LWMs is most effective for a given type of core. For example, aqueous OTC.HCl may be encapsulated in DEMs or SMs

(Chapter 2 and Chapter 4, Experiment 1). However, the double-emulsion process is typically more effective for encapsulating aqueous core materials than particulate (e.g., 1.83 mg OTC.HCl/100 mg lipid may be encapsulated in SMs compared to 3.15 mg OTC.HCl/mg lipid in DEMs; Chapter 2 and this experiment). Similarly, a spray process for producing LWMs is typically more effective for encapsulating dry particulate core materials than the double-emulsion process (e.g., four times more particulate riboflavin may be encapsulated in SMs than DEMs, Langdon and Buchal, unpublished data). The purpose of this experiment was to encapsulate each form of OTC using the most effective process for a given phase (aqueous or particulate) of the core material. Comparison of the encapsulation efficiency DEMs and SMs for the different OTCs illustrates the strengths and weaknesses of each encapsulation process, as well as demonstrating the best method for encapsulating the two forms of OTC.

Comparison of the effectiveness with which OTC.HEM was encapsulated in DEMs and SMs illustrates a limitation of the double-emulsion process of encapsulation. Resulting capsules (DEMs and SMs) containing OTC.HEM have fairly high retention efficiencies (both exhibit a retention efficiency of 65%). However since the solubility of OTC.HEM is very low (only 12 mg/ml in 0.2 M sodium carbonate) its maximum encapsulation efficiency in DEMs is 152 times less than SMs containing particulate OTC.HEM. Encapsulation of slightly soluble materials, such as OTC.HEM, as particulate core in SMs represents a much more effective encapsulation method than encapsulation of these materials as an aqueous core in DEMs.

Highly soluble materials such as OTC.HCl are more effectively encapsulated as an aqueous core by DEMs (3.15 grams core /100 grams lipid, Table 4.1) than as particulate core in SMs (1.00 gram core/100 grams lipid, Table 4.2). In addition, OTC.HCl is retained significantly better as an aqueous core by DEMs (30-44% retained, Table 4.1) than as a particulate core in SMs (13% OTC.HCl retained, Table 4.2) ($p<0.05$, Tukey HSD multiple range tests of core/capsule combinations).

The differences in the retention characteristics between DEMs containing OTC.HCl and OTC.HEM (encapsulated using similar starting aqueous core concentrations; Table 4.1, Figure 4.3a) and the differences between SMs containing OTC.HCl and OTC.HEM (encapsulated using the same percentages by weight of particulate core to lipid; Table 4.2, Figure 4.3b) are best explained by interactions between capsule materials (core and lipid) observed during the two different encapsulation processes.

OTC.HEM is encapsulated 4 times more effectively than OTC.HCl in lipid SMs (when starting with similar percentages by weight of particulate core to lipid, i.e., 15 %w/w OTC in lipid). The behavior of these OTCs in the encapsulating lipid differs throughout the encapsulation process. Particulate OTC.HCl when sonicated in molten lipid, causes a thickening of the lipid mixture (suspensions of >15%w/w OTC.HCl become too thick to spray). In the spray flask and during the spray process, OTC.HCl particles reaggregate into clumps, which can partially or completely block the spray tip. If a spray is aborted due to clogging, OTC.HCl may be observed to aggregate and fall out of suspension in the spray flask reservoir. The results of this aggregation behavior may be observed in the resulting SMs. Individual SMs contain aggregates of OTC.HCl which are sparsely distributed throughout the lipid bead.

In contrast, OTC.HEM particulate forms a very homogenous suspension when sonicated into molten lipid and pours and sprays evenly. As demonstrated, the starting percentage of OTC.HEM may be doubled with a similar increase in encapsulation efficiency (see Table 4.2). Resulting SMs contain individual crystals of antibiotic uniformly distributed throughout the lipid bead.

The aggregation of OTC.HCl particulate during the spray process may adversely effect encapsulation efficiency in several ways: core material may be lost from suspension before a spray is complete, effectively lowering the starting ratio of core to lipid. Aggregates of core in the spray tip may act to filter the lipid as it passes out of the

spray tip, lowering core concentrations in resulting SMs. Also, the core may separate from the lipid as it exits from the spray tip, the unencapsulated core then being lost in the collecting bath. In addition, the hydrophilic nature of the OTC.HCl particulate possibly results in a greater number of particles exposed at the surface of the SMs which, when removed by rinsing, could result in empty pockets and fissures in the SM wall. OTC.HEM particulate, being more insoluble, seems to be easily coated by lipid such that particles near the surface are well protected.

It was expected that an OTC.HCl core (aqueous and particulate) might leach more rapidly than an OTC.HEM core from both SMs and DEMs given OTC.HCl's higher solubility in the aqueous environment (solubilities in water at 25°C, 1 gram OTC.HCl/ml vs. 0.01 gram OTC.HEM/ml). It was also expected that differences in leakage of OTC.HCl between capsule types would favor SMs. However, the loss of OTC.HCl particulate from SMs (87% lost in 24 hours in seawater, Table 4.2) was significantly higher than the loss of aqueous OTC.HCl from DEMs (56-70% lost in 24 hours in seawater; Table 4.1) ($p<0.05$, Tukey HSD multiple range tests of core type/capsule type combinations). This difference between the 24 hour retention efficiencies of the two capsule types containing the same core material may be attributed to differences in the processes of SM and DEM formation.

As noted OTC.HCl crystals aggregate and separate from lipid during SM formation, resulting in surface exposed core. Removal of surface exposed particulate from SMs during the rinsing and filtration processes, may create empty pockets and fissures into the SM wall. In contrast, DEMs are formed when lipid completely surrounds a droplet of aqueous core. In the double-emulsion process this occurs when the primary emulsion (molten lipid containing micron-sized droplets of core) is homogenized in an aqueous solution of hot PVA. As the lipid is dispersed it forms lipid droplets containing smaller droplets of core material. Cooling of this solution by its addition to cold water yields DEMs. During the encapsulation process, unencapsulated

core droplets pass into the hot PVA and cooling water. Since encapsulation of an aqueous core in DEMs depends upon the lipid completely surrounding the core, there can be no surface-exposed core as may occur when a hydrophobic particulate is dispersed into a lipid bead. There should be less deformation of the capsule wall by rinsing and filtering of the capsules, since core material is simply lost (unencapsulated) during capsule formation, rather than partially encapsulated and then dissolved as in SM formation. DEMs might therefore be expected to have higher retention due to better wall integrity.

An additional factor common to all capsule preparations is the presence of phospholipid in the lipid wall. Phospholipids may form micelles and channels in the capsule wall, resulting in an increase in wall permeability (Chapter 2). While all capsule preparations were stored as wet pastes, DEMs containing aqueous core materials might favor complete hydration of phospholipids in the capsule wall. Hydrated phospholipids would be expected to more readily form channels in the lipid wall exposing core materials to the external environment (Chapter 2). This is not explicitly supported by the data, which suggest that retention efficiency within each capsule type is strongly effected by core type (Tables 4.1 and 4.2). The degree of channel formation and core leakage associated with phospholipid components of the capsule wall may be dependent on a more core specific, rather than phase specific, interaction between the core material and phospholipid

Results: experiment 2

Capsule type and storage method had a significant effect on long-term storage retention efficiency ($p<<0.0001$, one-way ANOVA storage/capsule combinations, $F_{3,8} = 549.54$; Table 4.3).

