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Encapsulation of a lipid precursor, the eicosapentaenoic acid, to study the development of the *Crassostrea gigas* oyster flavours

B. JOUZEL†, A.-L. PENNARUN†, C. PROST†, D. RENARD‡, D. PONCELET§ and M. DEMAIMAY†*

† Département des Sciences des aliments, ENITIAA, Rue de la géraudière, BP 82225, F-44322 Nantes Cedex 3, France
‡ Unité de Physico-Chimie des Macromolécules, INRA, Rue de la géraudière, BP 71627, F-44316 Nantes Cedex 3, France
§ Département du Génie des Procédés alimentaires, ENITIAA, Rue de la géraudière, BP 82225, F-44322 Nantes Cedex 3, France

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The present study is part of a larger project whose aim is to understand how the oyster *Crassostrea gigas* develops its aromas from a lipid precursor, the eicosapentaenoic acid (EPA), in glyceride form. The objective of this study is, therefore, to prepare an encapsulation process that will enable the bivalve to be supplied with this lipid precursor. The complex coacervation method was chosen as it gave the best compatible microcapsules with respect to the nutritional aspects of oyster (i.e. digestibility) and the environmental constraints (i.e. behaviour and stability in seawater). The aim of this study is to manufacture and optimize a process of complex coacervation, to obtain capsules made of gelatin and acacia gum with a size under 100 µm in diameter and containing very small drops of cod liver oil (rich in EPA). The preservation of these microcapsules in seawater has been confirmed.

Keywords: Pacific oyster, *Crassostrea gigas*, lipid, microencapsulation, complex coacervation.

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Introduction

Recent studies showed that the use of *skeletonema costatum* diatoms, which are rich in eicosapentaenoic acid (EPA; C20: 5w3), is responsible for the changes of the lipid and fatty acid composition of the adult oyster *Crassostrea gigas* (Piveteau *et al.* 1999). Moreover, this fatty acid (EPA) is an aroma precursor for fish (Josephson and Lindsay 1987). However, the precise role of EPA in the development of the aromas and in the changes of lipid and fatty acid composition of the oyster remains to be understood. To go further in the scientific understanding, microencapsulation technology can be used as it offers a potentially useful approach to deliver only this fatty acid, in glyceride form, to suspension feeders. The assimilation of the glyceride form is easier than the fatty acid form. The use of the microencapsulation technology requires, however, an understanding of the feeding behaviour of the oyster. The gills represent the main food collecting organs of this bivalve. They

* To whom correspondence should be addressed. e-mail: demaimay@enitiaa-nantes.fr

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collect and transport nutrient particles. This nutrient uptake is influenced by several factors: the size of food particles must be between 7–100 μm in diameter to be effectively retained (Winter 1978). The composition of the nutrients must be organic (Bayne 1993) and the density of the food particles must be equal to the seawater density. In addition, the nutrients must be digestible (Gabbot *et al.* 1976) and the composition of the artificial diet should not be altered in seawater.

Microcapsules must respect all these constraints to take advantage of the technology applied to oyster feeding. The characteristics of an 'ideal' microcapsule are linked to their preparation, biochemical content and performance in aqueous suspension (Soughate *et al.* 1992). Several types of microcapsules have been tested over the last 20 years, but only four of them have been the object of more advanced studies: protein-walled microcapsules, nylon protein, microgel particles and gelatin-acacia microcapsules. All these capsules, except protein-walled microcapsules, have been tested on oysters. Protein-walled microcapsules were, however, tested on fish and their size (153 μm) was too big to be applied in the oyster artificial feeding (Ozkizilcik and Chu 1996). Nylon-protein microcapsules, used in very few studies on molluscs, have a low level of digestibility in the case of *Crassostrea virginica* larvae (Chu *et al.* 1982). Microgel particles sediment themselves very easily at the bottom of water and this can be detrimental to the nutrition of molluscs (Robert and Trintignac 1997). On the contrary, the gelatin-acacia microcapsules obtained by complex coacervation are interesting for several reasons. They are easily digested by larvae *Crassostrea virginica* (Chu *et al.* 1982). They are stable in seawater and can be autoclaved for long-term storage (Chu *et al.* 1987). In some cases, microcapsules of 2–3 μm in diameter can be produced (Langdon and Waldock 1981). This size may be, however, too small for the adult oysters. The complex coacervation method to produce microcapsules is, therefore, difficult to reproduce, unless on a laboratory scale, and further studies need to be performed in order to optimize the process for oyster feeding applications.

