Liquid-core microcapsules: A mechanism for the recovery and purification of selected molecules in different environments

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Publications and presentations associated with this work

Publications

Whelehan M, von Stockar U, Marison IW. 2010. Removal of pharmaceuticals from water: Using liquid-core microcapsules as a novel approach. Water Research. 44:2314-2324.

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Whelehan M, Marison I. 2010. Capsular Perstraction as a Novel Methodology for the Recovery and Purification of Geldanamycin. Biotechnology Progress (Submitted).

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Whelehan M, O'Shea DG, Marison IW. 2010. Successful application of capsular perstraction as a novel in-situ product recovery technique: Removal and purification of geldanamycin from *Streptomyces hygroscopicus* cultures. Applied Microbiology and Biotechnology (Submitted).

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Poster presentations

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Summary

Liquid-core microcapsules can be described as miniature sized particles (< 1 mm in diameter) consisting of a liquid-(core) enveloped completely within a defined porous or impermeable membrane, and can be manufactured from a wide range of natural and/or synthetic materials. These structures usually take a spherical form and have been shown to have many exploitable characteristics in numerous processes. In this study, these particles were used as a tool for the recovery and/or purification of different molecules in their associated environments.

The first section of this study was devoted to the characterization of a methodology to manufacture microspheres/microcapsules using an alginate hydrogel as the encapsulation matrix. Due to its many advantages, such as an easy operation and the ability to control droplet size, the vibrating nozzle (jet) technique was chosen as the manufacturing technique. Using the single nozzle system, and a pressurized flow control system to delivery the polymer, it was possible to produce microspheres/spheres, under reproducible conditions, in a size range of 100 μm to 2 mm in diameter, with the produced structures having a standard size deviation of between 1-1.5%, for any size generated. Whilst the size was mainly dependant on nozzle diameter, it was identified that this feature could be altered further by increasing/decreasing the frequency of the vibration and/or the flow of the polymer.

For the concentric system, similar reproducible results were obtained, but due to the more complicated system, brought about by the two-flow mechanism to the nozzle, the size deviation increased to around ± 2.5% in most cases. Due to the separate flows of the outer and inner phases, it was possible to control the core and membrane size of the microcapsules during production, with either being able to occupy between 10-90% of

total capsule volume. It was shown throughout this work that this trait can be a very important property, as it can affect the numerous characteristics of capsules, such as: mechanical strength, buoyancy and most importantly for this study, the mass transfer (permeability) of external and/or internal compounds to and from the core of the microcapsules. For both arrangements it is possible to produce > 1.2 l/hr of microspheres and > 2 l/hr of liquid-core microcapsules, and this can be easily and naturally elevated to higher volumes by increasing the number of nozzles on the encapsulator.

Firstly the microcapsules were used as an innovative technique (known as capsular perstraction) to recover the commonly found pharmaceutically active compounds; sulfamethoxazole, metoprolol, furosemide, carbamazepine, clofibric acid, warfarin and diclofenac from water. The approach of preparing capsules with different solvents within their cores and combining them in water, contaminated with pharmaceuticals, enabled a rapid recovery of the drugs from this sphere. In addition the uptake of warfarin was examined to assess the conditions affecting mass transfer of the molecules into the capsules. It was subsequently determined that the stagnant organic layer was the main limiting factor. This part of the study emphasized how the characteristics (size and membrane thickness) of capsules can affect the removal rate of compounds into the liquid-core and also how the rate of extraction can be simply controlled by varying these parameters during the capsule manufacturing process.

In a second application, the capsular extraction technology was further developed by adopting it as an aide for the recovery and purification of the antibiotic geldanamycin from Bennett's medium. From this work it was shown how a small quantity of capsules was capable of rapidly extracting the molecule from the culture medium. Again the limitations to mass transfer were accessed, and it was discovered that the main rate-

limiting step was the external resistance outside of the capsules, which can be governed by controlling the outer turbulence. In a further development the capsules showed their potential to be used as a mechanism for concentrating, purifying and enabling crystallization of the extractant, using a very simplistic and straightforward procedure, which was not destructive to the microcapsules, hence enabling their continuous re-use. Finally the capsules were applied to a real-time situation, in order to examine the feasibility of using the simple, non-toxic and sterile technology as a novel in-situ product recovery technique, to improve the production and recovery yield of geldanamycin in cultures containing the bacterium *Streptomyces hygroscopicus.* Implementation of this approach resulted in the rapid removal of the metabolite from an environment which was causing its break-down and seriously affecting recovery yields. Extraction enabled the molecule to be transferred into a stable and secure domain, where it was protected from external influences. This removal improved overall net production by 30% compared to fermentations containing no capsules. Most importantly however, the capsule-facilitated recovery process acted as a methodology, which enabled high recoveries (> 53%) of the fermented geldanamycin to be obtained as highly purified crystals (> 97%) using a facile, inexpensive and reproducible process, which should be easily implemented at a lab-scale or industrial-level.

Keywords: Liquid-core microcapsules; Capsular perstraction; Alginate; Dibutyl sebacate; Oleic acid; Pharmaceutically active compounds; Geldanamycin; In-situ product recovery and Downstream processing.

Table of Contents

Chapter 1

Microencapsulation using Vibrating Technology

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Keywords: Microencapsulation; Production of microspheres/microcapsules; Vibrating nozzle encapsulator; Rayleigh's jet instability and break-up; Alginate; Monocentric and concentric nozzle; Multiple vibrating nozzle encapsulator and Heating nozzle/pulsating-head device.

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1.1. Abstract

For well over a half a century now, microencapsulation and encapsulated products have played a very important role in numerous industries like agriculture, chemical, pharmaceutical, cosmetic and the food industry, whereby protection of the core material from its active environment, improved handling properties and controlled release of the encapsulant were the main reasons behind performing the process. In recent decades these particles have been applied to numerous biotechnology and medical processes, including cell encapsulation for the generation of artificial implants and the production of high density cell cultures. This has opened up a whole new exciting field for the technology and resulted in the development of new production procedures to help manufacture the desired microspheres/microcapsules. Whilst for industries like agricultural, food and cosmetic, the prerequisite for the successful employment of the technology is usually only limited to high production rates at low cost, for application in medical and biotechnological processes the criteria is somewhat more stringent. Successful application in these areas requires a technology, which has the capability to produce mono-dispersed, homogenous shaped capsules, with a narrow size distribution, using a short production time, under simple conditions, with high efficiency and high production rates using many different materials, including highly viscous solutions. It should also enable different production sizes and many different core materials to be encapsulated, and finally if required, can be performed under sterile conditions. To-date no established or newly developed encapsulation technique can fully adhere to the aforementioned criteria and the limitations possessed by all, have slowed down and/or prevented the use of encapsulation technology at an industrial level for biotechnological and medical applications.

Production of microcapsules by the controlled break-up of laminar liquid jets using vibrating technology has gained significant interest mainly due to its simplistic approach to produce homogenous microspheres/microcapsules of the desired size. Certain limitations still exist for this methodology, which have yet to be fully re-solved, such as an inability to manufacture large quantities and process highly viscous fluids. However, promising solutions have being suggested and developed, such as the multiple nozzle system, which can help overcome these shortfalls.

This review initially discusses the importance of encapsulation and the reasons behind its implementation. The paper then sets out to discuss some of the mechanical techniques used to produce capsules for biotechnological and medical applications along with some of the more commonly used encapsulation matrices, discussing the pros and cons of each technique and material. A detailed description of the theoretical and practical know-how and aspects behind production of different capsules using vibrating technology is then given. Finally the challenges facing application of the technology at a large scale level are discussed.

1.2. Introduction

Microencapsulation can be defined as a process, which involves the complete envelopment of pre-selected core material(s) within a defined porous or impermeable membrane (shell) using various techniques, to give miniature sized particles ranging in size from 1-1000 μm[1]. Microcapsules can take many structural forms (Figure 1) and have proven to have many exploitable characteristics for application in many different processes (Table 1). They can be manufactured from a wide range of natural and/or

[1] Whilst no defined consensus exists, in general capsules < 1 μm in size are called nanocapsules, 1-1000 μm are termed microcapsules and > 1000 μm are referred to as macrocapsules.

synthetic materials, but are also commonly found in nature i.e. plant seeds, bacterial spores, egg shells etc.

The concept of encapsulating a material (also referred to as the encapsulant) within a defined membrane initially dates back to the 1930's in the food industry [1], whereby the process was mainly referred to as 'locking' rather than encapsulation [2]. The first significant industrial use occurred in the 1950's when microcapsules were incorporated into carbonless copy paper by the National Register Company [3], with the capsules produced by a complex coacervation process [4]. In this operation, capsules were filled with around 2-6% (v/v) of a leuco dye, which were coated on the underside of the top sheet of paper. Under compression the capsules burst, releasing the dye which is exposed to an acidic component that is coated on the lower sheet. The interaction of both of these components yields a dark dye (Sparks 1991). To date carbonless copy paper incorporating microcapsules is still employed commercially and is one of the most significant products utilizing microencapsulation technology with an annual worldwide consumption in the order of 10 million tons [5].

Early versions of microcapsules were impermeable and had to be broken apart, most often by mechanical means, for the inner ingredients to become active. Examples include controlled release of flavors, aromas, perfumes, drugs, detoxicants, fertilizers and precursors in textiles and printing [6]. In the following years and decades microencapsulation technology was applied successfully to the cosmetic, printing, chemical, pharmaceutical, agriculture and food industries where it has become well developed and accepted [7-9]. A large number of materials were encapsulated and modification of mass transfer characteristics and reduced interactions of the encapsulant with the environment were the main reasons for performing the process [10].

Encapsulation of flavor compounds by extrusion started in the 1950's [11] and has become a major technique in the aroma industry [12]. In the 90's a wave of patents appeared relating to aroma encapsulation, in which most of the patents covered special aspects of the encapsulation of flavor oils within a gelatin wall by complex coacervation [10]. In the US alone there are several hundred types of capsules being used as food additives [13]. Microcapsules have also been used as extraction aides for various compounds, such as warfarin [14], hexavalent chromium ions [15] and propionic acid [16]. In these cases, encapsulation prevented the formation of stable emulsions which subsequently reduces the settling time of an extraction process [10].

In recent times the technology has been applied to medical and biotechnological disciplines where the encapsulation of recombinant therapeutic proteins [17,18], cell implants [19-21] and mammalian cells [22,23] has taken place. They have also being applied to bioconversion [24,25] and fermentation processes [9,26,27] .

Due to its many existing and potential applications in many diverse areas, microencapsulation has received much attention from both academic and commercial bodies [28], and its further development is of a major interest both from an economic and scientific point of view. The growing interest is demonstrated by the exponential increase in the number of publications (non-scientific and scientific articles and patents) reporting on the subject over the decades since the 1950's. In the period between 1955-59, less than hundred publications were available, but this number has increased to nearly ten thousand for the period 2000-05 alone [29].

There are six main reasons for the encapsulation of a material within a membrane compared to its non-encapsulated species and these include:

1) Protection (stabilization) of the encapsulant from interactions with reactive environments an/or future surroundings;

2) Sustained (continuous), controlled or timed release of the core material;
3) Targeting of compounds to specific sites;
4) Enabling usage as extraction aides for product removal including in-situ product recovery;
5) Improved flow properties of the encapsulant for enhanced handling (inc. safety), usage and storage, and
6) Improved organoleptic properties of the core material.

Table 1 outlines some examples where the technology has been employed and also outlines the reasons for performing the process.

Table 1: Examples of some encapsulated species and the basis for performing the procedure.

Impetus (1-6)	**Examples**	**References**
1	**Cells (Prevention)**	
	Mammalian (immuno-response and cell	[20,30]
	damage due to agitation and aeration)	[31,32]
	Yeast (ethanol toxicity)	[33,34]
	Enzymes	
	Improved stability and reactivity	[35,36]
	Prevent denaturing	[37]
	Food additives and bioactives	
	Off-setting loss and deterioration by	
	High temperature food processing	[6]
	Passage through the gastrointestinal tract	[38]

	Oxidation	[7,39]
	Hygroscopicity	[6]
	Evaporation (aroma compounds)	[2,12]
	Recombinant Proteins	
	Improved stability and protection	[18]
2	Agrochemicals	[40]
	Bioactive ingredients	[41]
	Pharmaceuticals	[42,43]
	Vitamins	[44,45]
	Adhesives	[46]
	Flavors	[12,47]
	Fragrances	[48,49]
3	Antibodies	[50]
	Vitamin C	[45]
	Bioactive ingredients	[51]
4	**Environmental pollutants**	
	Extraction of pharmaceutical and	[14]
	Herbicides/pesticides	[52]
	Heavy Metals	[15]
	In-situ product recovery	
	Culture environments	
	Primary and	[26]
	Secondary metabolites	[27,53]
	Bioconversion processes	
	Lipase-catalyzed reactions	[25]

	Hydrolysis of Penicillin G	[24]
5	Pesticides Biosorbents Food (natural colorings)	[54,55] [56] [6]
6	Taste (shark liver oil) Appearance (tea bags)	[57] [58]

Structurally, microcapsules can be classified as five different types [2,6,10,12,59], which are shown in Figure 1.

Type I: Mononuclear: These are the simplest form of microcapsules, in which the core material (usually a liquid, in which case they can be referred to as liquid-core microcapsules [60]) is surrounded by a continuous defined membrane. The diameter of the core material and the membrane thickness can vary in size with either occupying between 10-90% of total capsule volume. These particles are also termed simple or single-core microcapsules.

Type II: Double/multi-shells (walls): Usually mononuclear microcapsules in which a second or multiply shells are added to the original capsule. The extra shells are added to modify the original stability and/or permeability characteristics of the microcapsules [61,62].

Type III: Polynuclear (multi-core): Microcapsules containing two or more separate cores [63,64] and are usually formed from emulsions.

Traditionally, microcapsules are seen as spherically shaped particles with a well defined shell and core structure (type's I-III) but other forms do exist.

Type IV: Microbeads/microspheres: Most common type of capsule and contain particles (including cells) entrapped within a solid matrix, which does not possess a distinctive

membrane [65]. Whilst these structures can be termed capsules they are usually referred to as microbeads [65] or microspheres [66,67]. In this review both terms are used interchangeably. They can be converted into type I by the addition of an outer shell and the subsequent liquefaction of the core, and is the basis for the production of the classical poly-L-lysine-alginate microcapsules as described in 1980 by Lim and Sun [68]. These particles can also be manufactured with no material trapped inside, and can be used as immobilization matrices.

Type V: Irregular or non-spherical shaped capsules. These particles can be mononuclear, polynuclear or solid particle entrapment. These are the most common type of capsules used in industry, but will not be discussed in this review.

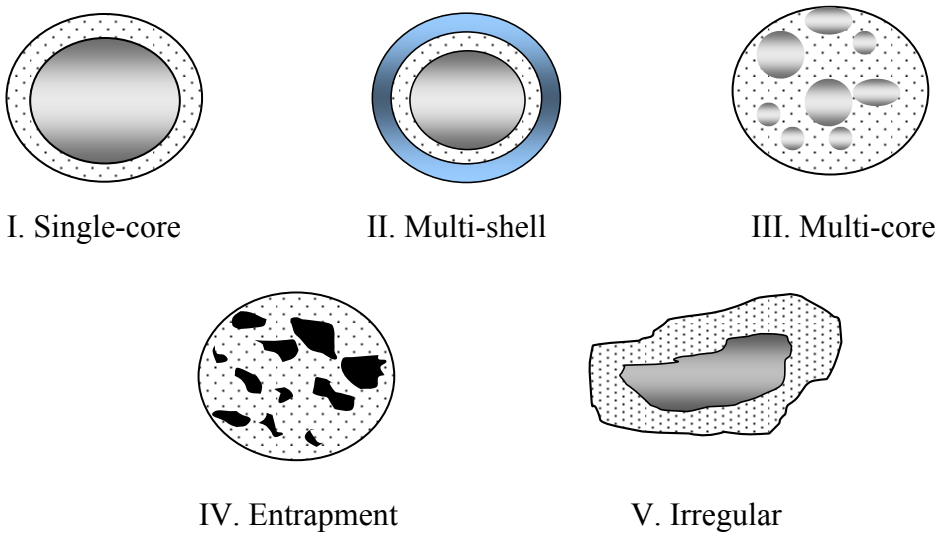


Figure 1: The five main structural forms of microcapsules (adapted from [2,6,10,12,59]). Type III – V can also have multiply shells added to modify stability and permeability of the capsules.

1.3. Production of microspheres/microcapsules

Many different techniques for the production of microsphers/microcapsules have been described [62,69]. For simplicity the methods can be categorized as chemical, physicochemical or mechanical processes [9] and include techniques such as: chemical; in-situ polymerization [70] and interfacial polymerization [71]; physiochemical; complex coacervation [72] and mechanical; spray-drying [59] and extrusion based methods [73].

1.3.1. Factors governing selection of an encapsulation procedure

The encapsulation process requires a technique for enveloping the desired product into capsules of the desired size and properties from the selected wall polymers. As suggested by Ubbink and Kruger [74] and Madene et al. [12], the technique selected for encapsulation should always depend on the end-use (which is governed mainly by the core material and to a smaller degree the shell material) of the encapsulated product. This is in contrast from normal practice, whereby the technique is usually chosen before the core and polymer materials (companies usually have set equipment already in place). Success (or failure) on adapting the core/polymer materials to the procedure, to enable production of the capsules, is then usually performed using an empirical (trial and error) approach. The major disadvantage of this approach is that from the outset it focuses on a specific technology to solve problems, which it may only be able to partially solve, or not at all [74]. This ultimately results in the production of an ineffective product. However, placing emphasis on final product application enables a clearer specification of the requirements to be fulfilled by a prospective technology before selection begins [74].

1.3.1.1. Requirements of encapsulation techniques for producing microspheres/ microcapsules for medical and biotechnological applications

Successful application and performance of microcapsules in medical and biotechnological applications requires a methodology capable of producing small (< 200 μm), mono-dispersed, homogenous and spherically shaped spheres/capsules, with a narrow size distribution, using a short production time, under mild and simple conditions and low costs, with high encapsulation efficiencies (% of product encapsulated) and production rates, from highly viscous solutions, allowing different production sizes and the ability to produce type I-IV microcapsules, which can all be performed under sterile conditions if required.

1.3.2. Mechanical procedures

This section describes some of the common mechanical procedures used for producing microspheres/microcapsules for medical and biotechnological applications. These operations use mechanical procedures rather than a well defined physical or chemical phenomenon to produce the desired particles [69]. They are based on the principle of generating a droplet(s) from a polymer extruded through a nozzle (orifice). They work using mechanical means (i.e. cutting or vibration forces) to increase the normal dripping process at the orifice, or they break-up an extruded liquid stream produced by the polymer when it is passed through the nozzle. After production, the droplets are immediately solidified to spheres/capsules by either physical e.g. cooling or heating, or chemical means e.g. gelation. Mechanical procedures are the major methodologies used for the production of microspheres for biotechnological and medical applications. Various coating and spray-drying methods are often used in industry, whereas the extrusion of a polymer through a nozzle(s) is used mainly at a lab scale [10,75]. Some

of the main mechanical technologies for fluid dispersion into droplets and subsequent conversion into capsules are: 1) spray-drying; 2) jet-cutting; 3) rotating disc; 4) electrostatic; 5) coaxial air-flow and 6) the vibrating nozzle. The principal of operation of these techniques will be discussed, along with their capabilities to match the criteria set out in section 1.3.1.1 and finally their ability to produce type I-IV microcapsules. Since these methods are primarily based on droplet formation at an orifice, an understanding of this process is required to enable an adequate understanding of each system, and this phenomenon will be discussed in the following section.