Table 4.3: Long-term storage retention efficiency of lipid-walled microcapsules. Encapsulation efficiencies (mg core/100mg lipid) and standard deviations of lipid spray microcapsules (SMs) and double-emulsion microcapsules (DEMs) measured after capsule preparation (T=Day 0) and after 5 weeks of storage. Capsules stored as wet pastes (wet-stored) or as freeze-dried powders (freeze-dried). SMs contained oxytetracycline hemicalcium salt (OTC.HEM). DEMs contained oxytetracycline hydrochloride (OTC.HCl).

Capsule type	Original encapsulation efficiency Day 0	Encapsulation efficiency after 5 weeks storage	Percentage of originally encapsulated material retained
wet-stored SMs	6.63±0.13	5.69±0.10	85.85±0.83
freeze-dried SMs	7.67±0.07	4.19±0.26	54.60±3.41
wet-stored DEMs	4.71±0.09	1.34±0.01	28.48±0.69
freeze-dried DEMs	4.60±0.09	1.78±0.03	38.77±1.26

Note: Maximum encapsulation efficiency of SMs for OTC.HEM (based upon the percentage of core material by weight of capsule materials) = 25 mg core /100mg lipid.

Maximum encapsulation efficiency of DEMs for OTC.HCl (based upon initial core concentration and ratio of core material added to lipid) = 10.5 mg core/100 mg lipid.

Table 4.4: Time course of storage retention efficiency of double-emulsion microcapsules (DEMs). Percentage (and standard deviation) of core material retained during storage. Retention calculated from amount of material originally encapsulated (T=Day 0) and T=0 hr measures of encapsulation efficiency on Day 1, 7, 14, and 28 of storage. Percentage of core material retained = [(T=0 hr encapsulation efficiency)/(Day 0 encapsulation efficiency)*100]. Capsules stored as wet pastes (wet-stored) or as freeze-dried powders (freeze-dried). DEMs contained oxytetracycline hydrochloride (OTC.HCl).

	Days of storage			
Capsule type	1	7	14	28
wet-stored DEMs	47.47±1.47	31.22±0.22	29.05±0.06	29.46±1.57
freeze-dried DEMs	28.50±1.02	30.68±1.06	36.27±2.73	35.45±1.69

OTC.HEM spray microcapsules stored for 5 weeks as a wet paste exhibited significantly higher retention of core material than freeze-dried SMs, wet-stored DEMs or freeze-dried DEMs ($p<0.05$, Tukey HSD multiple range tests, Table 4.3). The effect of freeze-drying on capsule performance appears to vary depending on capsule type (see Table 4.3): freeze-dried SMs exhibited lower retention of core (55%) than wet-stored SMs (86%) ($p<0.05$, Tukey HSD multiple range test), while freeze-dried DEMs retained significantly more material (39%) than wet-stored DEMs (29%) ($p<0.05$, Tukey HSD multiple range test).

Over time, storage retention efficiency of DEMs seemed to change such that wet-stored DEMs retained progressively less core material, while retention of core material by freeze-dried DEMs improved (Table 4.4; Figure 4.5). Retention of core material by wet-stored DEMs was significantly higher than by freeze-dried DEMs one day after storage ($p<0.05$, two-tailed t-test), approximately equal after one week, and then significantly less at weeks two and four of storage ($p<0.05$, two-tailed t-tests).

The same pattern in capsule performance was exhibited in measures of 24 hour retention efficiency of DEMs over time. The 24 hour retention efficiency of wet-stored and freeze-dried DEMs did not differ significantly one day after capsule preparation (two-tailed t-test). However, over time, the 24 hour retention efficiency of freeze-dried DEMs improved, while that of wet-stored DEMs decreased (see Table 4.5, Figure 4.6). The 24 hour retention efficiency of freeze-dried DEMs was significantly higher than wet-stored DEMs when tested after one, two, and four weeks of storage ($p<0.05$, two-tailed t-tests).

The 24 hour retention efficiency of both wet-stored and freeze-dried SMs did not appear to change over one month of storage (see Table 4.5, Figure 4.6). The 24 hour retention efficiency of wet-stored SMs was significantly greater than freeze-dried SMs when tested one day after capsule preparation ($p<0.05$, two-tailed t-test). This difference was maintained after one, two and four weeks of storage ($p<0.05$, two-tailed t-tests).

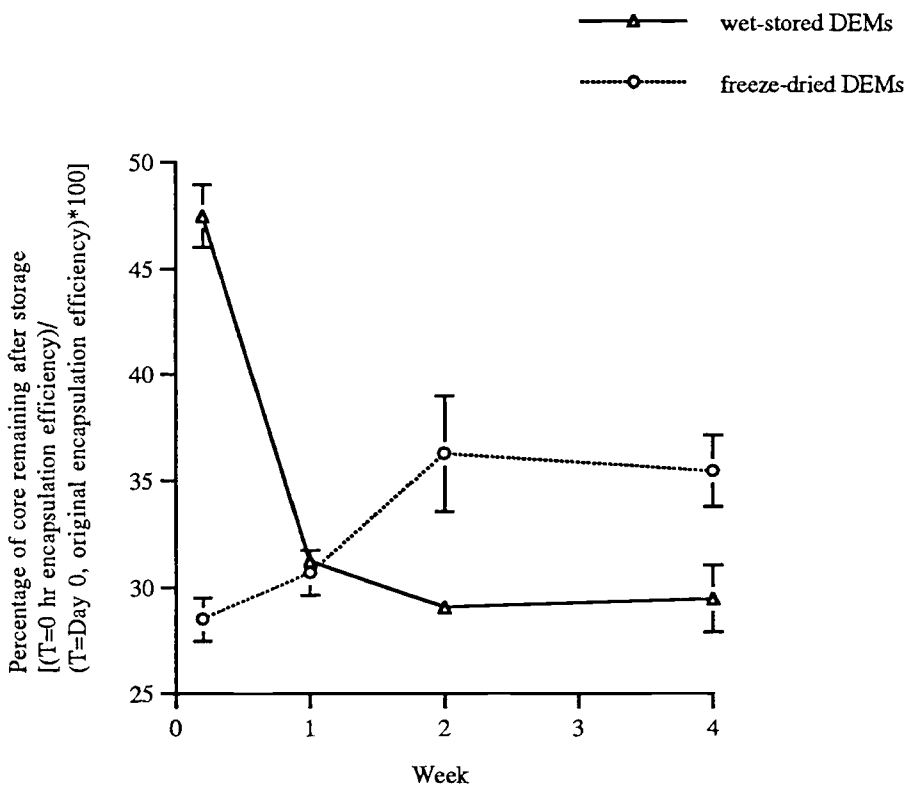

Figure 4.5: Storage retention efficiency of wet-stored and freeze-dried double-emulsion microcapsules (DEMs) containing aqueous oxytetracycline hydrochloride (OTC.HCl). Storage retention efficiency calculated on days 1, 7, 14, and 28 of capsule storage. Error bars = ±1 standard deviation.