Complex coacervation is a common method of oil microencapsulation (Burgess and Carless 1985) used to avoid the oxidation of the incorporated material. The coacervation phenomenon results from weak attractive and non-specific interactions between biopolymers, giving rise to the formation of soluble or insoluble complexes. In complex coacervation, two counter-charged polyelectrolytes interact and coacervate droplets are formed (Madan and Price 1972, Madan *et al.* 1974, Daniels and Mittermaier 1995). When all the functional properties of each component are combined together, these properties are generally improved, such as their surface properties. The system used in many investigations is gelatin/acacia gum and many research efforts were reported to establish the optimum conditions of coacervate formation (Ijichi *et al.* 1997). The ability to microencapsulate oil droplets by the complex coacervation method is known to be strongly influenced by the operating conditions, such as total concentration and protein-to-polysaccharide ratio, pH and ionic strength of both biopolymer solutions (Madan and Price 1972, Madan *et al.* 1974, Daniels and Mittermaier 1995). For an ionic strength near 10 mM and at a pH between 3.75–3.97, the two biopolymers carry exactly opposite net charges, resulting in a maximum electrostatic attraction (Daniels and Mittermaier 1995). In addition, maximum insoluble complexes are obtained for a ratio 1:1, corresponding to the maximal neutralization of charges carried out by each of the two biopolymers (Tolstoguzov 1986).

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The aim of the present study is, therefore, to prepare an encapsulation process based on the complex coacervation method enabling the bivalve to be supplied only with the fatty acid EPA in glyceride form. Optimization of the method of microcapsule preparation is, therefore, established in order to define the parameters of complex coacervation and to obtain capsules that are suitable for the constraints of the oyster physiology.

Materials and methods

Materials

Acid pig skin gelatin (IEP = 8.6, bloom grade = 275, Batch: 12597) was a gift from SKW Biosystems (Isle-sur-Sorgue, France). The temperature of the gel point is comprised between 25–35 °C.

Acacia gum (Instant Gum, batch: G9752) was a gift from CNI Company (Rouen, France).

Cod liver was use due to its high EPA content (9.54%), according to (Numaguchi and Nell, 1991), and was purchased from the 'Coopération pharmaceutique française' (Melun, France).

Aqueous sodium hydroxide (0.05 N) and hydrochloric acid (0.05 N) were purchased from Panreac Quimica SA (Barcelona, Spain) and were used in order to adjust the pH of the solutions.

Principles of microcapsule production

Gelatin solution. Gelatin (4 g) are dispersed into 200 ml distilled water at 20 ± 1 °C for 1 h. The aqueous solution was gently stirred at 50 ± 1 °C, until the complete dissolution of gelatin (15 min). This solution was then poured into a round-bottomed flask of 500 ml. The pH is adjusted to 7 by adding slowly 0.05 N NaOH.

Acacia gum solution. Biopolymer powder (4 g) was dispersed into 200 ml distilled water at 20 ± 1 °C, while stirring gently. This solution was heated at 50 ± 1 °C for 15 min. The pH is then adjusted to 7 by adding 0.05 N NaOH.

Reactor. The reactor was a round-bottomed flask of 500 ml, with a diameter of 15 cm. Mixing and dispersion were insured using an ultraturax (primary emulsion) and later on a six blade Rushton turbine with a diameter of 6.5 cm.

Microcapsule production. The complex coacervation process could be divided into four steps: the oil/gelatin primary emulsion, the acacia gum adding to the primary emulsion, the coacervation itself and the capsules maturation (figure 1). First, cod liver oil is added to the gelatin solution, in the reactor and then emulsified, with an ultra-turrax, to obtain very free lipid droplets. Secondly, while oil/gelatin primary emulsion is maintained by mixing (at 500 rpm), using a turbine reactor, the acacia gum solution is added. Thirdly, to obtain coacervation, the pH is reduced to 3.9 by the dropwise addition of 0.05 N HCl. Finally, the capsules formed are maturated under gentle stirring of 100 rpm at a temperature of 10 °C.