1.3.2.1. Theoretical background behind the formation of droplets by liquid extrusion through a nozzle

The extrusion of a liquid through an orifice results in one of five different droplet formation processes occurring at the discharge point of the nozzle (Figure 2), with the mechanism dependent on the velocity v of the extruded liquid [76]. The different mechanisms arise due to the interaction of gravitational, surface tension, impulse and frictional forces [77]. At very low velocities $(v < v_I)$, the extruded liquid sticks to the edge of the nozzle until the gravitational force is high enough to overcome the surface tension, resulting in the release of a drop (mechanism 1). A small rise in the velocity increases the number of droplets formed, whilst further escalation amplifies droplet formation (mechanism 2), and can result in coalescence of the droplets occurring, reducing mono-dispersity. The diameter of the droplet formed (d_d) during mechanism 1 and 2 can be estimated from equation 1 [10,62,78,79], which approximates the balance between the two main forces present; gravitational force pulling the drop down and the force of surface tension holding the drop pendant to the tip at the instant of drop detachment [80], and is as follows;

$$d_d = \sqrt[3]{\frac{6d_n\sigma}{g\rho}} \quad (1)$$

where d_N is the diameter of the nozzle, σ is the surface tension of the extruded liquid, g is the acceleration due to gravity and ρ is the density of the fluid.

Mechanism 1 and 2 are commonly used at a lab scale where only small volumes of droplets are required and this process is commonly known as dripping [80,81]. As seen in equation 1 the size of the droplets produced here is mainly dependent on the nozzle diameter.

However, for systems producing alginate drops which are subsequently gelified into spheres/beads by landing in a bath of $CaCl_2$, it must be noted that;

$$d_d \neq d_{cab} \quad (2)$$

in which d_{cab} is the diameter of the produced Ca-alginate bead.

Recently Chan et al. [82] extended equation 1 further by adding in an overall correction factor (K) which enables a good approximation of d_{cab} to be obtained.

$$d_d = \sqrt[3]{\frac{6d_n\sigma}{g\rho}} \cdot K \quad (3)$$

In their study Chan et al. [82] calculated that the overall correction factor varies from 0.73 to 0.85 under the conditions examined which correlated well with their experimental data, resulting in an average absolute size deviation of > 5%.

Further increasing the velocity causes the formation of an uninterrupted laminar jet (continuous stream), which eventually breaks-up into droplets by axial symmetrical vibrations and surface tension (mechanism 3). An additional escalation of the jet velocity $v > v_{II}$, leads to statistical distribution of the droplet size, which is caused by either spiral symmetrical vibrations (mechanism 4) or by the high frictional forces that are present, when the jet is sprayed (mechanism 5). As will be discussed in section 1.3.2.7., applying an additional frequency force to the laminar jet (mechanism 3), will result in the controlled break-up of it into uniform, mono-dispersed droplets of equal size. The size of the droplets can be pre-selected within a certain size range and depends on, the frequency applied. Whilst mechanism 2 and 3 enable the formation of mono-dispersed droplets of equal size only the latter is used at an industrial level, as it enables the production of high quantities of droplets (subsequently produced into spheres/capsules) when a vibrational frequency is applied to the extruded jet.

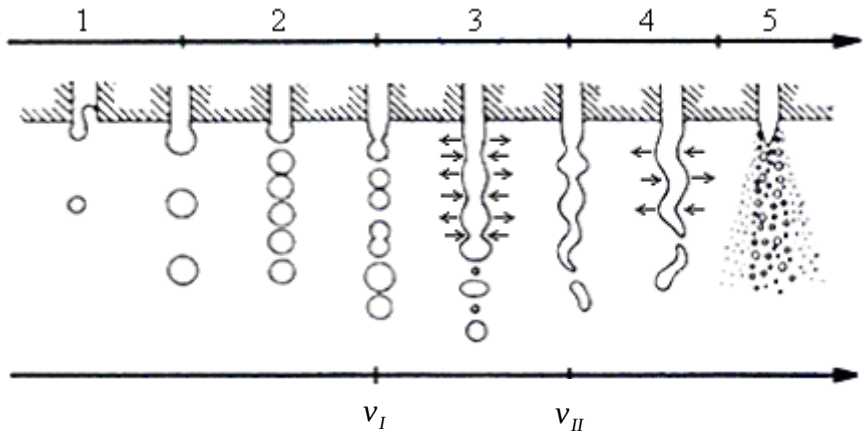


Figure 2: Different mechanisms of droplet formation as a function of jet velocities [76].

1.3.2.2. Spray-drying

Spray-drying is a unit operation in which a liquid product (containing the encapsulant and the shell material) is atomized by a hot gas current in a drying chamber, allowing the evaporation of moisture and formation of microspheres [59]. A double sized nozzle is employed, allowing air (an inert gas liked nitrogen is rarely used) from an annular geometry to atomize the liquid stream, which forms fine particles containing the microencapsulated product in a dispersed state. These particles are then dried by flash evaporation and pass through a cyclone and are collected in a holding chamber [83]. Depending on the starting feed material (viscosity, density etc) and operating conditions (atomization pressure, nozzle type) a very fine powder of capsules (10-50 μm) or large size particles (2-3 mm) can be obtained [59]. Spray-drying is the most commonly used and oldest microencapsulation technology in the food industry [39,84], due to low cost and the readily available equipment [59] and has mainly been employed to encapsulate flavors using gum acacia as the shell material [1].

The main advantages of spray-drying, is the ability to obtain large scale production with high encapsulation efficiencies, in continuous mode, at relatively low costs. The set-up is straightforward and is adaptable to commonly used processing equipment [83,84], and if required, can be performed under sterile conditions. The main disadvantages are that the produced capsules have an irregular shape and a large size distribution, whilst they also tend to form aggregates. Polar active agents with low boiling points such as ethyl acetate are also difficult to encapsulate. In the food industry a major limitation is the restricted number of materials available to be used as the shell of the particles [29].

The technique is mainly used to produce type IV capsules by spraying the encapsulant dispersed in the polymer matrix but can also produce type I and III by spraying

emulsions and coacervates [10]. In general the process is used for producing dry particles [9].

1.3.2.3. Jet cutting technique

The jet cutting technique is a relatively new process, which was developed by Vorlop and Breford in 1994 and is based on the mechanical impact of a cutting wire on a solid liquid jet [85]. When a polymer is forced through a special nozzle at high velocity, a solid liquid jet is formed within a very short distance of the nozzle. This jet can then be broken-up into equal cylindrical segments when passed through a cutting tool, which consists of several wires fixed onto a holder [85]. Due to surface tension, these cylindrical segments form spherical droplets/beads when passing through the air, the former forming spheres when dropped into hardening/polymerization solutions. The diameter of the resulting particles is dependent on: (1) the number of cutting wires; (2) the number of rotations of the cutting tool; (3) the mass flow rate through the nozzle and (4) the mass flow depending both on the nozzle diameter and the velocity of the fluid [86]. This simple and effective method enables the production of small mono-dispersed homogenous shaped beads, > 200 μm to several millimeters in diameter, with a narrow standard size deviation using viscous fluids at high production rates [85,87].

The main disadvantage of this method is the loss of material incurred during each cut of the liquid jet. This is known as the cutting loss and can be decreased by re-cycling or applying proper inclination of the cutting tool or nozzle and by also using smaller cutting wires [85]. Whilst these losses can be reduced to negligible amounts for the production of small beads, they do however increase with the increasing size of the produced particles (due to use of thicker cutting wires) and cannot be ignored if this method is to be applied at an industrial level. Other disadvantages include the inability

to produce beads < 200 μm in size, and to be performed under sterile conditions for large scale production. Although Schwinger at al. did encapsulate murine fibroblasts aseptically [88], it was achieved by placing the machine under a laminar flow safety bench, which would not be feasible for large scale production. The affect of this invasive technique on cells or other encapsulants within the polymer during the cutting process is yet unknown (i.e. within the cutting loss and their ability to be fully re-cycled for future encapsulation without any damage been caused). The jet velocities applied also have the potential to deform the spheres upon impact in the hardening/collection solution but this can be overcome using a soft landing method [89] or a pre-gelling step [85] instantaneously after jet cutting and before impact in the collection bath. However these add further complexity to the process. At present the technique is used mainly to produce Type IV capsules but has shown the capability to produce Type I microcapsules [85].

1.3.2.4. Rotating (spinning) disk atomization

Spinning disk atomization is a technique which is based on the disintegrating of a feed liquid performed on disc(s) to produce droplets [90]. When a liquid is dropped onto the surface of a rotating disk it is centrifugally accelerated to a high velocity and distributes as a thin film on the disc. Depending on the flow rate of the feed, droplets/microspheres are then released due to the centrifugal forces at the tip (teeth) of the rotating disk or from ligamentry streams released from the edge of the disk [91]. The size of the droplets produced is determined mainly by the rotation speed of the disk. This simplistic technique has shown the capability to produce microspheres ≥ 200 μm in diameter [91], with a narrow size distribution and is easily scalable, with possible production capacities of tons/day using a multi-disk system [28].

The main disadvantages of this system are the large surface area or volume required to collect the produced beads [90] and problems in ensuring sterility of this area during cell encapsulation. Other disadvantages include deformation of beads due to the high impact speed into the solution and the production of satellite beads which can affect the standard size deviation [91]. At present this systems seems capable of only making type IV microcapsules.

1.3.2.5. Electrostatic extrusion

The basis of electrostatic droplet generation is the acceleration of the normal droplet formation process using electrostatic forces to pull the droplets off the orifice (needle) at a considerably faster rate compared to the normal dripping process, whereby removal is based solely on gravitational force. The electrical potential, which can be static or pulsed [92] is applied to the extruded polymer solution by passing it through a charged needle, with the produced droplets subsequently falling into a collecting/hardening solution, which has being earthed or holds an opposite charge [93,94]. If the electrodes are parallel plates, a uniform electric field is generated with respect to direction and strength, thus a uniform force is applied to the droplets at the tip of the nozzle [95]. It has been reported that the strong electric fields do not cause cells to lose viability and activity during the producing (encapsulation) process [96]. This technique is capable of producing smaller microbeads compared to normal dripping ($\geq$ 50 μm in diameter), of uniform size and shape under reproducible conditions and can also be performed under sterile conditions [96]. The main disadvantages include low production rates due to the low polymer flow rate through the needle, although this can be improved by increasing the number of needles. However a study undertaken by Bugarski and coworkers showed that even when scaled-up to a system of 20 needles, it was still only possible to obtain a

low flow rate of 0.7 l/h (36 ml/h per needle) [94]. Furthermore, increasing cell and alginate concentrations hugely increase the size and size distribution of microbeads produced by this system [96]. At present this system has only shown the capability to produce type IV microcapsules.

1.3.2.6. Coaxial air-flow

The coaxial air-flow technique (also known as the concentric air-jet technique), like the electrostatic extrusion method, is also based on the acceleration of the normal dripping process at an orifice. However, instead of electrostatic forces, the technique uses a stream of compressed air to pull the liquid droplets from the nozzle at a faster rate compared to the normal gravitational force [89,97]. The coaxial concentric nozzle consists of an inner orifice, in which the polymer material is extruded and an outer orifice, through which the compressed air flows and strips the droplets formed at the tip of the internal nozzle [89]. The process is capable of producing capsules with diameters > 200 μm, of uniform size and shape, under reproducible and mild conditions and can be performed under sterile conditions [62,97-99]. The main disadvantage of the system, like electrostatic extrusion, is the low production rates due to the low flow rate of the polymer solution extruded through the needle. This can be overcome to some degree by increasing the air flow rate, but this can increase the trajectories of the produced drops resulting in a larger surface area being required, which could increase the complexity of the process. Even with these increases, significantly smaller throughputs are achieved in comparison to the other methods such as jet cutting and vibrating techniques [62]. This technique is also only able to produce Type IV microcapsules.

1.3.2.7. Vibrating-jet (nozzle) technique

The vibrating-jet technique, which is more commonly known as the vibrating nozzle technique or prilling [10,100,101], is one of the most widely used methods for the production of microspheres and microcapsules [28]. The technique is based on the principal of laminar jet break-up by the application of a vibrational frequency with defined amplitude to an extruded jet. As discussed in section 1.3.2.1., when a liquid is extruded through a nozzle at certain flow rates, it produces a laminar liquid jet which can break-up freely into short lengths by natural irregular disturbances (provided these perturbations reach a threshold, otherwise little or no break-up occurs). These segments then form spherical droplets in the air due to the surface tension of the extruded liquid. However, natural break-up can be irregular and is not possible to fully control and hence results in the formation of droplets which are not of equal size and shape [102].

Lord Rayleigh demonstrated that controlled break-up of laminar jets into small uniform droplets of equal size can be achieved, simply by applying a permanent sinusoidal force, at defined frequencies to the jet, resulting in the formation of one droplet per hertz of frequency applied (Figure 3). This highly regular and reproducible break-up occurs only at vibrational frequencies that are near the natural frequency for the break-up of the jet itself [102]. The characteristics of the drops formed are dependant on the nozzle diameter, the flow rate of the laminar jet, the size of the frequency at defined amplitude, and the viscosity of the extruded liquid [73] and will be discussed in the next section.

The sinusoidal force can be applied by either vibrating the nozzle (vibrating nozzle technique), pulsating the polymer in a chamber before passing through the nozzle (vibrating chamber technique), or periodic changes of the nozzle/orifice diameter during extrusion [61,62,75]. Whilst no set agreement exists, the authors suggest that collectively these different methods of applying the sinusoidal force to the laminar jet,

be termed the vibrating-jet technique. The choice of method used to apply the vibrational force is dependent on the system to which it is being applied to. For example, in liquid-liquid systems, it has being proven that the pulsation of the liquid is the optimal method, whilst for microsphere formation in a gas phase, all three techniques can be applied successfully [62].

The rest of this review will focus solely on the vibrating-jet technique, in which emphasis will be given on theoretical background, choice of membrane polymers for the procedure and the capability to produce type I-IV microcapsules. During the explanation of the production process, the ability of the methodology to match criteria set out in section 1.3.1.1. will be discussed and examined and where problems exist for meeting the criteria, solutions will be suggested, explained and their possible incorporation into the process, will be described.

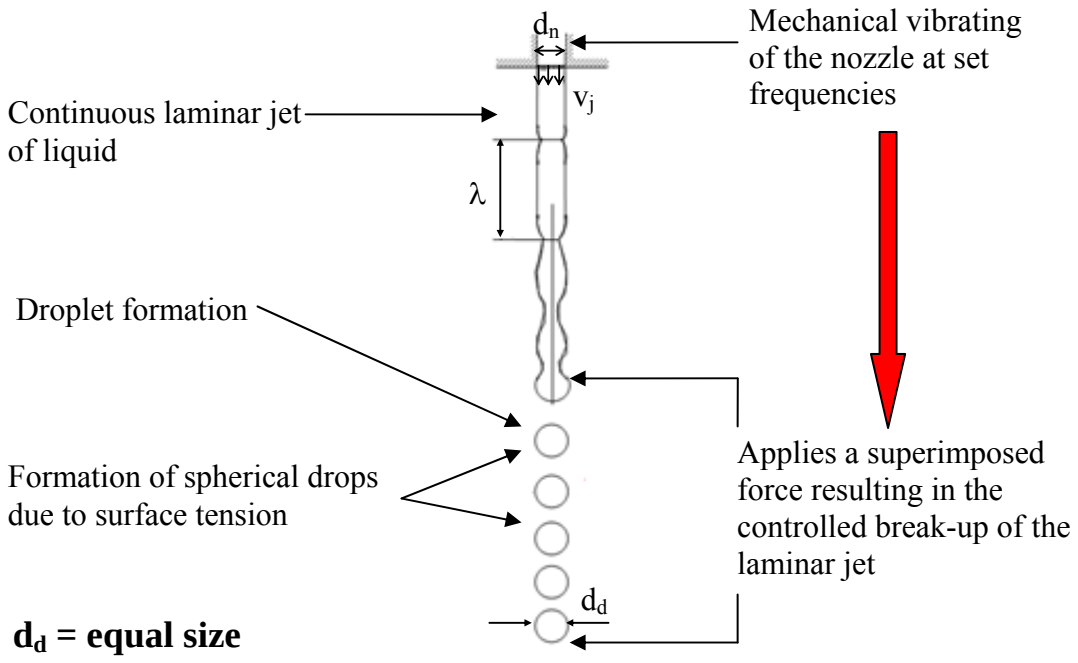


Figure 3: Controlled break-up of a laminar liquid jet into droplets of equal size. This is achieved by applying a sinusoidal force to the extruded jet, by mechanical vibrating the nozzle at a set frequency with defined amplitude.

1.3.2.7.1. Production of droplets by Raleigh's jet instability (theoretical aspects behind jet break-up)

At the end of the 19th century Lord Raleigh analyzed theoretically the aspects behind the instability of liquid jets and their ability to break-up into droplets due to axial symmetric disturbances [103]. Using a linearized stability analysis, Rayleigh showed that for wavelengths smaller than the circumference of the jet no break-up occurred [104]. For longer wavelengths the disturbances grow exponentially andl result in break-up. He proposed a mathematical description (equation 4) for inviscid and Newtonian fluids relating the optimal wavelength of the disturbance as a function of the jet diameter.

$$\lambda_{opt} = \pi\sqrt{2} \cdot d_j \tag{4}$$

where λ_{opt} represents the optimum wavelength for break-up of a jet of diameter d_j.

The frequency is related to the wavelength and the jet velocity by the following equation;

$$\lambda = \frac{v_j}{f} \tag{5}$$

in which v_j represents jet velocity and f the frequency vibration.

In 1931, Weber extended the equation further and included the effects of the physico-chemical properties of the fluid on determining the optimal wavelength for jet break-up and proposed the following equation [105];

$$\lambda_{opt} = \pi\sqrt{2} \cdot d_j \sqrt{1 + \frac{3 \cdot \eta}{\sqrt{\rho \cdot \sigma \cdot d_j}}} \tag{6}$$

where η is fluid viscosity. The size determination of the jet diameter has being based on different assumptions such as;

$$d_j = d_n \tag{7}$$

as proposed by Schneider and Hendricks d_n [106].

However, Brandenberger and Widmer [107] showed that for nozzles using a precision-drilled sapphire stone as an orifice the relationship between both d_j and d_n is a function of Weber's number of the nozzle (We_n) and is given by the following equation;

$$\frac{d_j}{d_n} = 4.33 \cdot We_n^{-0.337} \tag{8}$$

where

$$We_n = \frac{v_n^2 \cdot \rho \cdot d_n}{\sigma} \tag{9}$$

in which v_n is the velocity of the liquid in the nozzle.