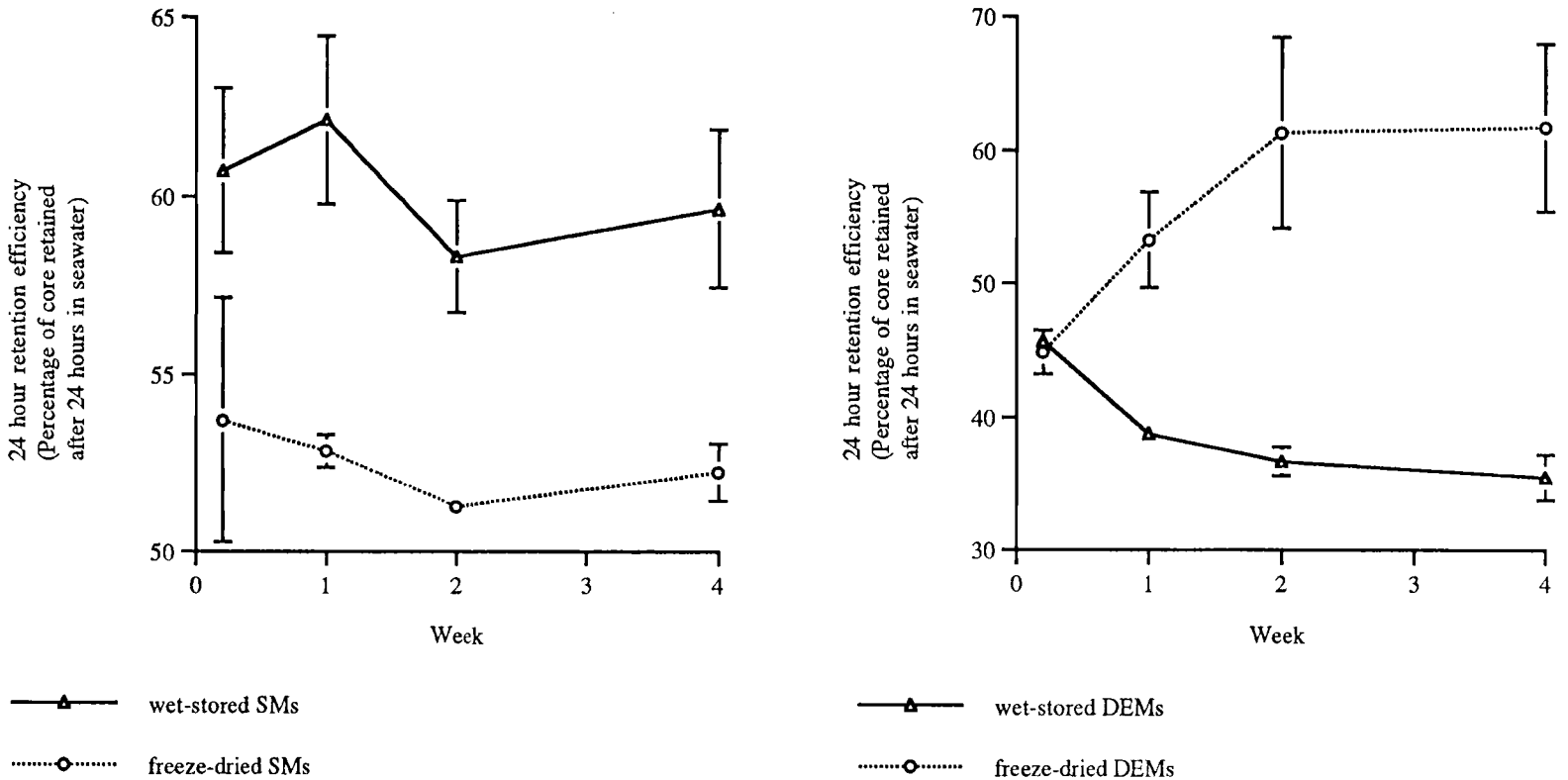

Figure 4.6: Twenty-four hour retention efficiency of spray microcapsules (SMs) and double-emulsion microcapsules (DEMs) containing oxytetracycline hemicalcium salt (OTC.HEM) and oxytetracycline hydrochloride (OTC.HCl), respectively. Capsules stored as wet pastes (wet-stored) or freeze-dried powders (freeze-dried). Twenty-four hour retention efficiency measures made on day 1 , 7, 14 and 28 of capsule storage. Error bars = ± 1 standard deviation.

Table 4.5: Twenty-four hour retention efficiency of lipid-walled microcapsules. Percentage (and standard deviation) of material retained after 24 hours suspension in seawater. Calculated from 24 hour retention efficiency experiments conducted on Day 1, 7, 14, and 28 of storage. Percentage retained = [(T=24 hr encapsulation efficiency)/(T=0 hr encapsulation efficiency)*100]. SM = spray microcapsules. DEMs = double-emulsion microcapsules. Capsules stored as wet pastes (wet-stored) or as freeze-dried powders (freeze-dried). SMs contained oxytetracycline hemicalcium salt (OTC.HEM). DEMs contained oxytetracycline hydrochloride (OTC.HCl).

	Days of storage			
Capsule type	1	7	14	28
wet-stored SMs	60.72±2.30	62.14±2.35	58.31±1.58	59.65±2.22
freeze-dried SMs	53.70±3.44	52.84±0.47	51.25±0.36	52.23±0.82
wet-stored DEMs	45.72±0.99	38.80±0.33	36.68±1.09	35.48±1.73
freeze-dried DEMs	44.88±1.65	53.31±3.62	61.33±7.14	61.69±6.27

Similarly, the percentage of originally encapsulated material which was delivered after 24 hours by wet-stored SMs (true delivery efficiency) was significantly greater ($p<0.05$, two-tailed t-test) than freeze-dried SMs one day after capsule preparation and over the subsequent weeks of storage (Figure 4.7). The amount of material delivered did not change for either storage method (Table 4.6)

Table 4.6: True delivery efficiency of lipid-walled microcapsules. Percentage (and standard deviation) of originally encapsulated material remaining after 24 hours suspension in seawater. Calculated from amount of material originally encapsulated (T=Day 0) and T=24 hr measures of encapsulation efficiency on Day 1, 7, 14, and 28 of storage. Percentage of originally encapsulated material remaining = [(T=24 hr encapsulation efficiency)/(T=Day 0 encapsulation efficiency)*100]. SMs = spray microcapsules. DEMs = double-emulsion microcapsules. Capsules stored as wet pastes (wet-stored) or as freeze-dried powders (freeze-dried). SMs contained oxytetracycline hemicalcium salt (OTC.HEM). DEMs contained oxytetracycline hydrochloride (OTC.HCl).

	Days of storage			
Capsule type	1	7	14	28
wet-stored SMs	59.26±1.88	59.02±1.75	57.74±1.43	61.03±0.26
freeze-dried SMs	49.56±3.14	48.06±0.16	46.70±1.00	50.69±1.41
wet-stored DEMs	21.70±0.51	12.11±0.27	10.65±0.50	10.44±0.17
freeze-dried DEMs	12.78±0.09	16.33±0.79	22.13±1.58	21.80±1.58