To obtain microcapsules with a diameter lower than 100 µm, the parameter influence (time, temperature, speed, component concentration) at each step have to be evaluated (table 1).

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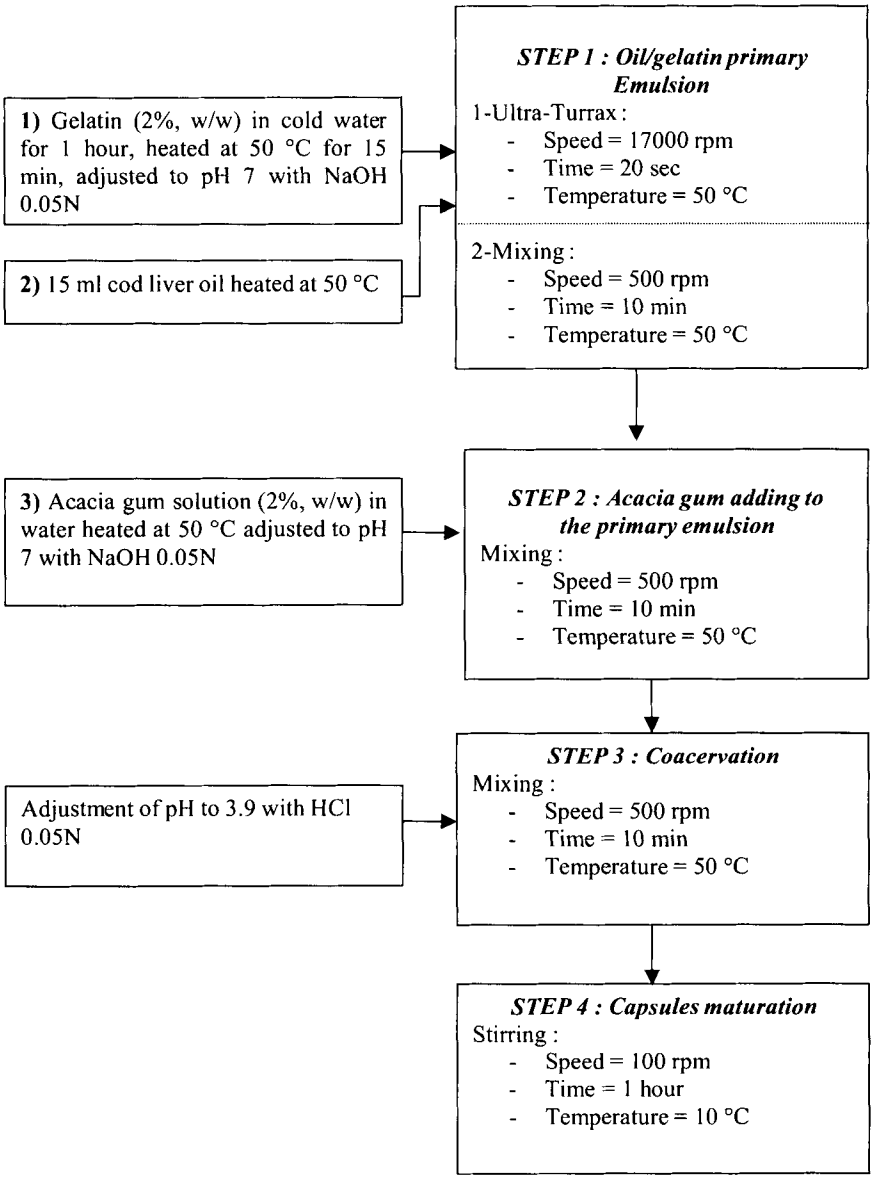

Figure 1. Diagram of microcapsules formation by complex coacervation.

Particle analysis

The emulsion and the microcapsule size distribution were characterized using a phase contrast optical microscope (Leïca HDMR, ×400 magnification) equipped with a CCD camera (Sony mono, Tokyo, Japan). Twenty microlitres of the emulsion or dispersion was placed between glass slides to examine the particle size and to determine the capsule shape.