Since one droplet is generated by each hertz of vibration, the drop diameter d_d, can be calculated by the following simple mass balance equation [73];

$$d_d = \sqrt[3]{6\frac{F}{\pi \cdot f}} \tag{10}$$

where F is the flow rate of the extruded liquid.

The vibrational frequency is itself linked to the wavelength by the following equation

$$f = \frac{F}{\lambda} \cdot \frac{4}{d_j^2 \cdot \pi} \tag{11}$$

This implies that the droplet diameter, as a function of wavelength and the jet diameter, is given by the following equation;

$$d_d = \sqrt[3]{\frac{3}{2} d_j^2 \lambda_{opt}} \tag{12}$$

Equations 4-12 imply, that for a given nozzle diameter, there are two main parameters to be determined to achieve optimal droplet formation: vibrational frequency and jet velocity. These two parameters trigger the production conditions and they have to be optimized within a certain range [62]. The equations also suggest that a range of frequencies exist around f_{opt} , whereby uniform sized droplets can be obtained, and is dependent on the nozzle diameter, rheology of the fluid and the surface tension [10]. Whilst this vibration system is theoretically based on liquids with Newtonian fluid dynamics it is also applied to non-Newtonian liquids such as alginate [73], to make uniform drops, which can be produced into microspheres by methods such as ionotropic gelation. The equations can be used to give an approximation of the frequency and flow

rates needed to break-up the polymers into the desired particles [62]. However, in most cases the required values for a given nozzle diameter, are obtained and determined experimentally using an empirical approach for each system, in which the calculated value is employed as a reference (starting) point [61,62,73]. For most polymers it must be again noted, as in equation 2, that the size of the droplet does not necessarily equal the size of the fabricated sphere/bead.

1.4. Membrane polymers

Whilst a limited number of encapsulation methods seem to exist to produce the desired capsules, there is however an enormous range of polymer materials available to produce microspheres/microcapsules. These include natural materials such as proteins (whey proteins), carbohydrates (starch and maltodextrin), lipids (hydrogenated fat), acacia (Arabic) gums, cellulose and synthetic polymers [6,12,108]. The description and assessment (to perform as an encapsulation matrix), of some of the most commonly used polymers to produce capsules using the vibrating-jet technique for biotechnological and medical applications will be discussed in this section.

1.4.1. Alginate

Alginate is a general term referring to a group of naturally occurring hydrophilic polysaccharides found abundantly in nature [109,110], and was first discovered in the late 19th century by Edward Stanford [43,111]. Commercial alginates (in which production began in the 1920s) are usually derived from the cell wall of various species of Marine brown algae found in coastal regions and includes extracting species such as *Macrocystis pyrifera* (also known as giant kelp), *Ascophyllum nodosum, and Laminaria hyperborean* [112]. Alginate-like polymers can also be extracted from two bacterial

genera (*Pseudomonas* and *Azotobacter*) which produce the alginate as an exopolymeric polysaccharide during their vegetative growth [111,113].

In structural terms (Figure 4), nearly all alginates are composed of un-branched homopolymeric regions of two sugars: D-mannuronate (donated as M-blocks) and L-guluronate (donated as G-blocks) separated by regions of alternating M- and G-blocks, the exception being alginates synthesized by *Pseudomonas,* which lack G-blocks [114]. The M to G ratio in alginate and their distribution throughout the polymer dictates the gelling and the mechanical properties of the gel produced during the gelation process [115,116].

The addition of an alginate droplet to a solution of divalent cations results in a mild ionotropic gelation reaction which produces a spherical porous hydrogel network (Figure 9 and 10) and is the most common methods to produce spheres/beads. For decades now, alginate gels have been used commercially in a variety of industrial processes, such as textiles, paper, water treatment, plastics, ceramics, pharmaceutical and the food industry [117] and in recent times it has being applied to medical and biotechnological processes mainly in the area of cell and tissue encapsulation. The vast amounts of literature available in the last two-three decades show how significantly important this compound has become in these research areas.

Since the pioneering study by Chang in 1964 [118], which suggested the idea of encapsulation for the immunoprotection of transplanted cells, and was followed by the ground breaking work of Lim and Sun in 1980 [68], alginate has become the basic matrix material for cell, tissue and macromolecules encapsulation for research into the treatment of many different diseases, which are caused by the bodies inability to produce critical molecules such as growth factors, hormones and enzymes [110]. Indeed, it has been estimated that > 85% of all articles published since Lim and Sun's

work, which deal with cell encapsulation involve modifications of their poly-L-lysine-alginate system [65]. This interest has mainly come about due to alginates ability to form heat stable gels using a simple, rapid, gentle and non-toxic gelation technique using divalent cations and relatively cheap and available materials[2]. Alginate is also biocompatible with most cells and does not have a detrimental affect on many different types of compounds.

Calcium and barium are mostly used as the complexation ions to produce the gelled structure because of their selectivity and co-operative binding to the G-Blocks [119]. Calcium is non-toxic and has been the most used ion for cell immobilization purposes. Whilst barium does form a stronger gel compared to calcium, use of high concentrations in the gelling process may lead to leakage of toxic ions. However, this can be overcome or reduced to a minimum amount, by using low concentrations of the ion and by also applying extensive washing after gelification [120].

The main limitations of using alginate as an encapsulation matrix is essentially due to the poor mechanical stability of the hydrogel in the presence of mono and divalent cations which can have an adverse affect on the structure by destabilizing the polyelectrolyte interactions involved in gel integrity. These ions are commonly found in cell culture media or the physiological environment surrounding the applied microcapsules [22] and this area will be discussed in greater detail in section 1.5.4.2.

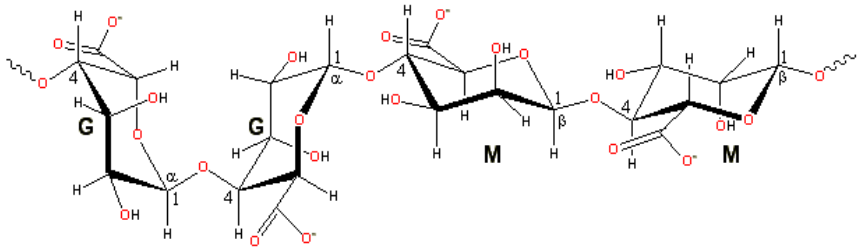


Figure 4: Molecular structure of alginate.

[2] Indeed, the successful production of alginate gels, only requires some alginate and calcium chloride solution, a syringe and a needle.

1.4.2. Chitosan

Chitin is a natural polymer extracted from crab shells, shrimps and other crustaceans by alkaline hydroylsis and can also be derived from the cell walls of fungi and the cuticle of insects [35,57,121]. Chitosan (Figure 5) is a biocompatible, biodegradable and non-toxic cationic linear polysaccharide which is rarely found freely in nature. It is composed of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine units and is obtained by the extensive deacetylation of chitin by hydrolyzing the amino-acetyl groups using NaOH [57,75]. It can form hydrogels by ionic [122] or chemical cross-linking [123] with the bi-functional agent glutaraldehyde and degrades via enzymatic hydrolysis [124]. Due to its weak mechanical properties and lack of bioactivity, chitosan is often combined with other materials to achieve more desirable mechanical properties for encapsulation. Chitosan has also being used to coat capsules to enhance stability and modify permeability instead of poly-L-lysine, due to it being regarded as more biocompatible [125,126]. It has also shown the ability to significantly exceed the stability of the poly-L-lysine-alginate complex in the pH range of 2-5 and also in the presence of salt [127]. Solubility is attained in aqueous solutions via protonation of its amine groups in acidic environments. Once, dissolved, chitosan can remain in solution up to a pH in the vicinity of 6.2. [122].

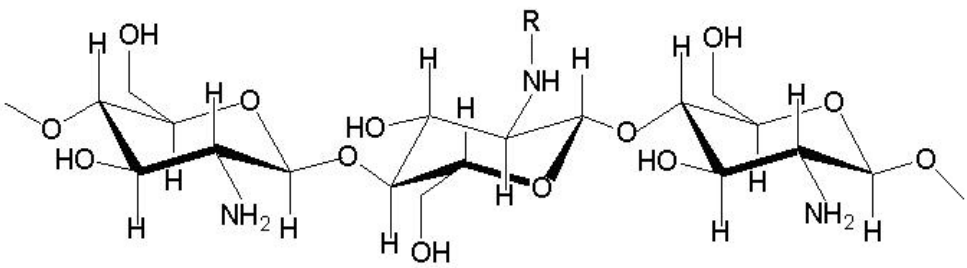


Figure 5: Molecular structure of chitosan.

1.4.3. Cellulose sulphate

Cellulose sulphate (Figure 6) is a biocompatible ester and is prepared in the laboratory by heterogeneous sulphation of cotton cellulose [128,129]. Linters (also known as cotton wool), which are a product of cotton processing is used as the cellulose raw material, and a mixture of sulphuric acid and n-propanol are used as the reaction solutions. As linters have a large ratio of volume to weight, substantially more reaction solution compared to cellulose is required during the sulphation reaction (in a standard reaction 1 kg of cellulose will require 20 kg of reaction solution to perform the process). This means that a large quantity of reaction solution is exhausted as waste after sulphation, which leads to high costs of production and a heavy payload for the environment [130]. However, improvements of the standard cellulose sulphate production process have being developed, in which the reaction solutions are regenerated and used repeatedly [130]. This method has the potential to reduce the cost of production and the corrosive environmental waste which is generated, thus enabling the polymer to compete with other cheaper materials.

Microcapsules are obtained from electrostatic interactions by dripping solutions of the polymer into the synthetic polycation poly(diallydimethylammonium chloride) [PDADMAC] (Figure 7). This results in a rapid reaction at the interface between the polymer and the polycation, cumulating in the formation of a mechanically stable membrane around the capsule core, which consists of un-reacted cellulose sulphate [129]. Materials to be encapsulated e.g. cells or enzymes or various compounds can be dissolved in the non-denaturing cellulose sulphate under relevant physiological conditions. During capsule formation, these materials get trapped within the core of un-reacted polymer, where they are protected from the PDADMAC [129]. Cellulose sulphate-PDADMAC microcapsules containing a liquid core have many advantages

over other polymers such as the ability to be manufactured by a simple single step process [50,129], which enables easy scale-up, they have excellent mechanical properties due to strongly interacting sulphate groups [50,128] and the membranes have a narrow pore size and a homogenous structure [129].

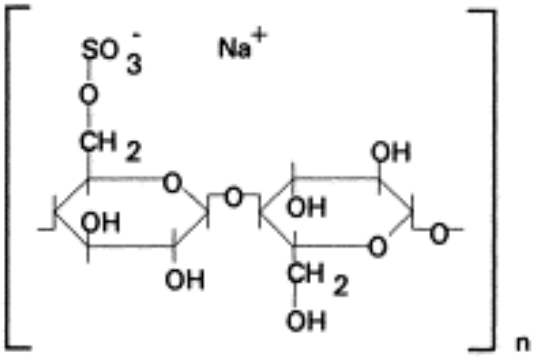


Figure 6: Molecular structure of sodium cellulose sulphate [129].

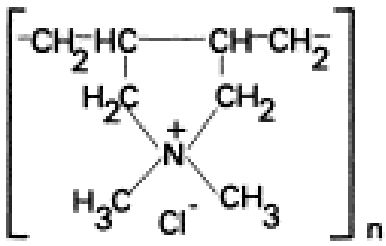


Figure 7: Molecular structure of PDADMAC [129].

1.5. Production of microcapsules (type I, III and IV) using the vibrating-jet technique

This section will discuss how type I, III and IV microcapsules are produced from a device based on the vibrating-jet technique, and emphasis will be based on the ability of the process to meet the criteria set out in Section 1.3.1.1. All experiments, unless specified, were performed on a lab-scale Encapsulator Model IE-50R from Inotech Biotechnologies, Basel, Switzerland (now EncapBioSystem, Greifensee, Switzerland) using sodium-alginate (Keltone LV and was obtained from Inotech) as the shell (polymer) material at concentrations of 2% (w/v) and a gelling (hardening) bath consisting of calcium chloride. All images of microcapsules were taking using a camera attached to a light microscope, interface to a PC and were taking at a magnification of 40X, unless stated differently. The aforementioned encapsulator enabled the production

of mono-dispersed and homogenous microspheres/microcapsules with a standard size deviation of ± 2.5% at alginate concentrations of between 1.5-2.5% (w/v) [results will not be discussed or shown]. This system is based on laminar jet break-up, induced by applying a sinusoidal frequency with defined amplitude to the liquid jet extruded through a nozzle. This encapsulator is designed to work with either a monocentric [single-flow] (Figure 8e) or concentric nozzle [two-flow] (Figure 12a-b) system. The chosen nozzle system depends on the type of microcapsules required [62].

1.5.1. General components of a vibrating nozzle encapsulator

Whilst different encapsulation devices, based on vibrating technology are available on the market e.g. Nisco Engineering AG, Inotech Biotechnologies AG and Brace Technologies, most consist of several central elements and can be assembled simply to make a lab scale encapsulator. These elements include:

- Pump mechanisms to feed the polymer(s) and/or core material to the nozzle(s).
- Nozzle(s) to create laminar liquid jet.
- Vibrating device and control system using signal frequency and amplitude to enable controlled break-up of liquid jet.
- Stroboscopic light to allow visualization of droplet chain and tuning of frequency.
- Agitated gelling bath to enable controlled gelification/polymerization of droplets to form microspheres/microcapsules and
- Collection device to enable easy recovery of produced particles.

In order to obtain the desired capsules at the required volumes, additional parts can be added to improve the process i.e. multiply the number of nozzles (section 1.5.4.1.) or high a frequency generator (section 1.5.2.1.) Figure 8 shows a schematic representation

of a lab-scale encapsulator with additional equipment and is based on the Inotech Encapsulator IE-50R. This system can be used to produce Type I, III and IV microcapsules using a single-flow or a concentric nozzle system.

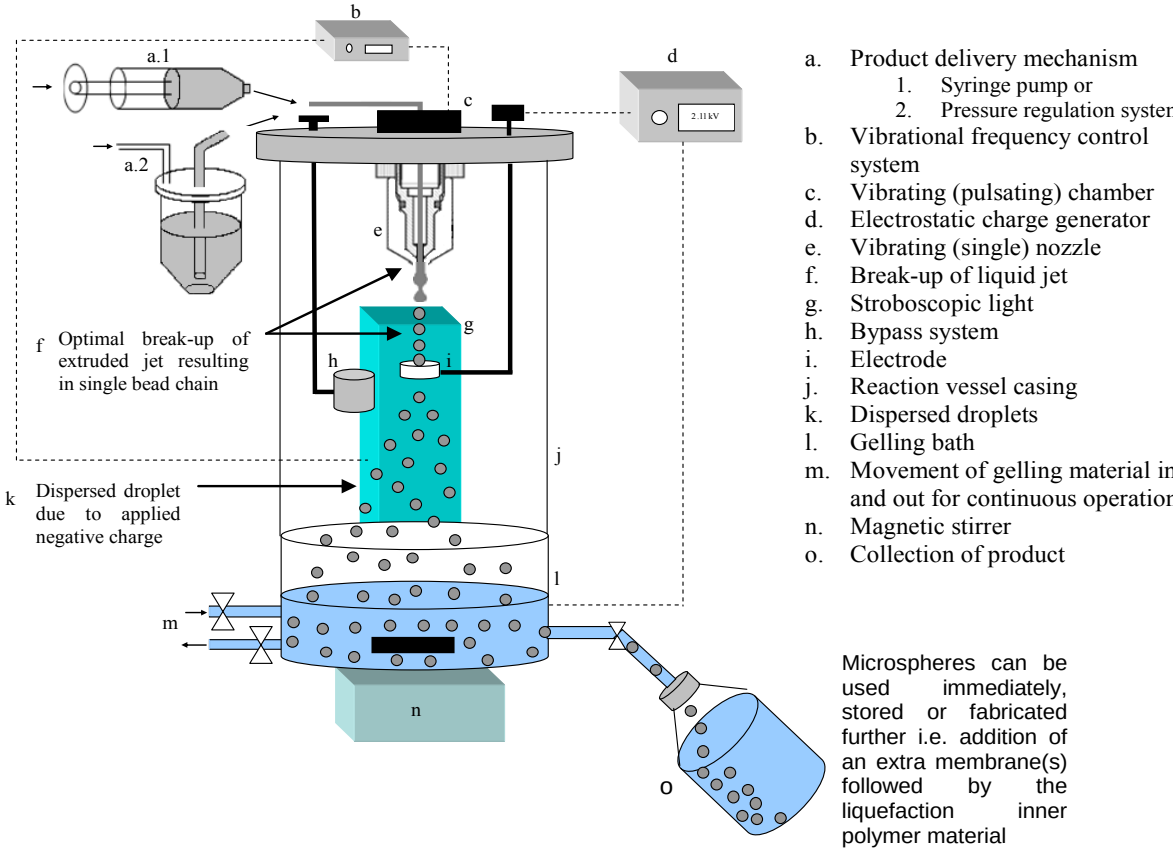


Figure 8: Schematic representation of an encapsulation device based on the principle of laminar jet break-up by vibrational frequencies. The schematic represents the Inotech encapsulator IE-50R and has a single-flow nozzle system (e) in place.

1.5.2. Monocentric nozzle system

The single-flow nozzle system can be used to directly produce type III and IV microcapsule/microspheres, but is mainly used to produce the latter in which cells or other solid particles are encapsulated within a polymer matrix. It consists of a single orifice (Figure 8e) in which the extruded polymer material passes through. The Inotech

encapsulator IE-50R uses a precision-drilled sapphire stone as the orifice on the tip of a stainless steel cone [107], although other materials have been used to create the precision orifice, but they usually suffer from limitations caused by imperfections in the nozzle geometry [107]. Nozzle diameters of between 50-1000 μm are available, which enable the production of particles in the size range of 100 μm to 2 mm in diameter.

1.5.2.1. Production of Type IV Microspheres using the Single-Flow Nozzle System

The product to be encapsulated is mixed with the polymer matrix before being placed in the delivery apparatus (Figure 8: a.1 and a.2). A very steady flow of the polymer through the nozzle at controllable rates is required to obtain the optimal break-up of the extruded liquid jet into droplets, which will enable the production of mono-dispersed microspheres with a very narrow size distribution. The flow of alginate to the nozzle can be achieved by one of two different systems[3], the selected one usually being dependent on the quantity of microspheres required. For the first mechanism, a pulsation free high precision syringe pump (a.1) is used for the extrusion of volumes of between 1-60 ml of polymer through the nozzle. For the second, an air pressure regulation system (a.2) is used. In this system compressed air is supplied to a vessel (which can be agitated if required) containing the polymer at a head pressure of between 0.1-2 bar and the desired flow rate is set using a pressure reduction valve. This system enables very large volumes to be delivered through the nozzle in a single process, and also produces more uniform and steadier flow rate of the alginate solution compared to the syringe pump method. This enables a narrower size distribution to be obtained for the produced particles compared to microspheres produced using the syringe pump

[3] Other pumping systems do exist for example Nisco Engineering uses a Lineasepta™ pumping bag system to delivery the polymer.

delivery mechanism. For the air pressure system, standard size deviations of 1-1.5% have been achieved compared to 1.5-3.0% for the syringe pump system.