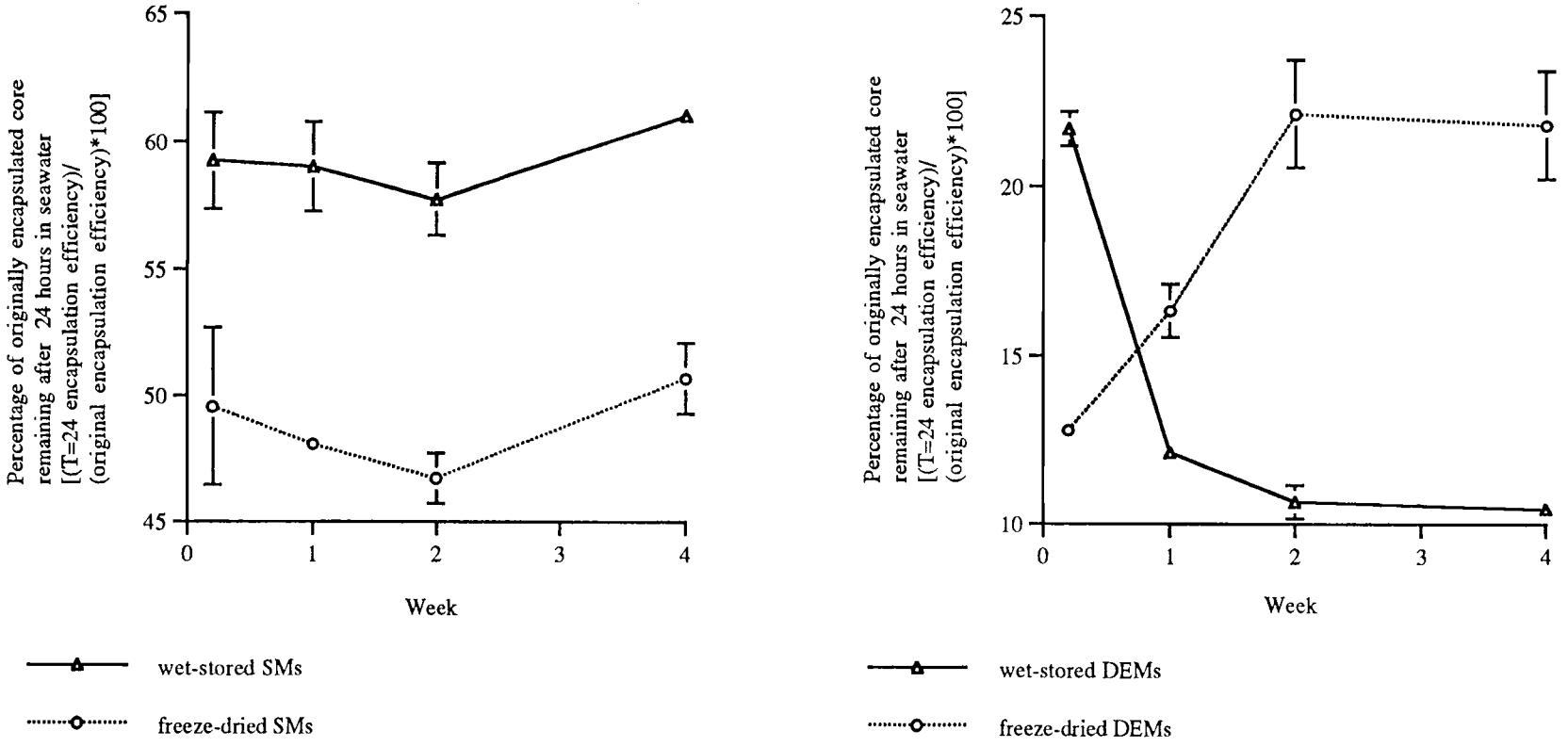

Figure 4.7: True delivery efficiency of lipid-walled microcapsules. Percentage of originally encapsulated core delivered after 24 hours in seawater by spray microcapsules (SMs) and double-emulsion microcapsules (DEMs) containing oxytetracycline hemicalcium salt (OTC.HEM) and oxytetracycline hydrochloride (OTC.HCl), respectively. Capsules stored as wet pastes (wet-stored) or freeze-dried powders (freeze-dried). Measures made on day 1, 7, 14, 28 of capsule storage. Error bars = ± 1 standard deviation.

Wet-stored DEMs initially delivered (true delivery efficiency) significantly more OTC.HCl than freeze-dried DEMs ($p<0.05$, two-tailed t-test; Table 4.6). However, after one, two, and four weeks of storage, wet-stored DEMs delivered significantly less core material ($p<0.05$, two-tailed t-test; Figure 4.7).

Discussion: experiment 2

The most effective method for encapsulating and delivering OTC is by lipid SMs containing particulate OTC.HEM. These SMs (wet-stored and freeze-dried) exhibit moderately good 24 hour retention efficiencies (66-75%, Table 4.2) and retain their core best when stored as a wet paste (86% of originally encapsulated core retained in capsules after 5 weeks storage, Table 4.3). While overall SM performance appears to be quite good, errors in SM testing protocol may have reduced measures of 24 hour retention efficiency.

The testing protocol for measuring the twenty-four hour retention efficiency of capsules required that core materials leached from capsules during storage be readily soluble in seawater. Capsules removed from storage were briefly suspended in seawater, filtered out of suspension, and rinsed with seawater. The encapsulation efficiency of the filtered capsules was calculated and used as the measure of T=0 hr encapsulation efficiency. It was assumed that all core material which had leached from capsules during storage would be effectively removed during the resuspension, filtration and rinsing of the capsules. The resulting measure of T=0 hr encapsulation efficiency was used as both a measure of the initial amount of core material contained within capsules at the start of the 24 hour retention efficiency measure, and as a measure of the amount of core material remaining after storage. Since, OTC.HEM does not rapidly dissolve in seawater, material leached from capsules during storage may not have been effectively removed by rinsing the capsules when the T=0 hr encapsulation efficiency measurement was taken.

As a result, the 24 hour retention efficiency values (Table 4.5) could reflect both losses of core material over the 24 hour test period and losses of during storage prior to retention measures. As a result, the twenty-four hour retention efficiency of these capsules may be higher than measured (those of storage retention efficiency would be lower).

For example, it is evident from the long-term storage retention measures conducted at 5 weeks that the 24 hour retention efficiency of wet-stored SMs at 4 weeks should have been around 75%, instead of the 60% measured during the 24 hour retention efficiency test (Table 4.5). Measures of long-term storage retention efficiency, employed an extensive solvent rinse (0.05 N NaOH) which should have effectively removed dissolved and crystallized core material leached from capsules during storage. After 5 weeks of storage (Table 4.3), 86% of the core material initially (Day 0) encapsulated in wet-stored SMs was retained (14% of the core material leached from capsules in storage). After 4 weeks in storage, the true delivery efficiency of these capsules [percentage of originally encapsulated core material (Day 0) remaining after storage and 24 hours in seawater] was 61% (Table 4.6; a combined 39% of core material leached from capsules during storage and 24 hours in seawater). Therefore, of the 39% of the originally encapsulated (Day 0) material lost , as much as 14% might be expected to have leached from capsules during storage, leaving 25% to be leached during the 24 hours of suspension in seawater.

In contrast, freeze-dried SMs retained 54% of their core material (46% of core material leached from capsules) during 5 weeks of storage (Table 4.3). Neither their 24 hour retention efficiency (Table 4.5) or true delivery efficiency (Table 4.6) changed appreciably during the initial four weeks of storage. After four weeks of storage, freeze-dried SMs delivered (true delivery efficiency) 51% of their originally encapsulated (T=Day 0) core (a combined 49% of the core leached from capsules during storage and 24 hours of suspension in seawater, Table 4.6). This suggests that these SMs experienced most of their core leakage during storage and that their 24 hour retention

efficiency was actually quite high, around 97% (49% of core leached from capsules during storage and 24 hours in seawater at four weeks - 46% of core leached from capsules after 5 weeks of storage).

The higher storage leakage of freeze-dried SMs may be an artifact of the freezing process which may rupture the lipid wall or interrupt the packing order of the lipids in the capsule wall. In SMs having a high proportion of evenly distributed particulate core material, such ruptures may remain upon returning to a higher storage temperature. These ruptures in the capsule wall may expose particulate core materials and increase leakage.

Since the 24 hour retention efficiencies (Table 4.5) and the amount of originally encapsulated core delivered in 24 hours (Table 4.6) by SMs in both storage regimes (wet-stored and freeze-dried) did not change over time, it seems likely that storage leakage did not increase after the first 24 hours following capsule preparation.