The size distribution of the oil/gelatin primary emulsion and the microcapsules dispersion were also determined based on the laser bean diffraction by the particles

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Table 1. Studied conditions of gelatin/acacia gum microcapsules.

Step of preparation	Parameters studied	Range studied
Oil/gelatin primary emulsion	Time (sec)	10–60
	Temperature (°C)	40–60
	Ultra turrax rotation speed (rpm)	7000–22 000
Acacia gum adding to the primary emulsion	Adding order of cod liver oil and acacia gum	
	Speed (rpm)	200–500
Coacervation	pH lowering time (min)	1–210
Capsules maturation	Speed (rpm)	100–500
	Time (hour)	1–5
Basic materials	Gelatin and acacia gum total concentration (%, w/w)	0.5–2
	Cod liver oil (ml)	15–25
	Acid	Acetic or hydrochloric

(Mastersizer S , Malvern Instruments, UK). $D_{v0.1}$, $D_{v0.5}$ and $D_{v0.9}$ are the characteristic values for which, respectively, 10, 50 and 90% (v/v) of the particles having a size lower than the measured diameter. $D_{v0.5}$ is used as a mean value. The homogeneity of the size is characterized by the span defined by the following relation:

$$\text{Span} = (D_{v0.9}D_{v0.1})/D_{v0.5}$$

To ensure a valuable reliability to the particle size analysis, for each experiment, three batches of microcapsules were produced in the same conditions. Every batch was simpled and analysed in triplicate.

Statistical analysis

Experimental designs are used to study two steps of the process: the parameters of the oil/gelatin primary emulsion and the quantities of basic materials. They were analysed by ANOVA using the StatGraphics plus software. The comparison of means was carried out using the Fisher PLSD test at a confidence level of 95%.

Results and discussion

In this process, encapsulation of lipids is based on four main steps. Two appear critical to control the size distribution: the oil/gelatin primary emulsion formation and the capsule formation.

Influence of mechanical factors on oil/gelatin primary emulsion formation

The emulsification parameters (temperature, emulsification time, ultraturax speed) are optimized in order to obtain the narrowest distribution of lipid droplets in the oil/gelatin primary emulsion. Figure 2 shows that between 10–60 s of

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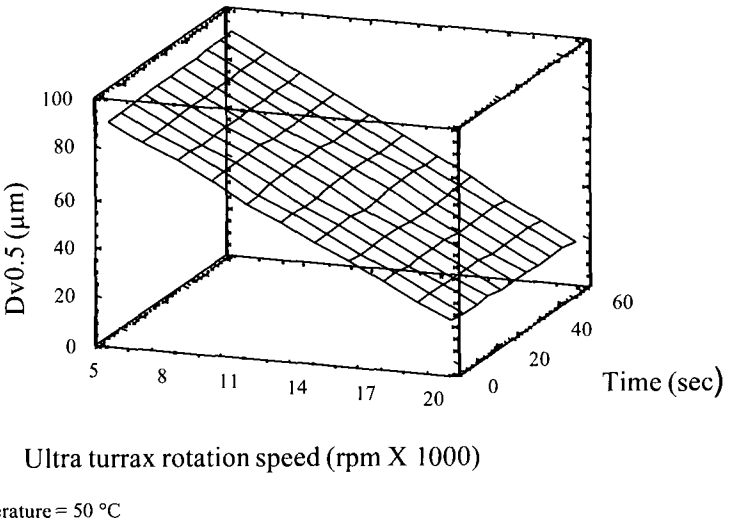

Figure 2. Effects of ultra turrax rotation speed and time on oil/gelatin primary emulsion droplet size.