The alginate, pumped through the nozzle, must be at a flow rate that is high enough to overcome the viscosity and/or the surface tension of the polymer solution, so that it can be extruded through the nozzle to form a continuous stable laminar liquid jet (mechanism 3 section 1.3.2.1.). However, the flow rate must not be too high, which could prevent jet break-up in a controllable manner and/or could result in the formation of a spray (mechanism 5 section 1.3.2.1.). High jet velocities will also increase the impact forces on the droplets when entering the gelification/collecting bath, resulting in deformation of the drops [62,89] whilst also increasing the occurrence of coalescence [102]. The size of this impact force on the droplets can be limited by reducing the distance between the nozzle and the impact site, or decreasing the jet velocity, but this leads to reduced production volumes. A simpler method is to add a surfactant such as Tween 80, which does not affect the sphere/capsule formation process [14] and can significantly reduce surface tension [91], preventing or reducing the occurrence of deformation and/or coalescence.

The extruded liquid jet is broken-up into droplets of equal size by the application of a vibrational frequency with defined amplitude to the laminar jet [73]. For the Inotech Encapsulator, the sinusoidal force is applied by passing the polymer through a pulsation chamber (Figure 8c) just before reaching the nozzle. This method enables more reproducible results to be achieved compared to vibrating the nozzle itself (vibrating nozzle experiments have been performed on an Inotech Encapsulator IEM, results are not shown). The size of the applied frequency to obtain optimal break-up of the jet can be estimated using equations 5 and 6, for a given nozzle diameter and flow rate [62]. The calculated frequency can be ± 20-40% of the required frequency for optimal jet

break-up, which is determined using an empirical approach as discussed in section 1.3.2.7.1. The value estimated using equations 5 and 6 acts as a starting point and as mentioned previously a range of frequencies exist (around f_{opt}) whereby uniform sized droplets can be produced. As seen in equation 12 the droplet diameter formed here is mainly dependent on the jet diameter, which is directly related to nozzle diameter (equation 8 and 9). A general rule of thumb for producing alginate microspheres (alginate concentration 1.5-2.0%) using the Inotech Encapsulator (IE-50R and IEM) is that the final bead diameter is approximately twice that of the nozzle diameter. Nevertheless, this size can be varied within a certain range by increasing/decreasing the frequency of the vibration and/or the flow rate of the polymer with higher frequencies and lower jet velocities enabling the generation of smaller droplets (equation 10). Application of higher/lower frequencies to the jet might not only decrease/increase droplet size but could also result in the coalescence of droplets [61,73] (higher frequencies) or the formation of one or more small droplets between the main ones called satellites[4] [61,62,73], which will again affect the overall standard size deviation. Satellites are usually formed at frequencies below the optimal frequency for jet break-up [62].

Optimal break-up of the jet results in the formation of a single bead chain (Figure 8f) which can be monitored using a stroboscopic lamp (Figure 8g) placed directly behind the chain. The frequency of the lamp is directly related to the frequency applied to the nozzle and this enables individual drops to be observed during break-up. The formation of droplets from a laminar liquid jet by vibrational frequencies can be so regular that they may appear to be slowed down, completely stopped or in reverse motion whilst

[4] Yuen could explain the formation of satellites by extending Rayleigh's linerized theory to a third order 131. Yuen MC: **Nonlinear Capillary Instability of a Liquid Jet**. *Journal of Fluid Mechanics* 1968, **33**:131-163.

being viewed through the lamp [102]. A video camera may also be installed to observe the drops and enables the direct measurement of their diameters.

A by-pass system (Figure 8h) is used to prevent polymer droplets, which may not be the required size or shape from entering the gelling bath, and also enables the retrieval of this polymer, which can be then re-used for future experiments. The apparatus consists of an electronically or manually controlled cup, which can be positioned directly underneath the nozzle when required. The by-pass system is usually operated during initial priming (setting optimal flow rate and frequency) of the nozzle with the polymer solution.

To prevent coalescence of the droplets from occurring, which results in loss of mono-dispersity and an increase in the standard size deviation [104], a strong negative charge is induced onto their surface during break-up using an electrostatic voltage system (Figure 8d). Coalescence in the air, can lead to the formation of particles with at least double the volume of other beads present (Figure 9a), or upon impact during entry into the hardening solution resulting in the production of irregular shaped particles known as doublets (Figure 9b). The voltage system applies an electric potential between the nozzle and an electrode (Figure 8i) placed directly beneath the nozzle. As droplets fall through the electrode, the induced charge on their surface causes the droplets to repel one another, resulting in dispersal of the chain into a cone like shape (Figure 8k) due to Coulomb forces [104]. Deflection of the droplets during break-up from their vertical position prevents them impacting one another in the air and results in entrance into the hardening solution occurring over a larger area. The size of the charge required for adequate separation of the jet in mainly dependent on, jet velocity, droplet diameter, concentration of the polyelectrolyte used, with higher values required for larger jet velocities and low polymer concentrations. The IE-50R is capable of applying an

electrical potential of 0-2.15 kV between the nozzle and the electrode. The main advantages of the system is that it enables higher frequencies to be used to produce smaller mono-dispersed beads for a given nozzle size [104] as well as allowing higher jet velocities for increased production capacity. The addition of a higher potential (> 2.15 kV) is not advised and can result in unstable droplets which can deform and/or burst [104,132].

Upon landing in the agitated bath containing calcium chloride, the alginate droplets are hardened by ionotropic gelation to form microspheres. A very critical point for the droplet is entry into the gelling solution. When a solution with a high surface tension is used such as calcium chloride [91], the polymer droplets can be held back partially at the surface of the hardening solution, during entrance, resulting in the formation of non-spherical droplets which are gelified before regaining there original shape (Figure 16a shows the outcome of this processes for the production of Type I microcapsules). As mentioned previously the problem of the surface tension can be overcome by the addition of a surfactant, which enables the formation of spherical microspheres (Figure 16b).

The Encapsulator may also be used for the continuous production of microspheres. In this system peristaltic pumps are used to control the movement of the hardening solution (Figure 8m) and newly formed microspheres from the reaction vessel to a collecting device (Figure 8o) and also add fresh solution to the vessel of the encapsulator. This keeps the volume at a constant level to ensure the impact height for all droplets is equal. After being given sufficient time to harden, microbeads are removed, and used immediately or modified further to produce the desired particles.

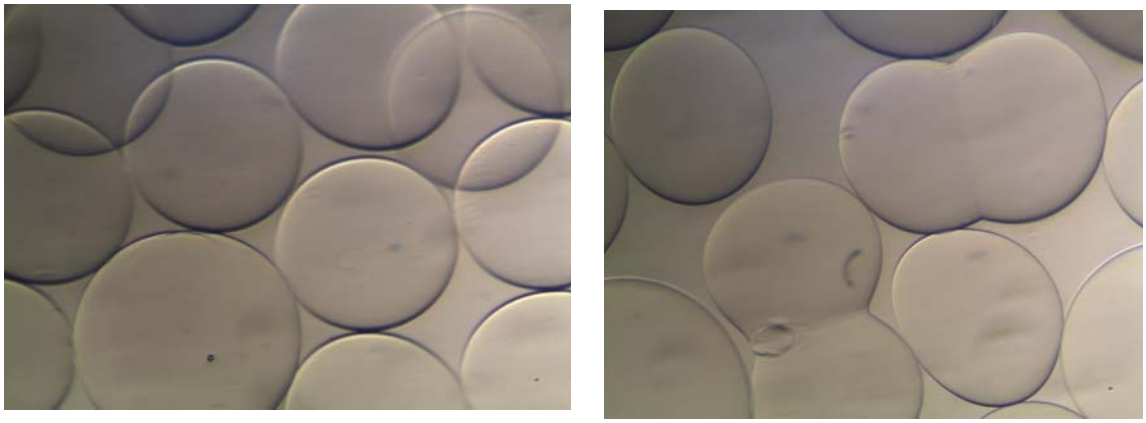
Once the optimal parameters have being obtained for particle production, a standard operating procedure should be defined on the parameters used, with slight tuning if

necessary, to enable the repeated production of the required microspheres on the Encapsulator IE-50R. Provided all other parameters are kept constant (i.e. alginate concentration, impact height etc), the size of the particles produced in each run will be in general ± 2.0 % of the original size, with a small amount of tuning of the frequency and/or flow rate enabling this to be reduced even further.

1.5.2.2. Production of type I and III microcapsules using the single-flow nozzle system

After production, Type IV microspheres can be converted into Type I microcapsules by addition of a further membrane followed by subsequent liquefaction of the now inner matrix incorporating the encapsulated product, to produce Type I microcapsules [22]. A well known example of this encapsulation technique is the production of alginate-poly-L-lysine microcapsules for the immobilization of mammalian cells and has being used since the 1960's [22,118,133]. Capsules produced using this approach can be seen in Figure 11a.

Type III microcapsules (Figure 11b) can be produced on the Inotech Encapsulator IE-50R by gently mixing the alginate with a hydrophobic substance such as oleic acid. For the capsules in Figure 11b, 1ml of oleic acid was mixed with 49 ml of alginate (although much higher amounts of oil can be used). The mixture is then produced into microcapsules using the same procedure for the production of type IV microcapsules. The main problem with this method is the difficulty in obtaining equal amounts of oil within each capsule. This predicament can be reduced by mixing at higher speeds for longer periods and extruding the liquid through the nozzle immediately after mixing. Figure 11b shows an example of type IV microcapsules produced by the aforementioned method.



(a) (b)

Figure 9: Light microscope images displaying droplet coalescence after jet break-up: (a) in the air, prior to reaching the hardening bath, resulting in double volume calcium-alginate microbeads and (b) during entry into the gelling bath resulting in the formation of doublets.

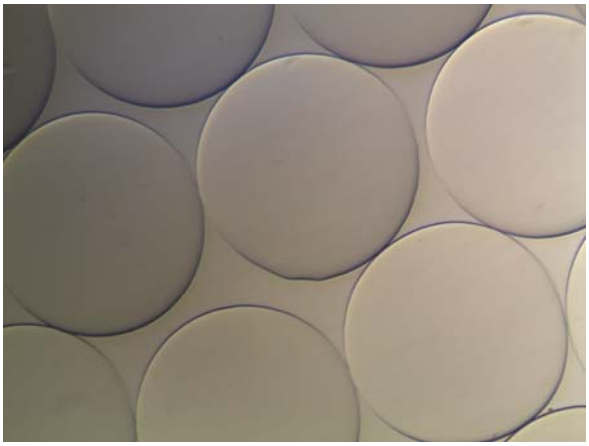


Figure 10: Light microscope image showing microspheres produced after the application of an electrostatic charge to prevent coalescence. Charge induction enabled the production of mono-dispersed, homogenous, spherical microspheres of equal size and shape. calcium-alginate microbeads where produced under the same conditions as spheres in Figure 9 and have a diameter of 763.02 μm ± 1.89%.

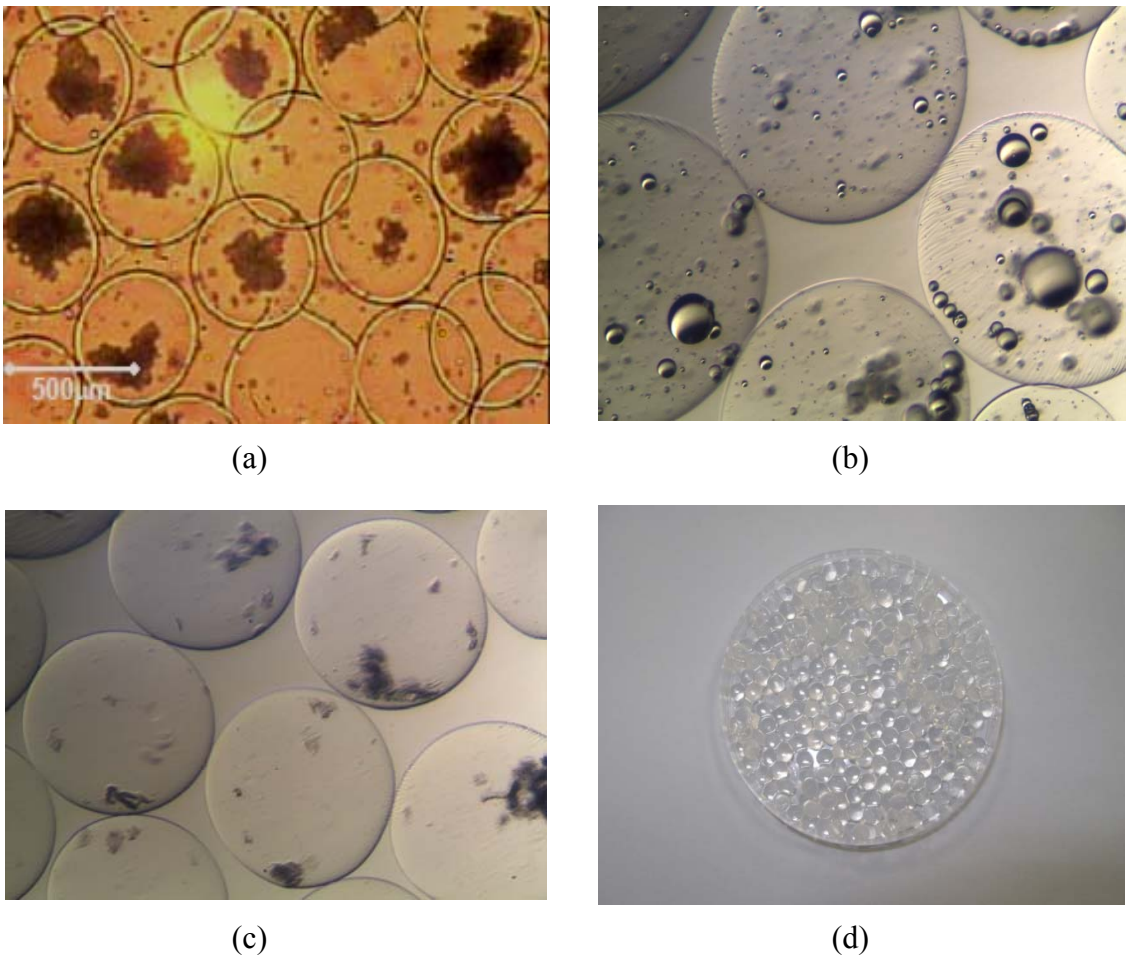


Figure 11: Light microscope images of microcapsules type I, III and IV produced by using a single-flow nozzle system on an Inotech encapsulator IE-50R or directly produced from these microcapsules: (a) Type I microcapsules produced from type III microcapsules as outlined in section 1.5.2.2. The capsules contain CHO cells and are enveloped in a poly-L-lysine-alginate membrane and have a diameter of around 500 µm; (b) Type III microcapsules, with a diameter of 953.81 µm ± 1.96%, containing multi-cores of oleic acid; (c) Type IV microspheres containing cells of *Streptomyces hydroscopicus* var. *geldanus*, which can also be produced into type I if required. Displayed microspheres had a diameter of 681.25 µm ± 2.51% and (d) Type IV

macrospheres with a diameter of 2.5 mm ± 2.01% and containing the anti-inflammatory drug diclofenac. This picture was taken to scale using a digital camera.

1.5.3. Concentric nozzle system

The Inotech Encapsulator IE-50R employing the concentric nozzle system is of similar set-up to the single-flow nozzle system as seen in Figure 8, the main difference involves the replacing of the single-flow nozzle with a concentric system (Figure 12a), which itself requires two feeds, one for the outer shell and the other for the inner core. The concentric nozzle system consists of two single nozzles termed an internal and an external nozzle, in which the inner nozzle is placed directly into the outer one (Figure 12b). Both nozzles can also be used on the Encapsulator as a single-flow system. The encapsulant is usually in the form of a liquid but if the encapsulation of a solid is required, this can be achieved by suspending the solid in a liquid, which is then extruded through the central orifice. For the Inotech system, diameters of 100-1000 μm are available for the outer nozzle and 50-900 μm for the inner one. Conventional wisdom tells us that the external orifice aperture must always be larger than the internal one to obtain the desired encapsulation of the core material within the chosen membrane. The external nozzle can be anywhere between 50-900 μm wider compared to the internal nozzle orifice diameter, which enables control of membrane size (membrane size can also be determined by varying the volume of the shell/core material and will be discussed in the next section). Type I microcapsules can be described as having an average diameter (d_m) , which is the sum of the core and the shell material diameters[5] and an average internal diameter (d_c) which consists solely of the

[5] For Type III and IV microcapsules d_m, is also taken as the diameter of the whole capsule.

measurement of the core diameter. The average size (thickness) of the microcapsule membrane (M_m) can then be obtained from the following equation

$$M_m = \frac{d_m - d_c}{2} \tag{13}$$

The concentric system is used mainly to produce type I microcapsules.

1.5.3.1. Production of type I microcapsules using the two-flow nozzle system

For the concentric system the two liquids required for production of the microcapsules can also be supplied to the nozzle using either a syringe pump or a pressure regulation system or a combination of both. The simultaneous supply of the two feeds to the concentric nozzle results in the formation of a co-extruded laminar liquid jet, which is subsequently broken-up into mononuclear drops, by the application of a vibrational frequency (Figure 12b and 12c). The produced droplets are then gelled into the desired mononuclear microcapsules. The effect of the two liquid-flows on producing liquid-core microcapsules using the Inotech Encapsulator IE-50R in comparison to producing microspheres will be discussed in this section.

The capsule diameter is mainly dependent on the diameter of the outer nozzle and like the single nozzle system; the size can be varied within a certain range by increasing/decreasing the applied flow rate and vibrational frequency. The diameter of the internal nozzle and the flow rate of the material will also affect the final capsule size with increasing diameters and volumes resulting in larger core volumes, hence bigger sized capsules. A general rule of thumb for the production of type I microcapsules is that the final d_m can vary between 1.5-2.5 times the size of the external nozzle diameter

with decreasing sizes resulting from higher frequencies and lower flow rates of the shell and core material. The diameter of d_c can vary between 1.0-2.5 times the diameter of the inner nozzle with increasing flow rates resulting in larger diameters. This implies that the thickness of the membrane material can be predetermined and controlled within a certain range[6] for a given concentric nozzle system by varying the ratio of the flow rate (volume) of the shell material to the volume of the core material in the extruded concentric liquid jet, during the microcapsule production process (Figure 13). The membrane thickness can be a very important characteristic of microcapsules as it has the ability to significantly affect the egress/ingress of compounds from microcapsules [14,27,52].

Unlike the monocentric nozzle system, no equations presently exist to help determine close values and/or an approximation of the optimal flow rates and vibrational frequency for the system and the values must be determined using an empirical approach. Similar to the single-flow nozzle system a range of frequencies around the f_{opt} value exist for the break-up of the jet into uniform droplets. In general, to obtain good production conditions the flow rate of the shell material is usually at least twice that of the core liquid; however this value can be reduced to obtain thin-membrane microcapsules as mentioned previously. Depending on the diameters of the internal and external nozzles and the flow rates of the two materials it is possible to obtain mononuclear capsules with a membrane diameter smaller than 50 μm and greater than 500 μm.