Double-emulsion microcapsules

Since OTC.HCl is highly soluble, it was possible to accurately calculate and track storage retention efficiencies and 24 hour retention efficiencies over time for wet-stored and freeze-dried DEMs using the T=0 hr encapsulation efficiency values from 24 hour retention efficiency measures. Since all of the OTC.HCl lost from capsules during storage dissolved rapidly when capsules were suspended in seawater, the measures of T=0 hr encapsulation efficiency accurately reflect the amount of initially encapsulated (T=Day 0) core material remaining after storage and the amount of core material initially encapsulated (T=0 hours) in DEMs at the start of the 24 hour retention measures. Indeed, the difference between the true delivery efficiencies (reflecting combined storage and 24 hour leakage losses; Table 4.6) and the 24 hour retention efficiencies (reflecting losses during 24 hours in seawater; Table 4.5), agree well with the storage retention efficiency

values calculated (T=0 hr encapsulation efficiency/ original (Day 0) encapsulation efficiency) and displayed in Table 4.4.

The differences between freeze-dried and wet-stored DEMs is less interesting than the pattern of leakage (storage and 24 hour losses) each preparation follows over time. DEMs stored as a wet paste exhibit very high leakage during the first day of storage (only 47.5% of the originally encapsulated material remains encapsulated; Table 4.4, Figure 4.5). These capsules seem to lose an additional 16% of their core material over the next 7 days, with leakage leveling off at approximately 71% after 14 and 28 days in storage (29% storage retention efficiency day 14 and day 28; see Table 4.4, Figure 4.5). Similarly, the 24 hour retention efficiency of the wet-stored DEMs decreases between days 1 and 7 and then levels out to approximately 36% on days 14 and 28 of storage (Table 4.5 and Figure 4.6).

In contrast, freeze-dried DEMs act in the opposite manner. The initial amount of leakage (T=Day 1) after freeze-drying and storage is extremely high, only 28.5% of the original core material remains encapsulated (Table 4.4 and Figure 4.5) . However, between day one and day fourteen of storage, storage losses actually decrease. This improvement in core retention seems to level off at day 14 (see Table 4.4 and Figure 4.5). This reduction in leakage is also reflected in the improved 24 hour retention efficiency of freeze-dried capsules between day one and day fourteen of testing (Figure 4.6). These improvements in both storage and 24 hour retention suggest that core material somehow becomes more effectively encapsulated during storage in freeze-dried LWMs.

Two mechanisms may explain this phenomenon. One explanation is that the OTC.HCl in the freeze-dried DEM preparation (either encapsulated or non-encapsulated) becomes less soluble or insoluble during the drying process or as a result of the PVA used in the drying process. For storage leakage and 24 hour retention efficiency measures to be accurate, OTC.HCl must be readily soluble in seawater. The solubility of the OTC.HCl does not appear to change with freeze-drying since the storage retention

efficiency of these capsules calculated at day 28 (Table 4.4) agree with the measures of long-term storage retention efficiency calculated after 5 weeks (Table 4.3). If there was a change in OTC.HCl's solubility in seawater, the 5 week storage retention measures (which employ an extensive solvent (0.2 N HCl) rinse in addition to a rinse of distilled water) should have resulted in different measures of leakage. Further, the encapsulation efficiency of unrinsed, freeze-dried DEMs calculated after 5 weeks was very close to that calculated originally (Day 0), indicating that the extraction procedure remained effective (0.2 N HCl was an adequate solvent) and that neither core degradation or a change in the absorbance spectra of the OTC occurred. An alternative mechanism is that the capsule walls actually became less permeable over time in the freeze-dried DEM preparation

Some steps in the freeze-drying process help to explain how these capsules may become less permeable over time. Freeze-dried DEMs are initially frozen in a 2% w/v aqueous solution of PVA and placed under vacuum. The process of freezing may interrupt the packing order of the lipids in the capsule wall, creating the opportunity for pores or channels to develop. In addition, the expansion of the aqueous core in DEMs as it freezes may rupture the capsule wall. During freeze-drying, the moisture from the aqueous core is drawn though the lipid wall. This process may create channels or weaknesses in the capsule wall. This tendency to form channels is likely to be even more pronounced since the lipid wall composition includes phospholipids present as contaminants in the FO portion of the lipid mixture (Appendix C). Hydrated phospholipids contained in the lipid wall might be expected to experience polymorphism and adopt a number of structural conformations (Cullis and Hope, 1985, Lindblom and Rilfors, 1992). Phospholipids in the triacylglyceride wall may form micelles and channels in the lipid wall and associate with the aqueous core and surface of the capsule (Figure 4.8; Chapter 2). Moisture drawn through the lipid wall is likely to exacerbate phospholipid channel formation. As moisture is drawn through the lipid wall, hydrated phospholipids and micelle structures within the lipid wall might be expected to orient

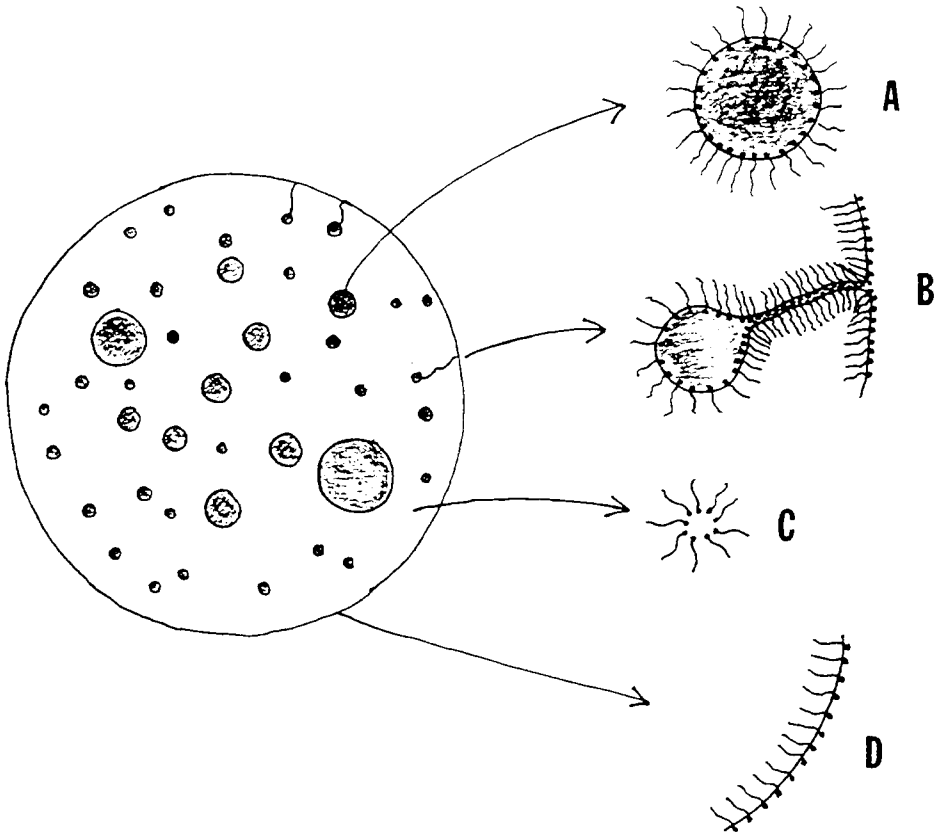

Figure 4.8: Possible behavior of phospholipids dissolved in a triacylglyceride (60%w/w tripalmitin/40% w/w fish oil) lipid-walled microcapsule (LWM) containing aqueous core:

A) Phospholipid association with aqueous core. Polar head groups of phospholipids insert themselves into core. Hydrophobic tails extend out into the lipid.

B) Phospholipid channel connecting aqueous core material to surface of the LWM. Phospholipids line the channel inserting their polar head groups into the aqueous core and channel environment. Hydrophobic phospholipid tails extend into the lipid.