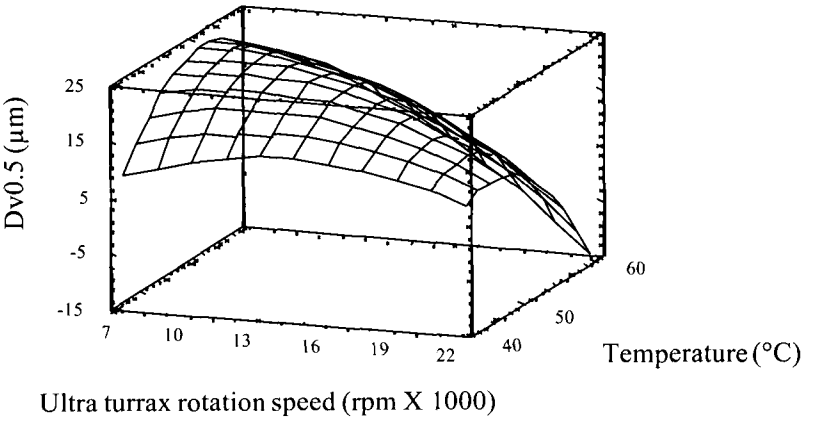

Figure 3. Effects of ultra turrax rotation speed and temperature on oil/gelatin primary emulsion droplet size.

emulsification, time of emulsification has no influence on the droplets size. Less than 10 s is necessary to get equilibrium.

Results in figure 3 show that the size of the droplets ($D_{v0.5}$) decreases when the ultra-turrax rotation speed increases at a temperature ranging between 40–60 °C. Nicest capsules were obtained for temperatures equal to or higher than 50 °C. In such conditions, the size of the lipid droplets decreases linearly with the ultra-turrax rotation speed (figures 2 and 3).

On other hand, size dispersion (span) is minimum at 50 °C and an Ultra-turax speed of 17 000 rpm (figure 4). Since, the microcapsules have to be as much as possible uniform to reach best reproducibility in the oyster feeding experiments (Madan and Price 1972), one then selects these conditions for further experiments. The oil droplets have a mean diameter of 20 µm.

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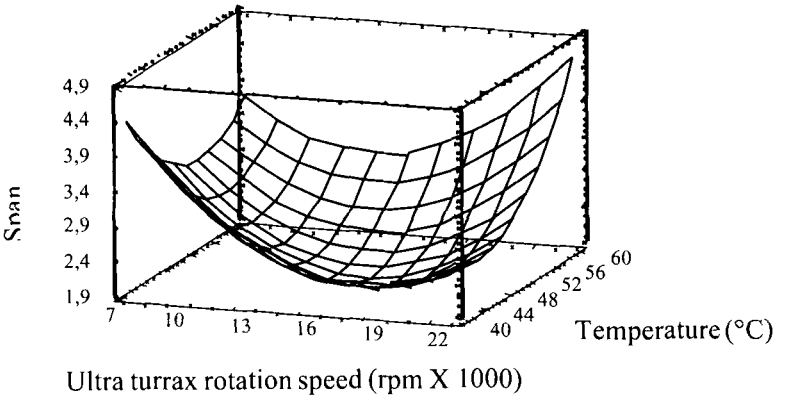

Figure 4. Effects of ultra turrax rotation speed and temperature on the polydispersity index (Span) of oil/gelatin primary emulsion droplet size.

Formation of the capsules or aggregates by coacervation

The second part of the process is the capsules formation. In fact, observing the capsules (figure 5) shows that many lipid droplets are encapsulated together by gelatin/acacia gum coacervates. They form aggregates more than membrane bound capsules (even for homogeneity to previous works, the term of capsules will be used below). To reduce the size of the aggregates, the coalescence of the droplets/coacervate complexes must be controlled. Each step is studied to reduce the size of these aggregates.