[6] For example, an external nozzle with a diameter which is 100 μm greater than the internal nozzle will general produces capsules with a membrane thickness size of around 100 μm. However, by increasing /decreasing the core/shell material volumes, the membrane thickness can be considerably reduced to < or > 100 μm in diameter (Figure 13).

The application of frequencies below the f_{opt} can again lead to the production of miniature sized satellites (Figure 14a and b). For the concentric nozzle system, these structures can have the following effects on the production processes: (1) they can form smaller mononuclear capsules during gelification, which are completely separate and independent of the larger desired microcapsules (Figure 14a), which will significantly increase the overall standard size deviation and (2) the small independent particles may become incorporated into the membrane of the large capsules. This may occur when small satellites collide with the larger droplets either during jet break-up and/or when entering the gelling bath. This results in the formation of a smaller independent core adjacent to the larger liquid-core and/or in some cases it joins onto the core material itself (Figure 14b).

A charge can also be applied to the mononuclear droplets to enable their dispersion and prevent coalescence occurring in the air and/or upon impact resulting in the formation of duplets and/or larger microcapsules (Figure 15). This charge must be applied at higher values compared to the monocentric nozzle system to enable similar droplet dispersion to be achieved. This is due to the smaller percentage of polyelectrolyte present in the droplet because of the core material[7]. Upon landing in the gelling bath drops can be again held back momentarily due to the high surface tension, which can again result in the formation of oval shaped capsules (Figure 16a). In some instances this delay can cause the droplet to burst, releasing the core material before gelification takes place (Figure 17). It is hypothesized that this bursting is caused by the movement of the core liquid out through the pre-hardened membrane material when capsules are been held back briefly at the surface of the hardening solution and hence results in release (bursting) of the core liquid. Bursting of the droplet can also be caused by high

[7] This problem can be overcome by increasing the alginate concentration, however as mentioned previously this will increase viscosity, which can prevent extrusion and/or jet break-up.

impact forces when the pre-gelified droplet hits the hardening solution. As mentioned previously surface tension can be reduced by the addition of a surfactant and/or by slightly heating (50-60 °C) the gelification solution. This will enable quicker entry of the drop into the solution preventing its deformation and resulting in an instantaneous gelification. Under ideal conditions a standard size deviation of < 2.5% and an encapsulation yield > 90% for the encapsulant can be obtained for capsules produced by this process, and depending on several variables, 200-2000 capsules per second can be produced. Most solvents can be encapsulated using the co-extrusion laminar jet break-up technique, provided a difference exists between the viscosity/surface tension of the membrane polymer and the liquid-core material [14].

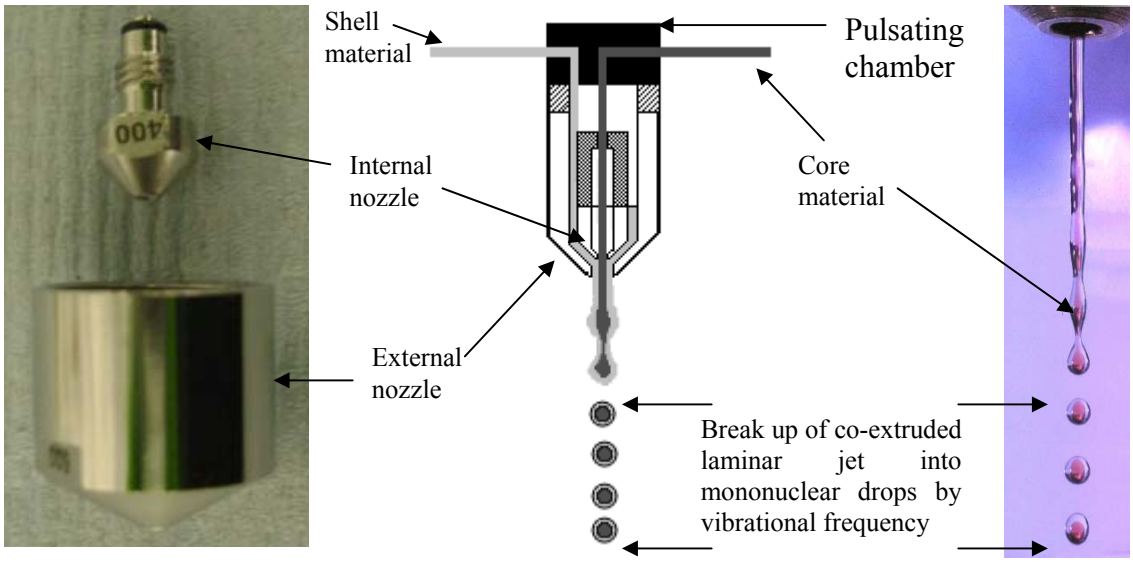


Figure 12: Images displaying the two-flow nozzle system used to directly produce type I microcapsules on the Inotech Encapsulator IE-50R: (a) Concentric nozzle with an internal diameter of 400 µm and an external diameter of 600 µm; (b) Schematic representation of the internal structure of the two-flow nozzle system and (c) Real-time image showing the laminar jet break-up of a co-extruded jet into mononuclear droplets.

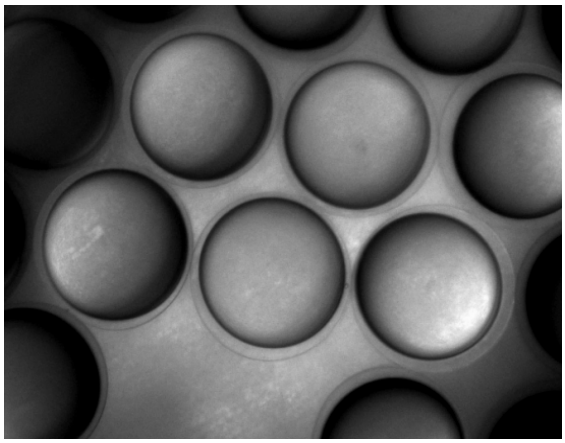


Figure 13: Light microscope image of mono-dispersed and uniform mononuclear microcapsules (type I) produced using the concentric nozzle system. The capsules displayed contain dibutyl sebacate as the liquid-core material and have a d_m of 616.92 µm ± 1.52%, a d_c of 551.81 µm ± 1.23% and a M_m of 32.55 µm ± 5.88%.

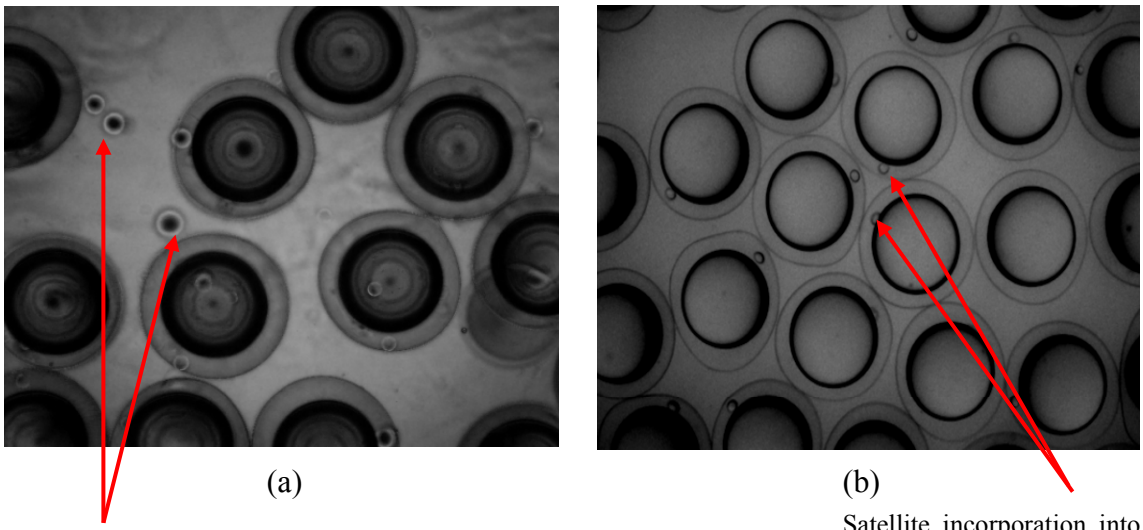


Figure 14: Light microscope images displaying the different types of satellites formed during the production of Type I microcapsules using the concentric nozzle system: (a) small independent microcapsules with a d_m of 123 µm ± 19.78% and a d_c of 85.38 µm ± 22.6%. The larger microcapsules have a d_m of 751.12 µm ± 4.29% and a d_c of 567.94 µm ± 2.7% and (b) satellite incorporation into the capsule membrane and/or the core material.

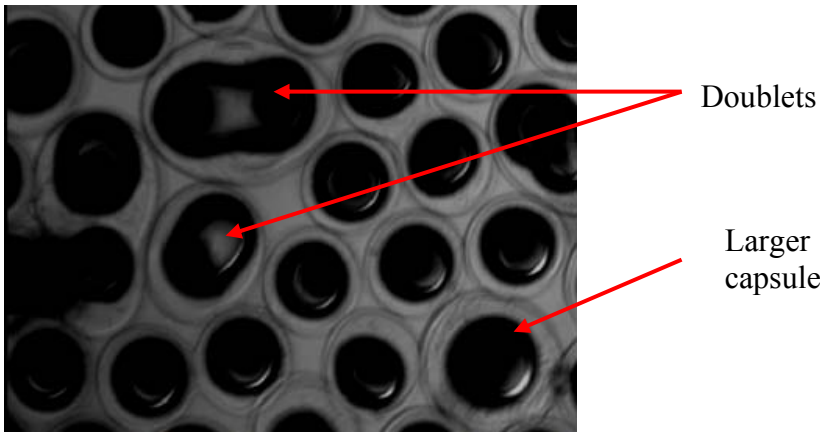
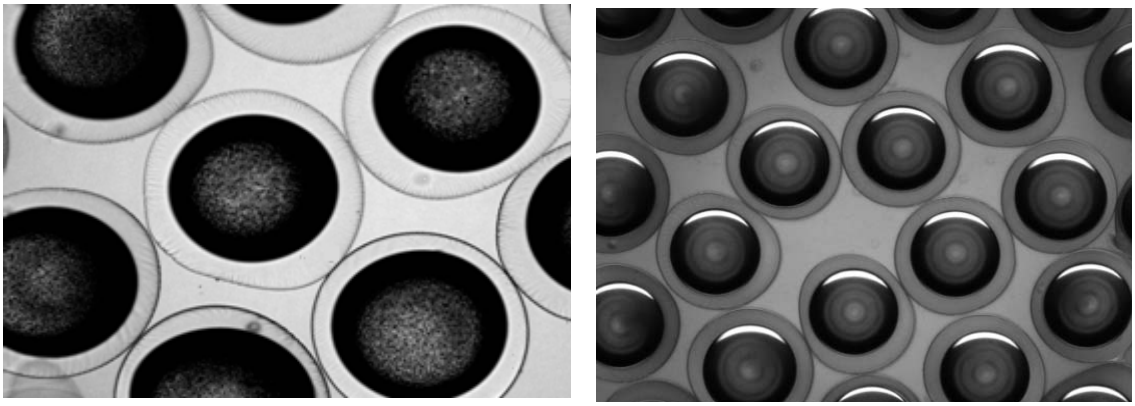


Figure 15: Light microscope image shown the affects of droplet coalescence after jet break-up and during entry into the hardening bath when using the concentric nozzle system. This can result in the formation of doublets and larger-sized capsules.



(a) (b)

Figure 16: Light microscope image of: (a) Non-spherical capsules formed due to the high surface tension of the gelling bath and (b) Improved structure and spherical shape as a result of heating the hardening solution which also contained the surfactant Tween 80.

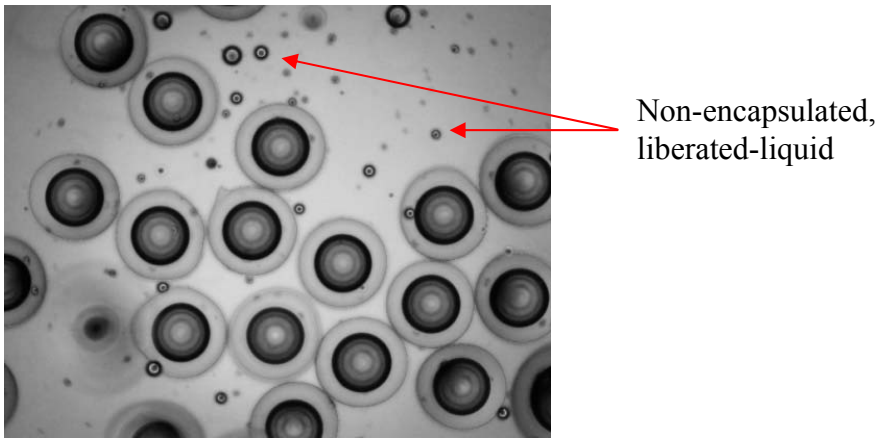


Figure 17: Light microscope image showing the release of the core liquid from type I microcapsules due to bursting of the capsules when entering (impacting) the gelling bath.

1.5.4. Challenges facing the vibrating nozzle system: possible solutions

As discussed and shown in the previous section, the jet break-up technique based on vibration technology has shown the capability to match most of the criteria set-out in section 1.3.1.1. However, it is still susceptible to two commonly known problems, which need to be addressed and overcome to enable further development of the process, and which will help facilitate its application towards a relevant industrial process. This section discusses the two main challenges and describes possible solutions to these problems.

1.5.4.1. Large scale production/scale-up

The vibrating technique using a monocentric or concentric nozzle system is usually limited to relatively small production yields of microspheres/microcapsules, as it only produces single droplets, one after another at any given time. The production flow rate is mainly dependent on the nozzle diameter with increasing diameters resulting in higher production volumes. However even for the largest orifice diameters, very low

production volumes are still achieved. For example, using the single-flow nozzle system on an Inotech Encapsulator IE-50R with an orifice diameter of 500 μm, only yields a maximum flow rate of 1.25-1.35 l/hr. For the concentric system (external nozzle of 600 μm and internal of 400 μm), a maximum flow rate of only around 2.1-2.7 l/hr can be achieved. It must also be noted that these flow rates do not equal production volumes, as losses will be incurred during initial priming of the system. Whilst these volumes are sufficient for lab-scale research and application, they fall well short of quantities (tons or cubic metres per day) required at an industrial level.

Brandenberger and Widmer [107] showed that the monocentric nozzle system can be scaled-up to achieve increased production quantities by simply adding more nozzles (from one to thirteen) for polymer extrusion. This enabled the simultaneous production of thirteen homogenize liquid jets which were broken-up and produced into microbeads of equal size and shape by the application of an equal vibrational frequency to all the extruded liquids. The flow rate of the jets was kept equal by using a concentric split, which enabled a relative flow difference between all nozzles of $< \pm 2.0\%$. Before extrusion through the nozzles the polymer solution passes through a vibrating chamber which transmits an equal disturbance to the solution hence resulting in the break-up of all jets into droplets of equal size (difference of droplet size of $< 0.7\%$ and was mainly attributed to small differences in the diameters of the nozzles). This is another reason for using a pulsating chamber to exert the perturbation onto the extruded jet instead of vibrating the nozzle, as to subject all liquid jets with the same sinusoidal force using the latter would pose a difficult engineering challenge.

By using the multi-nozzle system with a nozzle diameter of 200 μm, Brandenberger and Widmer [107] obtained a semi-continuous production capacity of up to 5 l/hr (0.385 l/hr per nozzle). This low production level was achieved due to the 1 litre capacity of the

polymer delivery apparatus. This can easily be overcome by increasing the volume of the delivery mechanism by using large pressurized vessels to control the flow. Due to the stop-start production initiated by the low delivery volumes, a clean-in-place (CIP) process using steam and filtered water was applied to the multi-nozzle system to prevent clogging of the nozzles by gelled alginate. The use of larger polymer volumes would reduce the need for this procedure, hence reducing the number of steps which would otherwise add cost and complexity to the process.

Using this system as an example, and the previously mentioned flow rates and nozzles diameters, it is estimated that a system (monocentric) incorporating a nozzle diameter of 500 μm, should be capable of reaching a production capacity of around 17 l/hr for the monocentric system, and for the concentric set-up a capacity of around 27-35 l/hr could be obtained. These quantities are still below what would be required for industrial applications, but as discussed previously by Heinzen et al. [62], provided the vibrational frequency, amplitude and flow rate are kept constant across all nozzles, larger quantities can be obtained simply by further increasing the number of nozzles which should still enable the production of spherical and equal-sized capsules. At present the authors are not aware of any research which has being published which discusses the maximum number of nozzles which can be placed on the same machine (nozzle plate) for optimal production.

The multi-nozzle system developed by Brandenberger and Widmer [107] did not employ an electrostatic voltage system to separate the droplets during jet break-up and this resulted in coalescence of the droplets, which increased the overall standard size deviation. It should be possible to apply a charge to the individual chains to enable separation. This could be applied using individual electrodes placed directly underneath each nozzle. Unlike other parameters, the charge applied to the each jet does not have to

be the equal, and needs only be applied at a minimum value which adequately separates the chain (higher values, [≤ 2.15 kV] would also not affect the production process and would just result in greater dispersions of the droplets). The multi-nozzle system has also shown the capability to be operated under sterile conditions [62,107]. It is envisaged that in the future, encapsulating devices (based on the vibrating-jet technology) applied at an industrial level could possibly contain several hundred nozzles which would enable the required quantities of tons/day to be produced.

1.5.4.2. Polymer Choice

Whilst encapsulation can take place using a large number of materials as the shell, the jet break-up technique, due to vibrational frequencies, has mainly focused on using alginate as the encapsulation matrix. Indeed the other mechanical processes described in this review also use the polymer as their number one choice. The use as an encapsulation material of non-Newtonian polyelectrolytes such as alginate, which exhibit pseudoplastic behavior [101], is limited in biotechnological and medical applications. This is on account of their naturally low mechanical stability (which will be discussed first) and their sensitivity towards chelating compounds such as such as phosphate, citrate, EDTA and lactate, or other anti-gelling cations like Na^{+} or Mg^{2+} [134]. These can reduce the mechanical stability of the hydrogel and in some cases cause complete dissolution of the gel network structure, releasing the encapsulant itself. These compounds are commonly found in biological and bioprocessing environments [135] and the sensitivity of alginates towards them is the main reason behind its limited success toward industrial and medical applications [136].

As shown in numerous studies increased alginate concentrations can result in microspheres with considerably improved mechanical properties. However, exponential

increases in viscosity are obtained with accumulating concentrations of alginate [101]. In some cases, depending on the source and purification procedure used, doubling the concentration will cause a 10-fold increase in the viscosity of the alginate solution [10,101]. As mentioned in section 1.5.2.1. very high viscosities can prevent extrusion of the alginate through the nozzle, especially when syringe pumps are being used, on account of their limited power. This problem can be resolved by pumping the solution under high pressure through the nozzle but the high jet velocities and/or viscosities can again prevent the break-up of the jet [135]. Using the Inotech encapsulator it was possible to extrude and break-up alginate solutions between concentrations of 2.5-3% (w/v). However concentrations ≥ 3% (w/v) could not be extruded and/or caused unstable jet break-up, leading to the production of hetero-disperse beads [136]. These results are somewhat in contradiction to results obtained by Prusse et al. [89], in which it was observed that microspheres could not be produced at alginate concentrations, in some situations at, and above 2% (w/v). This study also claimed then when production did occur at 2%, large standard size deviation was obtained and the spherical structure of the particles was compromised. This is again in contradiction to results obtained by our research group and is probably due to the use of different alginate solutions having higher viscosity/molecular weight range.