C) Phospholipid micelle. Phospholipids aggregate within the LWM, orienting themselves with their polar head groups at the center of the micelle and hydrophobic tails extending into the lipid.

D) Phospholipid association with LWM surface. Phospholipids aggregate at surface of the LWM exposing their polar head groups to the external aqueous environment. Hydrophobic tails extend into the lipid.

themselves into the configuration of a channel. This mechanism might be envisioned as the opening of a zipper, the phospholipids orienting to form the zipper teeth as moisture is drawn towards the outside of the capsule wall (Figure 4.9). If DEMs were not completely dry when removed from the freeze-dryer unit, moisture may still have been contained within the capsule walls and within phospholipid channels. The phospholipid channels would be kept open by residual moisture making the core more accessible the environment. Channel formation and residual moisture in the freeze-dried preparation may be the cause of the low (Day 1) storage retention of freeze-dried DEMs (Table 4.4). During storage over silica dessicant, the drying process may have been completed. Complete removal of moisture from the capsule walls, may allow the capsule walls to "heal", such that channels and inclusions produced by the drying process are eliminated and capsule integrity restored. As moisture is completely removed from the capsule wall, phospholipid channels may in effect zip closed and the dehydrated phospholipids reorient themselves more arbitrarily in the capsule wall (Figure 4.9).

While crystals of core material were observed in freeze-dried DEMs, it was noted during handling and testing that freeze-dried DEMs initially clumped together and appeared sticky. Over time in storage, freeze-dried DEMs became lighter in color and more flocculent . This observation suggests that the preparation was not completely dried when removed from the freeze-drying unit.

Conclusions

While DEMs appear to be less suited for delivery of OTC, compared to SMs, the long-term storage characteristics of freeze-dried DEMs suggest that the performance of this capsule type may be improved. Further testing of completely freeze-dried batches of DEMs is needed to demonstrate whether the observed improvements in capsule performance over time (storage losses and 24 hour retention efficiency) are related to

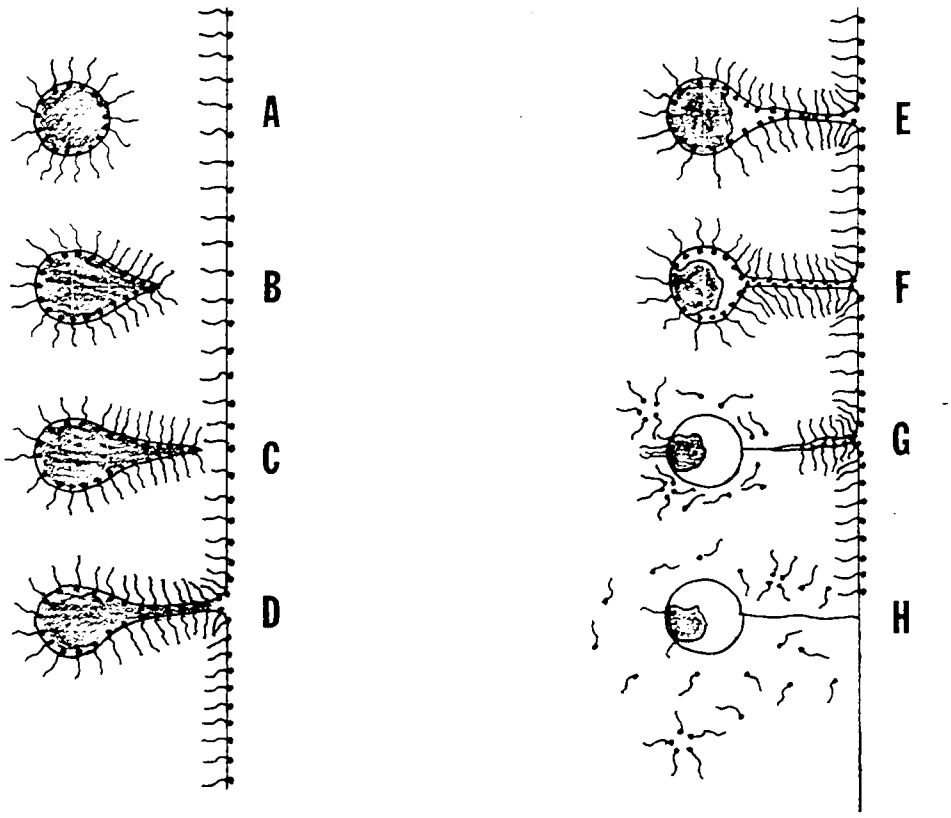

Figure 4.9: Phospholipid channel formation and collapse during freeze-drying and storage of a triacylglyceride (60% w/w tripalmitin/40% w/w fish oil) lipid-walled microcapsule (LWM) containing aqueous core:

A) Aqueous core droplet close to surface of LWM. Aqueous core surrounded by phospholipid. Polar head groups of phospholipids insert themselves into core. Hydrophobic tails extend out into the lipid wall. Phospholipids also aggregate at surface of LWM exposing polar head groups to the external aqueous environment. Hydrophobic tails extend into lipid wall.

B, C) As moisture is drawn through capsule wall, phospholipids follow forming a channel.

D) Phospholipid channel meets surface of LWM and phospholipids at surface merge with channel phospholipids creating an opening to the external environment. Phospholipids and channel stabilized by moisture within channel.

E, F) Drying of LWM continues. Core material is concentrated and begins to crystallize as moisture is removed.

G) As drying of LWM is completed, phospholipid channels are destabilized by absence of moisture. Channel collapses and dehydrated phospholipids disperse.

H) In the absence of phospholipid lining, the fissure in the LWM wall seals together.

removal of moisture remaining in the capsule walls. Further, it is evident that the majority of the storage losses for both DEMs and SMs, occur in the first 24 hours following preparation. Initial leakage losses from both capsule types may be minimized by eliminating steps in their preparation which expose them to the aqueous environment.

Currently, DEMs and SMs are immediately introduced into an aqueous environment after preparation. DEMs are solidified by addition of the secondary emulsion to cold distilled water, while SMs are collected in a bath of cold 0.2% w/v aqueous PVA. Both types of capsules are then screened, filtered with an aqueous solvent and water, and then stored as either a wet paste or freeze-dried in aqueous PVA. Initial leakage of DEMs may be reduced by eliminating the extensive screening and filtration steps following capsule preparation. After solidification in cold water, DEMs may simply be filtered dry on glass fiber filters and stored over dessicant or freeze-dried. Similarly, SMs may be produced by spraying the core/lipid suspension into dry chamber containing ambient or cooled air. The resulting powder of SMs may be collected and stored over dessicant or in the case of an aqueous core materials, freeze-dried. Controlled experiments to determine if freezing of the DEMs and SMs causes fracturing of the lipid wall are needed.

While dry DEMs and SMs from both modified procedures will contain unencapsulated or exposed core materials, these "contaminants" need not be removed immediately after preparation. Capsules must be resuspended prior to delivery to target bivalves. Removal of unencapsulated material may be accomplished at that time. The above modifications in capsule preparation and further investigation into storage methods and freeze-drying need to be examined to determine the best methods for maximizing the stability of lipid-walled microcapsules in storage.

These experiments demonstrate that SMs represent the most effective vehicle for encapsulating and delivering OTC. While treatment of adult populations of bivalves may be logistically difficult, bivalve larvae have been reported to be susceptible to *Vibrio* sp.

SMs containing OTC.HEM may be useful for the treatment of disease in bivalve larvae provided that they are digestible and that the OTC.HEM is released in an active form and demonstrates equivalent bacterialstatic properties as OTC.HCl.