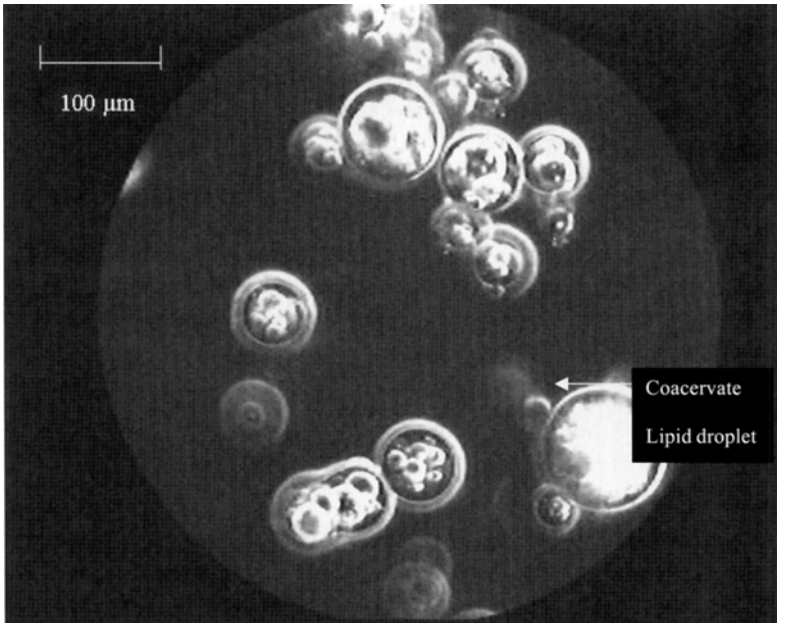

Figure 5. Gelatin/acacia capsules observation by phase contrast optical microscope (×400).

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Table 2. Effects of adding order of cod liver oil and acacia gum solution on the capsules size distribution.

Introduction of cod liver oil	Before Acacia gum[a]	After Acacia gum[b]
$D_{v0.5}$ (μm)	28	106
Span	3.12	3.67

[a] Mauguet *et al.* (1999) method.
[b] Langdon and Waldock (1981) method.

Table 3. Influence of the mixing rate on the median diameter of capsules during the capsules maturation step.

Mixing rate (rpm)	200	500
$D_{v0.5}$ (μm)	28	28
Span	5.07	3.12

Table 4. Time influence to reach the coacervation pH value.

pH lowering time	1 min	25 min	210 min
$D_{v0.5}$ (μm)	28	77	123
Span	3.12	1.63	2.38

Order of cod liver oil and acacia gum addition. An important factor is the order for the addition for cod liver oil and acacia gum in the primary emulsion. Table 2 shows that capsule size is lower when cod liver oil is added before acacia gum. This may be explained by the different structure of the interface in the primary emulsion and then coacervate first layer formation. Further experiments would be needed to verify this hypothesis.

Aggregate size vs mixing rate before and during coacervation step. The median diameter of the capsules ($D_{v0.5}$) was not dependent on the mixing rate during the coacervation step (table 3). However, the homogeneity of the capsule aggregates is the best when the mixing rate increases. The mixing rate of 500 rpm for 10 min is selected.

Time influence to reach the coacervation pH value. Table 4 shows that the time to reach the pH of coacervation (3.9) had a strong impact on the size of capsules. The addition rate of hydrochloric acid into the gelatin/acacia gum emulsion must be very fast to obtain small capsules/aggregates.

Capsule maturation step

During the maturation, the effect of stirring rate must be selected in order to limit aggregate coalescence.

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Table 5. Effects of stirring rate on the size and distribution of capsules.

Stirring rate (rpm)	100	500
$D_{v0.5}$ (μm)	28	61
Span	3.12	3.80

Stirring rate influence. The reduction of the stirring rate induces a decrease of the aggregates capsule size (table 5). This phenomenon can be explained because it exists a competition between the increase of coalescence due higher energy shocks between aggregates and the higher breaking shear effect while stirring speed is increased. Apparently, the coalescence is still the main effect for stiring speed under 500 rpm.

Stirring time influence. Capsule size distribution were measured after 1, 2 and 5 h of stirring. Results show that the maturation time has no influence on the size of capsules obtained. The relevant parameters for this step are the stirring rate value of 100 rpm and the stirring time of 1 h.

Impact of the chemistry on the capsule size distribution

Effect of the polymer and cod liver oil concentration. The concentration of polymer (gelatin plus acacia gum) vs cod liver oil has a significant effect for mean aggregate size. Figure 6 shows the low concentration of oil and polymers allow to obtaining of the smallest capsules. With the quantities of 15 ml cod liver oil, 4 g gelatin (or 2% w/w) and 4 g acacia gum (or 2% w/w), the capsule median diameter is lower than 100 μm.