This problem of only being able to use solutions, containing low concentrations of alginate, which only allows the production of particles with limited mechanical strength, can be resolved by reducing the viscosity of the alginate solution. This can be achieved by increasing its temperature during extrusion through the nozzle(s). Recently an apparatus has being developed (by EnCapBioSystems), for heating and/or maintaining polymers solutions at controllable temperatures as they pass through the pulsation chamber, before extrusion and break-up. This apparatus, termed a heating

nozzle/pulsating-head device (Figure 18), consists of a temperature control unit connected to a heating element placed adjacent to a circular aluminum casing, which conducts the heat from the element. The pulsating chamber (monocentric/concentric) with the nozzle holder attached sits within the heating device and is completely enveloped. The casing itself can be fastened tightly to the nozzle housing to improve heat transfer and enables a more precise temperature control. The heating apparatus can supply a controllable temperature of up to 60 °C ± 1 °C to the chamber which subsequently heats and/or maintains the temperature of the polymer during extrusion and jet break-up. The shell and/or core liquid can be heated in their reservoirs to the desired temperature using standard water baths before being pumped along insulted tubing to the pulsating chamber. At present only preliminary experiments have being preformed in regard to using this device and as of yet, no concrete conclusions can be made. However initial experiments have being performed and have shown the device to enable concentrations of alginate of ≥ 4% (w/v) to be successfully produced into microspheres which showed considerably improvements in mechanical strength compared to lower concentrations of alginates.

Whilst increasing alginate concentration can significantly improve mechanical strength, it does not however overcome its sensitivity towards chelating agents and other anti-gelling cations. In response to this predicament, numerous attempts have been made to help stabilize alginate gels using various hardening techniques. These include complexation with ions other than calcium i.e. barium [137] or additional complexation with other polymers [138]. Other methods include covalent cross-linking of alginate and a alginate-polyethyleneimine mixtures with glutaraldehyde [139,140], acrylamide [135] and epichlorohydrin [141]. Glycol alginate has itself been cross-linked with proteins [142]. Whilst the stability and strength of the gel structure improved significantly in

some instances, the gels did however still show sensitivity towards chelating agents, or in some cases the hardening step was not suitable for the encapsulated particles (i.e. cells) or the cross-linking component was itself affected when applied to the new environment i.e. hydrolytic enzymes [142]. Long term stability is still a major problem and most be overcome to enable use of alginate for encapsulation of artificial organs and for incorporation in biotechnological processes [143].

Another sizable problem with using alginate relates to structural differences. The overall composition and the sequence of the D and G regions of the polymer (Figure 4) can vary extensively between different batches of alginate and is mainly dependant on the species of origin, location of alginate in the plant and the time of year in which harvesting was performed. This variable composition has many negative affects on reproducibility of experiments (especially for cell encapsulation) and is still one of the limiting factors for large scale use of alginate at an industrial level [144]. However, certain bacterial strains do produce alginate homogenously. Bacterial alginate fermentations mainly use *A. vinelandii* as the producing organism but are not usually available commercially due to low production volumes (around 4 g/l), which make the product commercially non-viable [111,112]. However research has being undertaken to control and increase the production of alginate by certain bacteria [112]. Provided high enough concentrations can be manufactured, this type of research could enable the continuous production of alginates with a constant structure, which could potentially solve the consistency problems plaguing applicability in biotechnological and medical applications.

At present very few polymers seem to be available to produce the desired capsules according to the criteria in section 1.3.1.1. However the heating device previously mentioned has the potential to increase this number of polymers by enabling very

viscous polymers and gums, which only form liquids above room temperature to be extruded (i.e. gelatin) to produce microspheres/microcapsules. However, subjecting the polymer to high temperatures (up to 60 °) has the potential to adversely affect the encapsulant which could be counterproductive to the processes itself. This certainly would be the case for the encapsulation of most animal cell lines, probiotic bacteria as well as the entrapment of heat sensitive pharmaceuticals. Most aroma compounds are themselves very volatile and the process could result in evaporation of large amounts during the capsules production process.

Whilst alginate has proven to be a very versatile and interesting compound to work with and has provided the encapsulation community with a vast amount of interesting results and ideas for nearly 50 years, the authors feel that in the coming decades, for encapsulation technology to reach its full potential in biotechnological and medical processes, a shift from the extensive research on alginate is required toward other polymers.

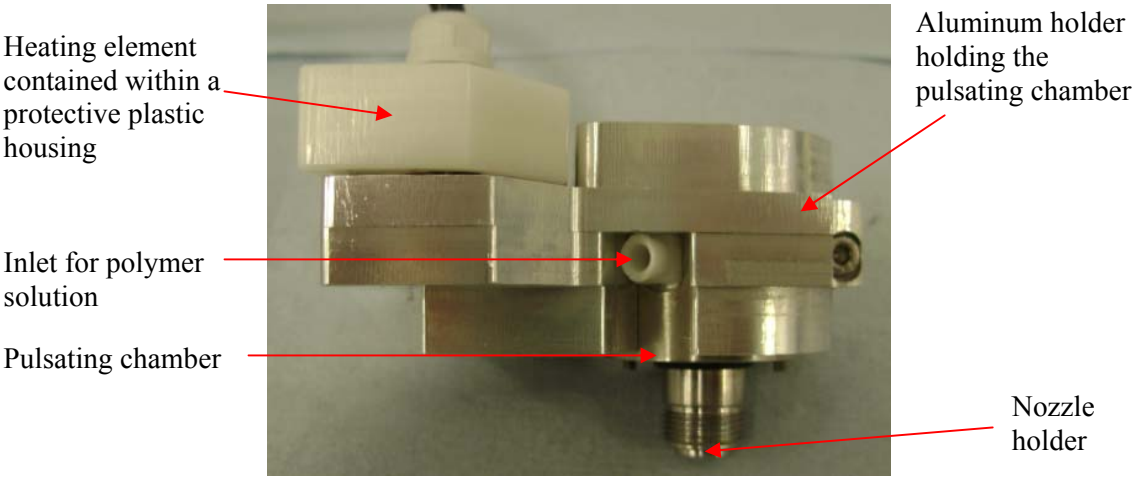


Figure 18: Image of the heating nozzle/pulsating-head device for the Inotech Encapsulator IE-50R. The nozzle holder displayed belongs to a monocentric system.

1.6. Nomenclature

Table 2: List of abbreviations and symbols, which are listed according to their appearance in the text.

Abbreviation/Symbol	Definition	Unit
v	Liquid velocity	m/s
d_d	Droplet diameter	µm
d_n	Nozzle diameter	µm
σ	Surface tension	N/m
g	Acceleration due to gravity	m/s^2
ρ	Fluid density	kg/m^3
d_{cab}	Diameter of Ca-alginate bead	µm
K	Overall correction factor	-
λ_{opt}	Optimal wavelength	µm
d_j	Jet diameter	µm
λ	Wavelength of perturbation	µm
v_j	Jet velocity	m/s
f	Vibrational frequency	1/s
η	Fluid viscosity	kg/ms
We_n	Weber's number	-
F	Liquid flow rate	m^3/s
v_n	Liquid velocity through the nozzle	m/s
d_m	Diameter of microcapsule	µm
d_c	Diameter of core of microcapsule	µm
M_m	Size of microcapsule membrane	µm

M-block (region)	D-mannuronate	-
G-block (region)	L-guluronate	-
PDADMAC	poly(diallydimethylammonium chloride)	-
CIP	Clean in place	-

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Chapter 2

Removal of Pharmaceuticals from Water: Using Liquid-Core Microcapsules as a Novel Approach

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2.1. Abstract

In recent years ever-increasing amounts of pharmaceuticals are being detected in the aquatic environment and in some cases, they have even been discovered in drinking water. Their presence is attributed mainly to the inability of sewage treatment plants to adequately remove these compounds from the sewage influent. The aim of this study was to investigate the feasibility, kinetics and efficiency of using liquid-core microcapsules as a novel methodology, termed capsular perstraction, to remove seven pharmaceuticals commonly found in the environment, from water. The process involves the envelopment of pre-selected organic solvents within a porous hydrogel membrane to form liquid-core microcapsules, which can be used to extract a large range of compounds. Results indicate that this novel approach is capable of extracting the seven chosen compounds rapidly and with a variable efficiency. The simultaneous use of both dibutyl sebacate and oleic acid liquid-core microcapsules at a liquid volume ratio of only 4% (v/v) resulted in the following extractions within 50 min of capsule addition to contaminated water: furosemide 15%; clofibric acid 19%; sulfamethoxazole 22%; carbamazepine 54%; warfarin 80%; metoprolol 90% and diclofenac 100%.

The effects of different agitation rates, microcapsule size and membrane thickness on the rate of mass transfer of warfarin into the liquid-core (dibutyl sebacate) of microcapsules was also examined. Results showed that the main rate-limiting step to mass transfer was due to the stagnant organic film (microcapsule size) within the core of the microcapsules. A volumetric mass transfer coefficient of 2.28×10^{-6} m/s was obtained for the smallest microcapsules, which was nearly 4-fold higher compared to the value (0.6×10^{-6} m/s) obtained for the largest microcapsules used in this study. Even with this resistance liquid-core microcapsules are still capable of the rapid extraction of

the tested compounds and may provide a platform for the safe disposal of the pharmaceuticals after removal.

2.2. Introduction

The presence and fate of pharmaceutically active compounds (PhACs) in ground, surface and drinking waters is now recognized as an emerging environmental issue [1]. This topic has been frequently reported in the last decade with a plethora of studies showing the widespread detection of these compounds in the aquatic environment at significant concentrations [1-4].

Several studies carried-out globally have concluded that treated wastewater from conventional sewage treatment plants (STPs) is one of the main point sources for entry of pharmaceuticals into environmental waters [5-12]. This ingress occurs mainly via human excreta (urine or stool), as either parent molecules or subsequent metabolites [13]. STPs were not designed to specifically remove pharmaceuticals from sewage influent [6] and their elimination in these plants can sometimes depend only on their ability to adsorb to solid particles (activated sludge) or to biodegrade naturally [14]. Pharmaceuticals not readily degraded in STPs are being discharged into receiving waters as changed or unchanged forms, usually forming complex mixtures of many compounds in treated effluents. This results in the contamination of rivers, lakes, estuaries and in some instances ground and drinking water [2].

The ecotoxicological implications of chronic exposure to sub-therapeutic levels of PhACs in the aquatic environment on humans and wildlife to date is yet largely unknown and is the focus of much on-going debate [15]. Pomati et al. (2006) showed how a mixture of thirteen drugs commonly found in the environment at sub-therapeutic

levels inhibited the growth of human embryonic kidney 293 cells (HEK293) by as much as 30% compared to controls [16]. It was concluded that the mixture of drugs at environmental exposure levels inhibited cell proliferation by affecting the physiology and morphology of the HEK293 cells [16].

The inability of primary and secondary methodologies in STPs to remove adequately some or all PhACs present in STPs effluents, has lead to extensive research into developing tertiary methods to remove these compounds. Tertiary methods used to remove pharmaceuticals from water include photocatalysis [17], flocculation [18], ozonation [19], advanced oxidation [20] membrane filtration processes [21] and adsorption by activated carbon [22]. Unfortunately, these methods are used rarely, mainly due to high costs [6] and in most cases are only financially viable when very dilute streams have been pre-concentrated. Processes used to pre-concentrate dilute streams are themselves very expensive, labour intensive and require large amounts of energy [23].

Liquid-liquid extraction is a relatively cheap and robust method which has been used for over a century in the chemical industry for the separation and pre-concentration of compounds and has also been used as an important separation process in water treatment [24,25]. Unfortunately this process is susceptible to several problems, including being unfeasible for large volumes of dilute streams due to the need for an extremely high contact area. However, these obstacles can be overcome by increasing agitation speed and/or the volume of the solvent used, but this can lead to elevated costs and/or the formation of stable emulsions, which are difficult to remove and prevent the re-cycling of the solvent for future use. The extraction solvent may also contaminate the water being treated due to direct contact of the two phases.

As discussed previously these limitations may be overcome by encapsulation of the solvent within a hydrogel membrane [26,27]. This membrane prevents direct contact between the two phases, which stops the formation of stable emulsions and also enables rapid extraction of compounds due to the large interfacial contact area provided by microcapsules. This enables a reduction in solvent volume and agitation speed compared to solvent extraction processes. This novel methodology (termed capsular perstraction) of encapsulating a hydrophobic organic solvent within a hydrogel has been successfully and efficiently demonstrated for the extraction of a range of pesticides and herbicides from aqueous solutions [27]. In this work, the extraction efficiency of the process improved with increasing hydrophobicity of the compounds recovered. Similar results were also observed by Boucher et al. (2008), who showed that the partition coefficient of five compounds between water and the oil-core of naturally occurring microcapsules from rapeseed was linearly correlated with their hydrophobicity [28]. It was also shown that microcapsules not only recover pollutants but also provided a mechanism, which enabled the subsequent bio-degradation of the recovered compounds [29].

In this study, the feasibility, kinetics and efficiency of using liquid-core microcapsules as a novel methodology for the recovery of seven PhACs from aqueous solutions were investigated

2.3. Materials and methods

2.3.1. Chemicals

Methanol, water, ammonium acetate, dichloromethane and dichlorodimethylsilane were of high performance liquid chromatography grade and were all obtained from Sigma-

Aldrich (Dublin, Ireland). Formic acid, calcium chloride, 3-(N-Morpholino) propanesulfonic acid (MOPS), sodium chloride (NaCl) and Tween 80 were obtained from Fluka (Buchs, Switzerland) and were of at least analytical grade. Clofibric acid, sulfamethoxazole, furosemide, warfarin, carbamazepine and metoprolol tartrate salt were all obtained from Sigma-Aldrich (Dublin, Ireland) and were of a purity > 95%. Sodium alginate was purchased from Inotech Biotechnologies (Basel, Switzerland). Dibutyl sebacate (Sebacic acid dibutyl ester, CAS No. 109-43-3) was of analytical grade and obtained from Fluka (Buchs, Switzerland). Oleic acid (*cis*-9-Octadecenoic acid, CAS No. 112-80-1) was of analytical grade and was obtained from Sigma-Aldrich (Dublin, Ireland).

Stock solutions of the pharmaceuticals were prepared in water using methanol as a co-solvent [30] to facilitate the solubility of each drug in the aqueous (water) phase. Initially the drugs were dissolved in methanol and were then added to the water, with a final methanol concentration of 5% (v/v), to obtain the desired concentration of the pharmaceuticals. The compounds were present in the aqueous phase at a concentration of 20 mg/l for all experiments, except diclofenac, which was used at a concentration of 8 mg/l. The presence of methanol at the employed concentration had a negligible affect on the mass transfer. Stocks were filtered under vacuum using 0.2 μm nylon membranes (Sigma, Dublin, Ireland). When salts were used, stocks were prepared in terms of the parent analyte. Standard solutions for calibrations were prepared in methanol.

Alginate stock solutions [2% (w/v)] were prepared by dissolving alginate powder in MOPs buffer [10 mM MOPs, 0.85% (w/v) NaCl pH 7.0] and mixing overnight on a magnetic stirrer at 4 ºC. After solubilisation the alginate solution was filtered using a 0.45 and 0.22 μm cellulose acetate filter membrane (Whatman, Dassel, Germany) under a pressure of 2 bar.

2.3.2. Methods

2.3.2.1. Glassware preparation

Prior to use, all glassware was silanised by rinsing thoroughly with a 10% (v/v) solution of dichlorodimethylsilane in dichloromethane, followed by rinsing twice with dichloromethane and twice with methanol.

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

RP-HPLC was used throughout this work using an Agilent 1100 series unit (Agilent Technologies, USA). The analytes were separated on a reversed-phase C_{18} Luna (2) column, dimensions 150 mm X 4.60 mm X 5 μm with an associated security guard cartridge system (Phenomenex, Cheshire, UK). The mobile phase was a binary gradient mixture of 0.1% (v/v) ammonium acetate, adjusted to pH 5.5 by the addition of formic acid and methanol. The gradient started at 10% (v/v) methanol, maintained isocratically for the first 5 min, thereafter the methanol content was raised linearly to 90% (v/v) within 13 min and was maintained isocratically for 20 min. This enabled complete and separate elution of all pharmaceuticals. The total run time was 30 min and retention time was used for peak identification. Samples (20 μl) were injected onto the column, which was maintained at 25 ºC, using a mobile phase flow rate of 0.8 ml/min and all pharmaceuticals were detected at a fixed wavelength of 270 nm. All samples were diluted in methanol and filtered using 0.20 μm nylon filters (Sigma, Dublin, Ireland) before analysis. Internal and external standards were run to ensure accurate results. The quantification of compounds was based on the external standard method using chromatogram peak areas. For standard calibrations a linear regression (r^2) value of at

least 0.998 was obtained for all pharmaceuticals and a limit of detection of > 0.1 mg/l was achieved for each drug.

2.3.2.3. Preparation of liquid-core microcapsules

Mono-disperse and mono-nuclear microcapsules of different sizes were prepared using the co-extrusion laminar jet break-up technique (Inotech encapsulator IE-50-R, Basel, Switzerland). The encapsulator was fitted with one of two different sized concentric nozzles. The nozzles consisted either of an internal diameter of 200 μm and external diameter of 300 μm or an internal diameter of 400 μm and external diameter of 600 μm. Alginate was supplied to the external nozzle using an air pressure regulation system which enabled flow rates of 10-30 ml/min to be generated using a maximum head pressure of 0.5 bar. The desired flow rate was set using a pressure reduction valve. The organic phase was supplied using a precision syringe pump connected to the inner nozzle to supply the organic phase at flow rates of between 5 and 15 ml/min. Spherical microcapsules were obtained by the application of a set vibrational frequency, with defined amplitude, to the co-extruded liquid jet consisting of alginate and the core solvent. The resulting concentric jet broke up into microcapsules which fell into a magnetically stirred gelling bath 15 cm below the nozzle. The gelling bath consisted of 32 g/l $CaCl_2$, 10 mM MOPS pH 7.0 and 0.1-0.2% (v/v) Tween 80, which was added to reduce the surface tension of the gelation solution. To prevent coalescence of the microcapsules during jet break-up and/or when entering the gelling bath, a high negative charge was induced onto their surface using an electrostatic voltage system which applied an electrical potential of 0-2.15 kV between the nozzle and an electrode, placed directly underneath the nozzle. As microcapsules fell through the electrode, they were deflected from their vertical position resulting in their impact occurring over a

larger area in the gelation solution. This enabled mono-disperse microcapsules with a standard size deviation of less than ± 2.5% to be manufactured. Microcapsules were allowed to harden for at least 30 min to ensure complete gelation and were then washed and filtered using a porous mesh to remove any un-reacted components.