While the FDA's Center for Veterinary medicine is currently involved in studies to approve OTC.HCl for use with bivalves (Moore *et al.* 1993), OTC is currently approved for use with crustaceans and finfish. The described SMs containing OTC.HEM are very robust and should be useful as additives to cold-extruded pelleted feeds for finfish and within cold-gel alginate particles used for artificial feeds in the shrimp industry. Effective encapsulation and reduction of leakage losses during feed presentation should improve feed palatability, allow more controlled and accurate dosing, and reduced discharge of antibiotics into the environment.

The investigated capsule types also have a wider application in nutritional research on bivalves and larger aquaculture species. Methods for controlled delivery of water-soluble vitamins and amino acids should allow more rigorous investigation of the nutritional requirements of aquatic organisms. Current research suggests that SMs may be the best method for encapsulating these materials provided that slightly insoluble forms or salts of the materials are assimilated by target organisms.

Chapter 5
Summary

Spray microcapsule development

A new process for producing lipid-walled microcapsules for delivering both aqueous and particulate core materials to marine bivalves was developed. Spray microcapsules (SMs) entrap aqueous and particulate core materials within a triacylglyceride bead. Spray microcapsules containing either aqueous polymeric dye, aqueous oxytetracycline hydrochloride (OTC.HCl), or particulate riboflavin were prepared using one of three mixtures of triacylglyceride wall material. The performance of resulting SMs was compared on the basis of the quantity of core material encapsulated (encapsulation efficiency), the quantity of core material retained after 24 hours of suspension in seawater (retention efficiency), and the amount of core material which might be delivered after 24 hours of suspension in seawater (delivery efficiency).

SMs composed of the triacylglyceride, tripalmitin (TP), efficiently encapsulated, retained and delivered core materials of an aqueous polymeric dye [poly(vinylamine) sulfonate (Poly-R, Sigma Chemical Co., Poly-R 478)], an aqueous antibiotic (oxytetracycline hydrochloride; OTC.HCl), or a particulate vitamin (riboflavin). Addition of lower melting point triacylglycerides, either triolein (TO) or fish oil (FO), to the TP wall in order to improve spray microcapsule digestibility, significantly reduced the performance of SMs containing aqueous core materials.

Addition of either TO or FO to the wall of SMs reduced encapsulation of each aqueous core material. Encapsulation of Poly-R was reduced from 1.81 mg Poly-R /100mg lipid in TP-walled SMs to 0.90 and 0.82 (mg Poly-R /100mg lipid) in SMs softened with TO or FO, respectively. Similarly, encapsulation of OTC.HCl was

reduced from 4.56 (mg OTC.HCl /100mg lipid) in TP-walled SMs to 1.19 and 1.83 (mg OTC.HCl /100mg lipid) in SMs softened with TO or FO, respectively.

Retention of aqueous core materials by SMs was also significantly reduced by addition of either TO or FO to the capsule wall. Retention of Poly-R was reduced from 94% in TP-walled SMs to 69% and 71% in SMs softened with TO and FO, respectively. Retention of OTC.HCl was reduced from 99% in TP-walled SMs to 36% and 21% in SMs softened with TO and FO, respectively.

Particulate riboflavin was encapsulated (4.41 mg/100 mg lipid), retained (98% retained after 24 hours in seawater), and delivered (4.31 mg/100 mg lipid delivered after 24 hours in seawater) well by SMs composed of TP. While addition of softer lipids to the capsule wall slightly increased the encapsulation efficiency of SMs for riboflavin (encapsulation efficiency = 4.62 mg/100 mg lipid and 4.82 mg/100 mg lipid for SMs softened with TO and FO, respectively), retention efficiency of SMs for riboflavin was reduced (retention efficiency = approximately 85% for SMs softened with TO or FO). Delivery of particulate riboflavin (ranging from 3.94 to 4.31 mg/100 mg lipid) by SMs composed of any of the three triacylglyceride mixes was not significantly different.

The results of this research suggest that TO and FO have similar effects on SM performance across core materials and that the more nutritionally valuable fish oil may be used in place of TO to soften the SM wall. Delivery of aqueous materials by SMs may be improved by modification of the technique for preparing SMs and use of higher purity lipids. SMs may be most efficient for delivering slightly soluble particulate materials, since the delivery efficiency of SMs containing a particulate core of riboflavin is not greatly reduced by addition of softer lipids to the capsule wall.

Digestibility of lipid-walled microcapsules

Lipid-walled microcapsules (LWMs) produced by a spray process (spray microcapsules; SMs) or a process of double-emulsion (double-emulsion microcapsules; DEMs) were fed to Manila clam (*Tapes philippinarum*) spat to assess whether capsule wall materials were digested and encapsulated core materials released. SMs and DEMs were prepared using a wall material of the triacylglyceride, tripalmitin (TP), or TP softened with 40% w/w fish oil (FO).

Spray microcapsules were stained red with the lipid soluble tissue stain Sudan III. Sudan III-stained SMs composed of either of the two triacylglyceride mixtures, TP or TP softened with FO, were fed to Manila clam spat and resulting fecal strands collected. SMs contained within fecal strands were counter-stained to determine if the triacylglyceride bead wall had been enzymatically reduced to free fatty acids. Only SMs softened with FO and contained within fecal stands were observed to accept counter-stain, changing in color from red to blue. The physical appearance of these SMs was also altered suggesting exposure to both physical and enzymatic digestive processes.

Double-emulsion microcapsules containing a high molecular weight, aqueous polymeric dye (Poly-R) only released their core material when softened with FO. These softer-walled DEMs emerged in fecal strands eroded and fractured. Fecal strands from clams fed on DEMs softened with FO often had a diffuse pink coloration, suggesting that aqueous dye had been liberated from DEMs and was bound in the fecal strand material. Softer-walled DEMs present in fecal strands also contained markedly less core material that TP-walled DEMs contained in fecal strands, further suggesting that release of core material is improved by addition of FO to the capsule wall.

SMs containing particulate oxytetracycline hemicalcium salt also only lost core material when the capsule wall was softened with FO. Softer-walled SMs appeared distorted and eroded in fecal stands. The amount of core material remaining within these

softened SMs was reduced (based upon the fluorescence of the OTC.HEM core material) in comparison to TP-walled SMs.

The results of these feeding experiments suggest that a triacylglyceride wall of tripalmitin is not enzymatically digested unless softened with FO. DEMs appear to deliver their aqueous core as a result of both enzymatic digestion and physical rupture of the capsule walls. SMs appear to remain largely intact, however, hydrolysis of the triacylglyceride wall to free fatty acids appears to be sufficient to allow release of particulate core material. Both aqueous cores and particulate cores may be delivered by lipid-walled microcapsules composed of TP softened with FO. The assimilation efficiency for materials delivered by these types (SMs and DEMs) of lipid-walled microcapsules needs to be addressed in a quantitative manner.

Optimal method for encapsulating and storing oxytetracycline

The most effective method for encapsulating and delivering oxytetracycline in LWMs was investigated. Two forms of oxytetracycline, oxytetracycline hydrochloride (OTC.HCl) and oxytetracycline hemicalcium salt (OTC.HEM), were encapsulated as either aqueous cores in DEMs or as particulate cores in SMs composed of 60%w/w TP/ 40%w/w FO. OTC.HCl was encapsulated (3.15 mg OTC.HCl/100 mg lipid), retained (30% retained after 24 hours in seawater), and delivered (0.94 mg OTC.HCl/100 mg lipid delivered after 24 hours in seawater) best as an aqueous core in DEMs. OTC.HEM was encapsulated (7.42 mg OTC.HCl/100mg lipid), retained (66% retained after 24 hours in seawater), and delivered (4.85 mg OTC.HCl/100 mg lipid delivered after 24 hours in seawater) most effectively as a particulate core in SMs.