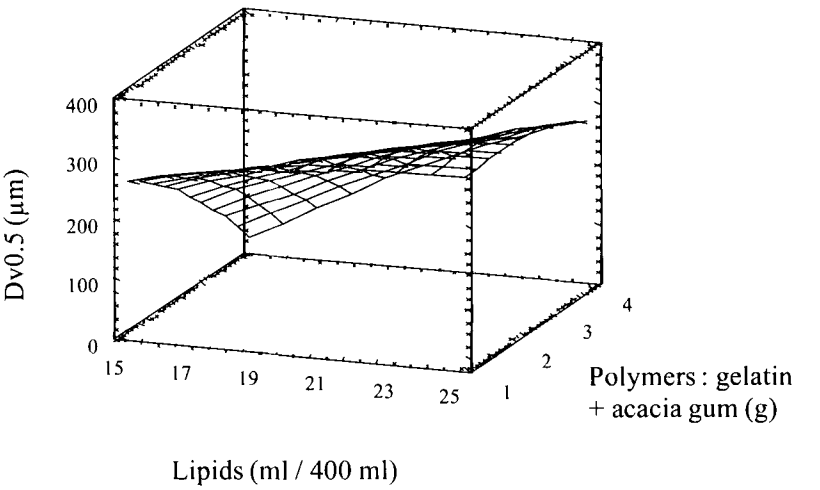

Figure 6. Effect of the quantity of cod liver oil and polymers (gelatin + acacia gum) on the median diameter ($D_{v0.5}$) of capsules.

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Table 6. Capsules size distribution, obtained from the diagram shown in figure 1.

Step of preparation	Parameters studied	Optimum parameters retained	Size of lipid droplets and capsules
Oil/gelatin primary emulsion	Time (s)	20	*Lipid droplet distribution:*
	Temperature (°C)	50	$D_{v0.1} = 4\,\mu m$
	Speed (rpm)	17000	$D_{v0.5} = 20\,\mu m$
			$D_{v0.9} = 42\,\mu m$
			Span = 2
Acacia gum adding to the primary emulsion	Adding order of cod liver oil and acacia gum	Cod liver oil before acacia gum	*Capsules distribution:*
			$D_{v0.1} = 9\,\mu m$
			$D_{v0.5} = 28\,\mu m$
	Speed (rpm)	500	$D_{v0.9} = 178\,\mu m$
Coacervation	pH lowering time (min)	1	Span = 3
Capsules maturation	Speed (rpm)	100	
	Time (hour)	1	
Basic materials	Gelatin/acacia gum total concentration (%, w/w)	2	
	Cod liver oil (ml)	15	
	Acid	Hydrochloric acid	

Selection of the acid. Two different acids have been tested for reducing the pH during the coacervation step. The capsule median diameter obtained with hydrochloric acid is lower than those obtained when using acetic acid. These differences are, therefore, statistically not significant but confirm Daniels and Mittermaïer's conclusions.

Final diagram for the capsule production

Based on this study, the optimum parameters retained and the corresponding distribution of the capsules obtained, from diagram showed in Figure 1, are presented in table 6. Figure 5 presents capsules observed through a phase contrast optical microscope. Micrograph shows that gelatin/acacia gum coacervates contain lipid droplets without any leakage. Capsules have a spherical shape and their size distribution is quite narrow.

Microcapsule storage

The storage of capsules is studied in four different conditions with the same trial. Results show that the size of the capsules is stable for at least 12 days, when the trials are diluted in seawater (whatever the temperature) or not diluted but at low temperature (results not shown). However, when the trial is not diluted in seawater and the temperature of the water is at 25 °C, the size of the capsules is not stable. The increase of capsule size could be due to the beginning of gel-sol transition of gelatin at this temperature; the microcapsules could, thus, aggregate

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more easily. In these conditions, microcapsules would be too large to be ingested by oysters. The efficiency must, therefore, be improved in the following ways:

- The ratio 1:1 of gelatin/acacia gum involves the maximum formation of insoluble coacervates (Tolstoguzov 1986) that can further bind together by hydrogen bonds. On the contrary, if one of the two biopolymers is used at a higher quantity than the other, some soluble coacervates are obtained.
- Another way to reduce capsule size and polydispersity is to use a chemical cross-linking agent. The interfacial walls reinforced by covalent cross-linking allow the individualization of microcapsules and avoid by this way aggregation (Lévy and Andry 1989). However, chemical cross-linking agents such as formaldehyde or glutaraldehyde could be toxic to oysters and not digestible.

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