Microcapsules can be described as having an average outer diameter (d_m) consisting of the alginate shell and liquid-core and an average internal diameter (d_{lc}) consisting solely of the liquid-core (Figure 1). Five different sized liquid-core microcapsules containing either dibutyl sebacate or oleic acid were used in this study and are described as follows. Dibutyl sebacate liquid-core microcapsules: (1) d_m 621.01 µm ± 1.53%, d_{lc} 546.4 µm ± 1.07%; (2) d_m 737.36 µm ± 2.39%, d_{lc} 569.91 µm ± 2.45%; (3) d_m 1106.04 µm ± 1.97%, d_{lc} 765.21 µm ± 1.23%; (4) d_m 1535.77 µm ± 2.32%, d_{lc} 1065.08 µm ± 1.99%. Oleic acid liquid-core microcapsules: (5) d_m 682.61 µm ± 1.75%, d_{lc} 502.57 µm ± 1.86%.

2.3.2.4. Measurement of microcapsule size

The size and size distribution of microcapsules were determined using a camera (model DP30BW, Olympus, Japan) attached to a light microscope (model, BX-51, Olympus, Japan) interfaced to a PC operating with Cell^F image analysis software (Olympus, Japan). Up to 100 microcapsules where measured for each batch manufactured using a magnification of 40X and the mean size and standard deviation were determined.

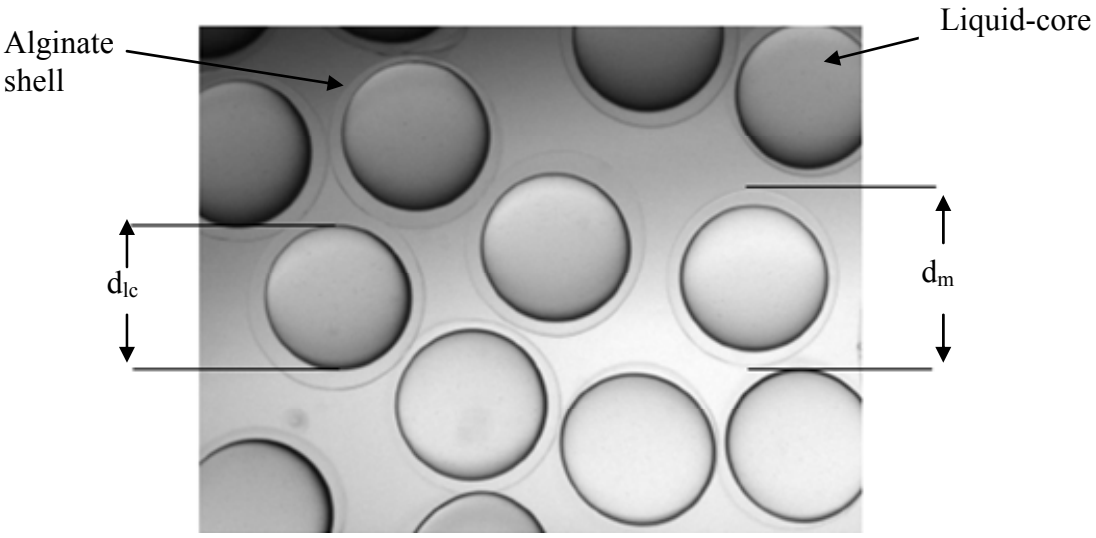


Figure 1: Light microscope image of mono-disperse and mono-nuclear dibutyl sebacate liquid-core microcapsules used in section 2.4.5.1. and 2.4.5.2. to extract all seven pharmaceuticals from water. Capsules displayed have a d_m of 621.01 µm ± 1.53 % and a d_{lc} of 546.4 µm ± 1.07%.

2.3.2.5. Determination of mass transfer

Mass transfer of the pharmaceuticals into the microcapsules and their resistance to mass transfer was calculated using the following simple global mass transfer equation:

$$-\frac{dC_{aq}}{dt} = k_L a\left(C_{aq} - C_{aq}^{e}\right) \qquad (1)$$

In this equation, C_{aq} is the drug concentration in the aqueous phase at time t. $k_L a$ is the overall mass transfer coefficient, which can be estimated from kinetic measurements. C_{aq}^{e} is the concentration of the pharmaceutical in the aqueous phase when equilibrium has been reached and can be determined from the following equation:

$$C_{aq}^{e} = \frac{C_m}{K} \tag{2}$$

where C_m is the drug concentration in the microcapsules. The partition coefficient K defined as the ratio of the pharmaceutical in the microcapsule to the concentration in the aqueous phase at equilibrium and was obtained from equation (3).

$$K = \frac{C_m^e}{C_{aq}^e} \tag{3}$$

where C_m^e is the drug concentration in the microcapsules when equilibrium has been obtained. The concentration of the drug inside the microcapsule at t was obtained from the following mass balance;

$$V_{aq}\left(C_{aq}^{0} - C_{aq}\right) = V_m\left(C_m - C_m^{0}\right) \tag{4}$$

where V_{aq} is the volume of the aqueous phase (bulk liquid) and is calculated by taking into account its dilution by water contained within the membrane of the microcapsules. V_m is the volume of the added microcapsules.

Substituting equations (2) and (4) into (1) results in

$$-\frac{dC_{aq}}{dt} = k_L a \cdot C_{aq} - \frac{k_L a}{K} \cdot \frac{V_{aq}}{V_m}\left(C_{aq}^{0} - C_{aq}\right). \tag{5}$$

Integration of equation (5) yields the following equation;

$$-\frac{1}{1+\frac{V_{aq}}{KV_m}}\ln\left[\frac{\left(1+\frac{V_{aq}}{KV_m}\right)\cdot C_{aq}-\frac{V_{aq}}{KV_m}\cdot C_{aq}^{0}}{C_{aq}^{0}}\right]=k_L at \qquad (6)$$

The volumetric mass transfer coefficient k_L was determined from the graph of equation 6 in which a is the specific interfacial mass transfer area per unit volume between the two phases.

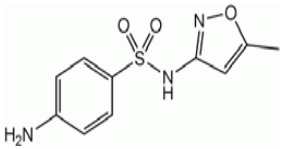
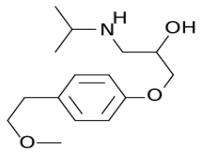
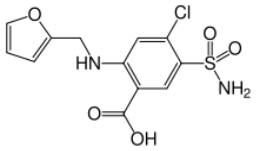
2.3.2.6. Capsular perstraction of pharmaceuticals from aqueous solutions

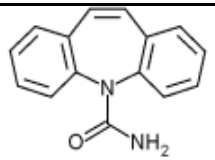
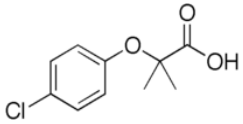
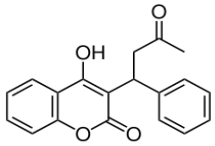
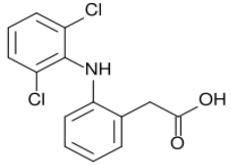
All capsular perstraction experiments were performed at 25 ºC in 100 ml Duran bottles, containing a constant volume of the aqueous phase [100 ml water with dissolved pharmaceutical(s)] and the organic phase (2 ml) within the microcapsules. This enabled a direct comparison of results obtained. Since experiments required five differently sized microcapsules to be added to the aqueous phase during experiments, the volume of the organic solvent was kept constant by adding different amounts of microcapsules using graduated tubes. Microcapsules were added to 100 ml of water containing PhACs and placed in a thermostatted rotary water-bath shaker (Infors AG, Bottmingen, Switzerland), after a sample was taken from the aqueous phase to determine the initial concentration (C_{aq}^{0}) of the pharmaceutical(s). Samples (0.1 ml) were drawn using a graduated 1 ml syringe (Sigma, Dublin, Ireland) and bevelled 23G needle (Microlance, Fraga, Spain) to prevent removal of microcapsules. Sampling from the aqueous phase

was preformed at defined intervals and the samples were analyzed using RP-HPLC to determine the extraction kinetics of the pharmaceuticals into the microcapsules. For all experiments the pharmaceuticals concentration removed due to sampling had a negligible affect on mass transfer. Control experiments containing only aqueous phase and the pharmaceutical(s) were ran for all extraction experiments to ensure that any decline in pharmaceutical concentration was due only to removal by microcapsules and not by degradation.

2.4. Results and discussion

Table 1: Characteristics of PhACs, which were used to help chose the compounds for the work undertaken in this study. Drugs are listed according to increasing hydrophobicity ($LogP_{oct}$).

Compound (CAS No.)	**Therapeutic Class**	**Chemical Structure**	**$LogP_{oct}$**	**Position in sales list[o]**	**% Removal in STPs**	**Conc. in environmental waters (ng/l)**	**Sphere**
Sulfamethoxazole (723-46-6)	Antibiotic (bacteriostatic)		0.89[a]	N.P.	17-71[k]	410[f] 1000[m] 13-45[p]	Ground water Surface water Drinking water
Metoprolol (37350-58-6)	Beta-blocker (receptor)		1.9[b]	66	0-83[n]	2200[j]	Surface water
Furosemide (54-31-9)	Loop diuretic		2.03[c]	8 and 32	8-54[k]	1.72-255[q]	Surface water

Carbamazepine (298-46-4)	Anticonvulsant (antiepileptic)		2.45[b]	77	0[k]-7[h]	1100[f] 1100[h] 30[f]	Ground water Surface water Drinking water
Clofibric acid (-)	Metabolite of three lipid regulators[d]		2.57[b]	N.P.	34-51[k] 50[g] 0-91[n]	4000[f] 175-185[b], 1075[f] 270[f]	Ground water Surface water Drinking water
Warfarin (81-81-2)	Anticoagulant		3.0[e]	9	-	1.0[l]	Surface water
Diclofenac (15307-86-5)	Analgesic/Anti-inflammatory		4.50[b]	21 and 56	0-75[n]	15-489[m]	Surface water

[a][31]; [b][32]; [c][30]; [d][33]; [e][34]; [f][1]; [g][35]; [h][8]; [i][36]; [j][37]; [k][6]; [l][38]; [m][3]; [n][2]; [o](HSE 2004); [p][39] and [q][40]. N.P., Not present. – Not available.

2.4.1. Capsular perstraction

To examine the capability of capsular perstraction to recover pharmaceuticals in this study, seven different compounds (Table 1) were chosen for extraction and selection was based on their varying $LogP_{oct}$ values. Drugs were also pre-selected on their ability to match the following criteria: (1) presence in the top 100 most commonly prescribed medicinal products in Ireland [53]; (2) specific mode of action (different therapeutic classes); (3) inability to be adequately/fully removed by STPs and (4) presence in the aquatic environment. Table 1 highlights the values for the pre-selection criteria.

Dibutyl sebacate and oleic acid were the solvents chosen for individual encapsulation within an alginate hydrogel for extraction experiments. This selection was based on: (1) their very high hydrophobicity ($LogP_{oct}$ = 6.2 and 7.7, respectively), which ensures a negligible diffusion of both solvents through the microcapsule wall; (2) their ability to extract a range of hydrophobic compounds from aqueous environments [26,27,41,42] and (3) their high affinity for some of the drugs (results not shown).

2.4.2. Capsular perstraction of selected pharmaceuticals using dibutyl sebacate liquid-core microcapsules

To assess the efficiency of capsular perstraction using dibutyl sebacate as the core extractant to recover pharmaceuticals from aqueous solutions, microcapsules with a d_m of 737.36 μm and a d_{lc} of 569.91 μm were incubated in water containing all seven pharmaceuticals and agitated at 300 rpm. From Figure 2 it can be seen that both warfarin and diclofenac are rapidly extracted from the aqueous phase, with both reaching an equilibrium concentration after 90 min with over 80% of the original drug extracted. The

higher concentration of each drug extracted compared to the other compounds could be attributed to the higher $LogP_{oct}$ values of these two compounds (3.0 and 4.5, respectively) in comparison to the other five drugs. Their very hydrophobic nature and limited solubility in water, makes the hydrophobic liquid-core of microcapsules a more suitable environment for these compounds [25,43]. Rapid extraction of all other pharmaceuticals was also obtained with equilibrium reached after 10 min for all compounds with 10-20% of initial concentration removed. There is no direct correlation between the amount of these compounds extracted into the organic phase with their $LogP_{oct}$ [44]. This is evident as both sulfamethoxazole and metoprolol have the lowest $LogP_{oct}$ values yet greater quantities are extracted compared to the other three compounds (furosemide, clofibric acid and carbamazepine) in this group. This implies that the partitioning of these drugs is not only based on their hydrophobicity, but might also depend on other factors, such as charged functional groups, steric effects and size. [25,45]. Higher levels of extraction of these compounds can be simply achieved by increasing the number of microcapsules used.

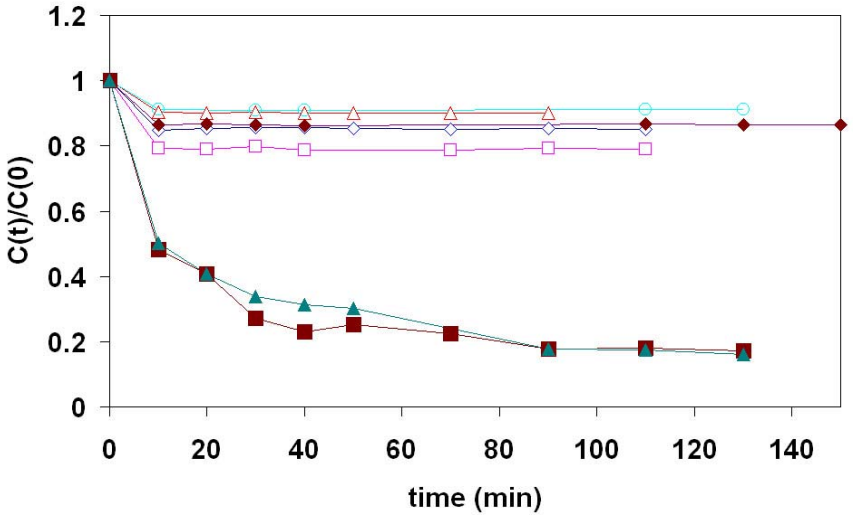


Figure 2: Capsular perstraction of all seven pharmaceuticals from water using dibutyl sebacate liquid-core microcapsules. Symbols: Sulfamethoxazole (open diamonds), metoprolol (open squares), furosemide (open triangles), clofibric acid (open circles), carbamazepine (closed diamonds), warfarin (closed squares) and diclofenac (closed triangle). C(t)/C(0) represents the ratio of the concentration at time t to the initial concentration C(0).

2.4.3. Capsular perstraction of selected pharmaceuticals using oleic acid liquid-core microcapsules

Oleic acid containing liquid-core microcapsules with a d_m of 682.61 µm and a d_{lc} of 502.57 µm were examined for their ability to extract all seven pharmaceuticals from water. The extraction conditions were the same as those for the dibutyl sebacate liquid-core microcapsules, such that results could be compared directly. From Figure 3 it can be seen that metoprolol is rapidly extracted with less than 50% remaining after just 10 min and equilibrium reached after 40 min with only 30% of the initial concentration remaining.

High quantities of diclofenac and carbamazepine are also extracted, 35% and 40% respectively, with equilibrium times of 30 and 50 min. Warfarin is not extracted using these microcapsules, which is in complete contrast to the results obtained with dibutyl sebacate liquid-core microcapsules, where over 80% was extracted (Figure 2). These results highlight the fact that extraction efficiency is not solely based on the hydrophobic characteristics of the compound that is extracted, but may also be dependent on the properties of the extractant, such as functional group, solubility etc. Of the remaining three compounds tested, all are extracted rapidly (equilibrium obtained after 10 min) but only low amounts were removed (between 5 and 10%).

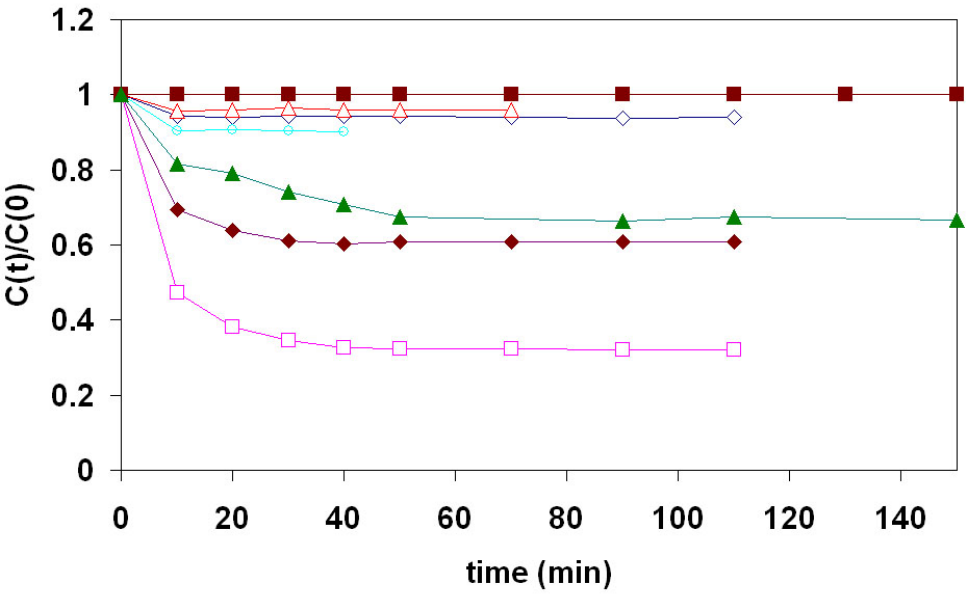


Figure 3: Capsular perstraction of all seven pharmaceuticals from water using oleic acid liquid-core microcapsules. Symbols: Sulfamethoxazole (open diamonds), metoprolol (open squares), furosemide (open triangles), clofibric acid (open circles), carbamazepine (closed diamonds), warfarin (closed squares) and diclofenac (closed triangle). C(t)/C(0) represents the ratio of the concentration at time t to the initial concentration C(0).

2.4.4. Capsular perstraction of selected pharmaceuticals using dibutyl sebacate and oleic acid liquid-core microcapsules simultaneously

There are numerous reports which state that water effluents from STPs can contain a large collection of different PhACs at varying concentrations [8,13,38]. It is envisaged that to be viable, any methodology used to treat waters containing PhACs, must be able to remove high levels of many different types of pharmaceuticals simultaneously. Since oleic acid and dibutyl sebacate liquid-core microcapsules showed different selectivities, it was decided to use both types of microcapsules concurrently in order to establish if effective recovery of all seven pharmaceuticals from water could be achieved. Extraction experiments were performed under the same conditions as those with the individual microcapsules and with a total solvent concentration of 4 ml (2 ml each for both dibutyl sebacate and oleic acid). From Figure 4 it can be seen that diclofenac, metoprolol, warfarin and carbamazepine are rapidly and effectively extracted with respectively around 100, 90, 80 and 50% of the drugs removed at equilibrium, all obtained within 40-50 min. The remaining three drugs, furosemide, clofibric acid and sulfamethoxazole, were rapidly extracted (equilibrium time of 10 min) with between 15 and 22% removed at equilibrium. In many cases the percentage of the pharmaceutical(s) removed using this novel approach is higher compared to recovery of the same drug(s) by conventional STPs, with increases in some instances of 50-100% being obtained (Table 1, data was unavailable for the removal of warfarin).