The effect of long-term storage on capsule performance was assessed for DEMs containing aqueous OTC.HCl and SMs containing particulate OTC.HEM. DEMs and SMs were stored as wet pastes or freeze-dried powders for 5 weeks. The effect of

storage on 24 hour retention efficiency and delivery efficiency was assessed after one day, one week, two weeks, and four weeks of storage. In addition, changes in storage retention efficiency over time was assessed for DEMs containing an aqueous core of OTC.HCl. Long-term storage retention efficiency of both capsule types was assessed at 5 weeks.

Spray microcapsules containing particulate OTC.HEM and stored as a wet paste exhibited the highest retention of core material after 5 weeks of storage (86% of originally encapsulated OTC.HEM was retained by SMs). Freeze-drying SMs caused a significant increase in initial leakage of SMs ; 24 hour retention efficiency and delivery efficiency were significantly reduced on day one of storage in comparison to wet-stored SMs (24 hour retention efficiency 7% less; delivery efficiency 9% less). Despite initial reductions in 24 hour retention efficiency and delivery efficiency, these measures did not decrease further over time and freeze-dried SMs appeared to be as stable in storage as wet-stored SMs.

Freeze-drying had a marked effect on the 24 hour retention efficiency and storage retention efficiency of DEMs containing OTC.HCl. Initially on day one of storage, freeze-dried DEMs retained less of their core material during storage than wet-stored DEMs (29% versus 46% of initially encapsulated core retained during storage, respectively). DEMs stored by both methods retained equivalent amounts of core material during 24 hour retention efficiency measures (45% versus 48% of core material retained over 24 hours in seawater, wet-stored and freeze-dried DEMs, respectively). Retention of core materials by freeze-dried DEMs, both during storage and during 24 hours retention efficiency measures, improved after weeks one and two of storage. By the second week of storage, retention of core material by freeze-dried DEMs during storage (36% of core material retained) and during 24 hour retention efficiency measures (61% of core material retained) was significantly higher than by wet-stored DEMs (29% of core

material retained during storage; 37% of core material retained over 24 hours in seawater).

Fluctuations in DEM performance during storage were attributed to incomplete drying of the freeze-dried capsule preparation prior to storage. Improvements in the performance of freeze-dried DEMs during storage were attributed to a completion of the drying process. Freeze-drying of DEMs may represent an effective method for reducing leakage of core materials and improving long-term storage of these lipid-walled capsules. Further experiments with freeze-drying of DEMs as well as modifications of the DEM preparation process need to be conducted to reduce opportunities for core leakage prior to and during storage.

Conclusions

Lipid-walled microcapsules may be produced using a spray process. The process is very effective for encapsulating slightly soluble materials such as riboflavin and the hemicalcium salt of oxytetracycline. While SMs may also be used to encapsulate aqueous core materials, LWMs formed by a process of double-emulsion (DEMs) may be more effective for this type of core material.

While SMs containing particulate core appear to be stable when stored as a wet paste for periods of over a month, retention of aqueous core materials by DEMs may be improved by freeze-drying capsules prior to storage.

Lipid-walled microcapsules composed of 60% w/w TP/40% w/w FO are digested by Manila clam spat and may be used to deliver water-soluble materials encapsulated as either aqueous or particulate cores. Assimilation of materials delivered by these capsules needs to be verified and quantified for a variety of marine bivalves and bivalve larvae.

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APPENDICES

Appendix A

Table A.1: Fatty acid composition (% of sample weight) of lipids used in the preparation of lipid-walled microcapsules. Fatty acid composition determined by hydrolysis of lipids and separation of fatty acids by GC (Analysis courtesy of Dr. Fu-Lin Chu, Virginia Institute of Marine Science, College of William and Mary).

Carbon structure	Tripalmitin (reported purity: >95% pure by manufacturer's GC analysis)	Triolein (reported purity: >65% pure by manufacturer's GC analysis)	Fish Oil (Dale Alexander's Fish Oil Concentrate)	Triolein (reported purity: >99% pure by manufacturer's GC analysis)
14:0	0.1	1.3	5.9	
15:0			0.1	
16:0	99.6	4.9	16.7	1.2
16:1w7		4.6	10.4	
16:3			0.7	
16:4			0.7	
17:0?			2.0	
18:0		0.8	2.8	
18:1w9		51.4	10.4	96.3
18:1w7		21.8	6.2	
18:2w6		11.0	3.9	1.7
18:3w3			0.7	
18:4w3			1.5	
20:1w9		0.8	0.8	
20:4w6			0.6	
20:4w3			0.5	
20:5w3			19.7	
22:1			1.9	
22:5w3			2.2	
22:6w3			11.8	
unidentified	0.3			

Appendix B

Table B.1: Effect of addition of soy phospholipid (L-alpha-phosphatidylcholine; Commercial Grade: 14% pure by manufacturer's analysis; Sigma Chemical Co.) on encapsulation and retention efficiencies (±standard deviations) of tripalmitin-walled spray microcapsules (SMs) containing riboflavin (Langdon and Buchal, unpublished data).

SM wall composition	encapsulation efficiency (mg core/100 mg lipid)	24 hour retention efficiency (percentage of intial core retained after 24 hours in seawater)
100% w/w tripalmitin	10.47±0.22	82.87±10.49
99% w/w tripalmitin/ 1% w/w phospholipid	10.49±.028	84.83±6.74
95% w/w tripalmitin/ 5% w/w phospholipid	11.22±0.14	65.03±0.81
90% w/w tripalmitin/ 10% w/w phospholipid	9.76±0.37	46.99±1.26

Note: Maximum encapsulation efficiency possible = 11.11 mg core/100 mg lipid

Appendix C

Table C.1: Purity of triacylglycerides used in lipid-walled microcapsules (LWMs). Lipid composition [average percentage(±standard deviation) of sample weight represented by lipid class].

	Lipid Class		
Lipid and reported purity	Triacylglyceride	Cholesterol	Phospholipid
Tripalmitin >95% pure by manufacturer's GC analysis	83.80±0.93	16.20±0.93	not detected
Triolein >65% pure by manufacturer's GC analysis	76.50±0.75	16.12±0.63	7.39±0.12
Fish Oil (Dale Alexander's Fish Oil Concentrate)	92.20±0.94	not detected	7.80±0.94
Triolein >99% pure by manufacturer's GC analysis	100.00±0.00	not detected	not detected

Appendix D

Table D.1: Effect of triolein (TO) purity on encapsulation efficiency (mg core/100 mg lipid) and storage retention efficiency (percentage of originally encapsulated core retained after 24 hours storage as a wet paste at 5°C) of double-emulsion (DEMs) microcapsules (wall composition, 60% w/w tripalmitin/40% w/w TO) containing aqueous oxytetracycline hydrochloride (OTC.HCl) (Buchal and Langdon, unpublished data).

Purity of triolein	Encapsulation efficiency (±standard deviation)	Storage retention efficiency (±standard deviation)
>65% triolein[1] by manufacturer's analysis	9.09±0.35	23.67±0.94
>99% triolein[2] by manufacturer's analysis	0.46±0.15	72.65±3.63

Maximum encapsulation efficiency (based on aqueous core concentration and ratio of core to lipid used in preparation) = 10.5 mg OTC.HCl/100 mg lipid

Note: Measured lipid composition
1) 76.50% triacylglyceride, 16.12% cholesterol, 7.39% phospholipid
2)100% triacylglyceride