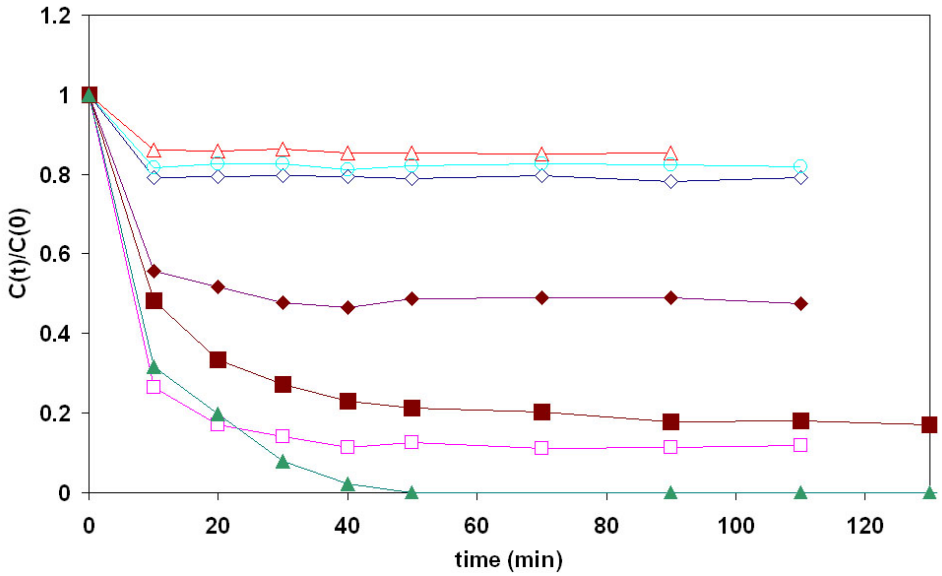


Figure 4: Capsular perstraction of all seven pharmaceuticals from water using a combination of dibutyl sebacate and oleic acid liquid-core microcapsules. Symbols: Sulfamethoxazole (open diamonds), metoprolol (open squares), furosemide (open triangles), clofibric acid (open circles), carbamazepine (closed diamonds), warfarin (closed squares) and diclofenac (closed triangle). C(t)/C(0) represents the ratio of the concentration at time t to the initial concentration C(0).

2.4.5. Effect of varying operating and microcapsule parameters on extraction rate: Factors affecting mass transfer of pharmaceuticals into microcapsules

Microcapsules can display three main resistances to mass transfer (Figure 5) when extracting compounds into the liquid-core [26,27,46]. To determine the dominating mass transfer resistance(s), the following parameters were varied: (1) agitation speed (stagnant aqueous film); (2) membrane thickness (alginate membrane) and (3) size of microcapsule (stagnant organic core). Warfarin was chosen as the test compound and extracted using

microcapsules containing a liquid-core of dibutyl sebacate. The effects of varying parameters on the extraction of warfarin were measured by removing samples from the aqueous phase at defined time intervals, and the effects were calculated using equations 1-6.

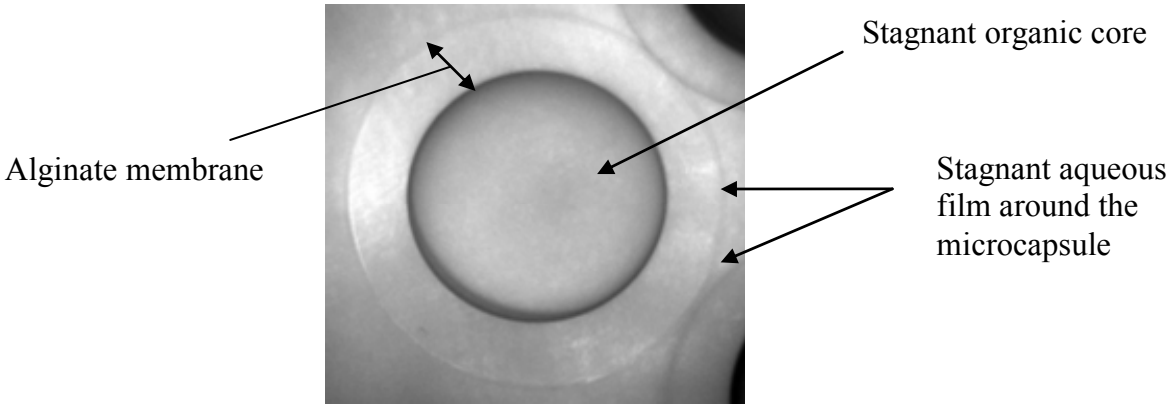


Figure 5: Light microscope image of a single dibutyl sebacate liquid-core microcapsule (d_m of 1106.04 μm and a d_{lc} of 765.21 μm), displaying the three main resistances to mass transfer.

2.4.5.1. Effect of agitation speed on the extraction rate of warfarin

Initially mass transfer takes place through a laminar sub-layer or stagnant aqueous film, which is located immediately adjacent to the interface between the two different phases. It is anticipated that high stirring speeds (which initiate an elevated degree of turbulence), will increase the mass transfer, since eddies will penetrate into the stagnant aqueous film and reduce the width. To show the effects of agitation speed on the extraction of warfarin into the liquid-core of microcapsules, experiments were conducted where equal volumes of capsules with a d_m of 621.01 μm and a d_{lc} of 546.4 μm were placed into aqueous solutions

of warfarin and the bulk liquid mixed at different agitation rates. From Figure 6 it can be seen that for agitation rates at and above 150 rpm, the diffusion rate of warfarin into the liquid-core of microcapsules was constant. It can be deduced that the external (bulk liquid) mass transfer resistance is not the rate-controlling phenomenon provided agitation speeds of ≥ 150 rpm are applied. Similar observations have been reported in [46] and [27].

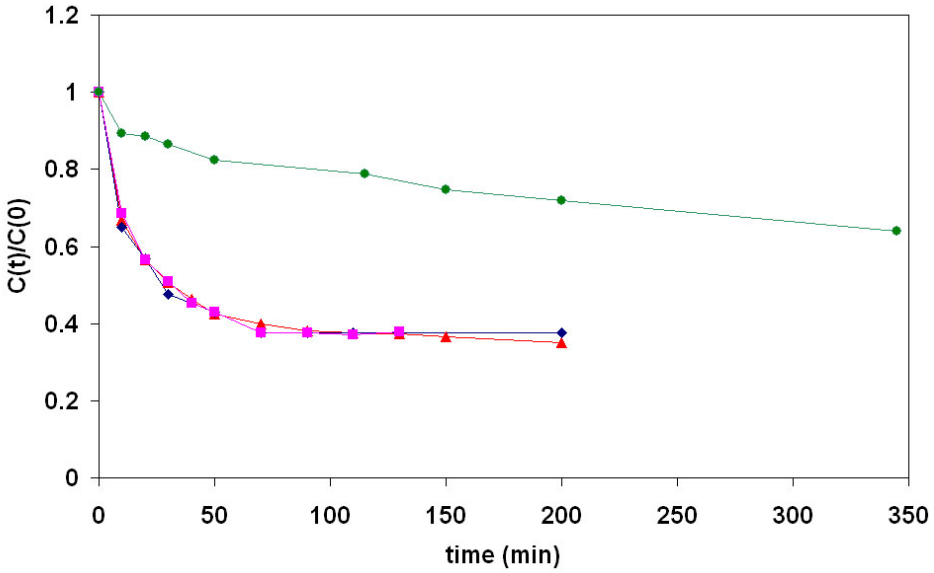


Figure 6: Effect of agitation speed on the extraction rate of warfarin from water into the dibutyl sebacate liquid-core of microcapsules. Symbols: 0 rpm (circles), 150 rpm (triangles), 300 rpm (diamonds) and 400 rpm (squares). C(t)/C(0) represents the ratio of the concentration of warfarin at time t to the initial concentration C(0).

2.4.5.2. Effect of alginate membrane on the extraction rate of warfarin

The hydrogel membrane of the microcapsules used in this study is mainly composed of water and alginate complexed with calcium ions. Due to its hydrophilic nature, negative charge and occurring regions of polymer chains, it was anticipated that these characteristics

might have an affect on the mass transfer of the hydrophobic warfarin into the liquid-core. Previously, numerous authors established that alginate hydrogels can hinder the diffusion of compounds [47-49]. Resistance to mass transfer due to the calcium-alginate in the membrane was observed by Wyss et al. (2004) when hydrophobic methylparathion was extracted from water using a similar type of microcapsule [27]. To test this hypothesis microcapsules with two different membrane thicknesses of 83.72 μm (d_m 737.3578 μm, d_{lc} 569.913 μm) and 37.3 μm (d_m 621.01 μm, d_{lc} 546.4 μm) were manufactured and incubated in aqueous solutions of warfarin, with a constant agitation speed of 150 rpm. As can be seen in Figure 7, the membrane thickness did not appear to affect significantly the transfer of warfarin into the organic phase of microcapsules since warfarin reached an equilibrium concentration after 90-100 min for both types of microcapsule. A similar outcome was reported by Stark (2001) for the extraction of phenylethanol into dibutyl sebacate liquid-core microcapsules, whereby reducing the membrane thickness resulted in a small increase in the extraction rate [46]. It can be therefore concluded that the rate-limiting step for mass transfer is not due to the alginate membrane. Due to the very high structural porosity of calcium alginate gels [50], which possess a large pore size distribution, ranging in size from 5 to 200 nm [51], the diffusion of low molecular weight molecules through this structure is unlikely to be limiting, providing there is no chemical and/or physical interaction with the alginate. Thus, it has been reported that low molecular weight compounds such as glucose, can diffuse relatively unhindered through the gels when low concentrations of alginate are used [52]. Whilst the extraction of warfarin through the thicker membrane was slightly reduced (Table 2), the very porous shell of alginate gels means that it is still possible to extract hydrophobic compounds at a rapid rate.

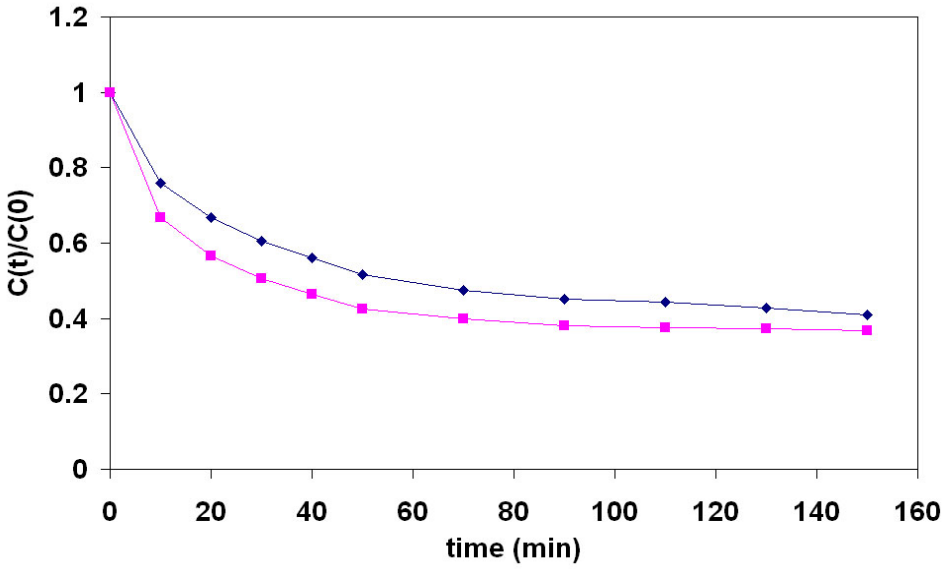


Figure 7: Effect of the alginate membrane on the extraction rate of warfarin from water at an agitation speed of 150 rpm using dibutyl sebacate liquid-core microcapsules. Symbols: membrane thickness 83.72 μm (diamonds) and membrane thickness 37.3 μm (squares). C(t)/C(0) represents the ratio of the concentration of warfarin at time t to the initial concentration C(0).

2.4.5.3. Effect of microcapsule size on the extraction rate of warfarin

Due to the relatively high viscosity of dibutyl sebacate (10 times higher than water) and the absence of turbulence within the microcapsules, diffusion within the liquid-core is assumed to occur mainly by convection. To establish this effect on the mass transfer of warfarin into the liquid-core, microcapsules with different sizes were incubated into aqueous solutions of warfarin and agitated at a constant rate of 150 rpm. The capsules used had a d_m of 737.36 μm, 1106.04 μm and 1535.77 μm and were termed small, medium and large, respectively. From Figure 8 it can be seen that for small microcapsules, equilibrium was reached after 90

min compared to 150-200 min for medium sized microcapsules and greater than 200 min for large microcapsules. These results clearly indicate that the main resistance to mass transfer is located within the stagnant core of dibutyl sebacate. These results are further emphasized by the k_L values obtained for each microcapsule size (Table 2).

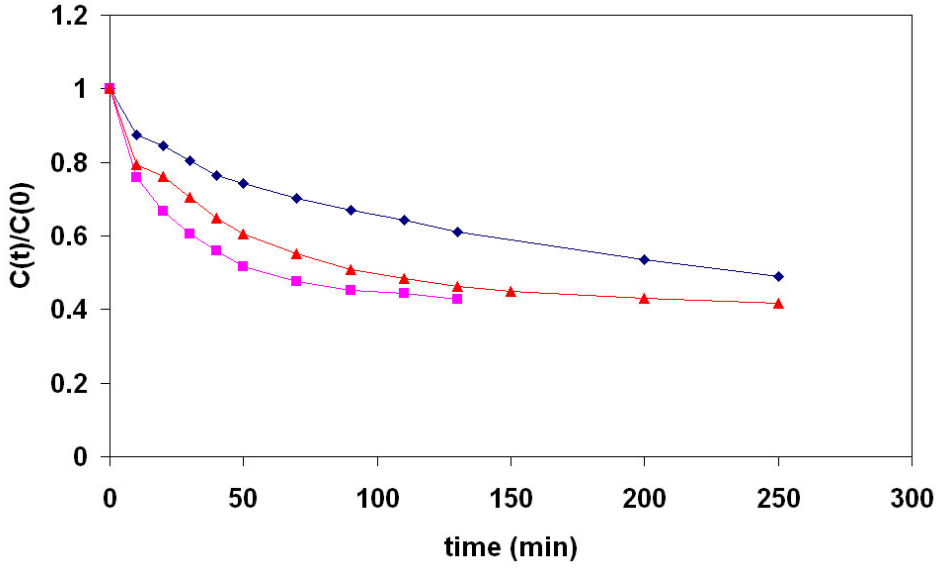


Figure 8: Effect of microcapsule size on the extraction rate of warfarin from water at an agitation speed of 150 rpm. Symbols: large microcapsules (diamonds), medium sized microcapsules (triangles) and small microcapsules (squares). C(t)/C(0) represents the ratio of the concentration of warfarin at time t to the initial concentration C(0).

2.4.5.4. Volumetric mass transfer coefficient (k_L)

Table 2: The k_L values for the extraction of warfarin into the dibutyl sebacate liquid-core of microcapsules. During experiments either agitation speed of the bulk liquid, the membrane thickness or size of microcapsules were varied, whilst all other parameters were kept constant. Values were obtained experimentally (Figures 6-8) and using equations 1-6.

Varied parameter	**Value of varied parameter**	**k_L (10^{-6} m/s)**
Agitation speed (rpm)	150	2.28[1]
	300	2.26[1]
	400	2.3[1]
Membrane thickness (μm)	83.722	2.05
	37.3	2.27
Microcapsule size (r_c μm)	737.3578	2.04
	1106.043	1.2
	1535.777	0.6

All agitation speeds were at 150 rpm, except where specified otherwise.

[1]Microcapsule size: d_m 621.01 μm and d_{lc} 546.4 μm.

Table 2 highlights the k_L values calculated for each parameter varied during capsular perstraction experiments for warfarin removal. As seen in Figure 8, these values demonstrate how the dominant resistance to mass transfer is the stagnant organic core within microcapsules. A k_L value for the smallest microcapsules used (d_m 621.01 μm) of 2.28 x 10^{-6} m/s was determined, which is nearly 4-fold higher compared to that of the largest microcapsules (d_m 1535.77 μm), in which a value of 0.6 x 10^{-6} m/s was obtained. A

graph of microcapsule size versus k_L shows a linear correlation between the two parameters at the values examined in this experimental work (Figure not shown). When extracting phenylethanol, Stark (2001) also observed that the limiting resistance to mass transfer was due to the stagnant organic film within microcapsules [46]. The k_L values obtained in this study are of a similar order of magnitude to the values obtained by [46] and [27]. The slightly reduced mass transfer of warfarin into the microcapsules, due the stagnant organic core did not significantly limit the extraction process. The results obtained show that very high extraction rates can be obtained due to the large interfacial contact area between the microcapsules and the aqueous phase containing the warfarin. This internal mass transfer resistance cannot be overcome, but can be lowered significantly by reducing the size of the capsules as much as possible. In summary, microcapsules with a porous hydrogel shell/membrane material, such as alginate, are capable of the rapid extraction of small molecules even if they possess significant hydrophobic characteristics.

2.5. Conclusions

Adequate treatment of water contaminated with PhACs requires a methodology which has the ability to remove a large amount of different pharmaceuticals, preferably in one single step. In this study this objective was achieved using capsular perstraction as a novel approach and involved using microcapsules consisting of either a liquid-core of dibutyl sebacate or oleic acid. The simultaneous use of these two types of microcapsules resulted in between 15 and 100% of the seven compounds been extracted rapidly (within 50 min of capsule addition) from the aqueous phase. Higher amounts of the drugs can be extracted by

simply increasing the amount of the organic solvent by the addition of more microcapsules to the water, which would also result in an increased rate of extraction.

Capsular perstraction has many advantages over the more traditionally used methods such as liquid-liquid extraction which includes (but not confined to): (1) the ability to perform rapid extractions at low agitation rates; (2) the avoidance of direct contact between the aqueous and organic phases during extractions; (3) simple recovery of microcapsules (solvent) from the water by sedimentation or flotation. The buoyancy of the microcapsules can be predetermined by varying the ratio of the alginate volume, to the volume of the core material in the extruded concentric liquid jet, during the microcapsule production process; (4) as previously shown, the stable microcapsules could be re-cycled for continuous use after the removal of the recovered compound by back-extraction into a separate phase; (5) the ability to control the selectivity of the extracted compounds by incorporating different liquids within the core during the manufacturing process. Most solvents can be encapsulated using the co-extrusion laminar jet break-up technique, provided a difference exists between the surface tension of the membrane polymer and the liquid-core material and (6) the prevention of membrane fouling by the envelopment of liquid-cores with membranes which are not prone to fouling. These advantages will result in more efficient extractions by increasing extraction speeds, reducing the volume of solvent required, preventing stable emulsion formation and contamination of the water being treated by the extraction solvent. Liquid-core microcapsules have also shown the capability to be used as a platform for the safe disposal of the recovered pollutants. These advantages all have a positive affect on the economic viability of the process.

Finally this work also emphasized how the characteristics (capsule size and membrane thickness) of capsules can affect the extraction rate of compounds into the liquid-core, and how the rate of extraction can be controlled by varying these characteristics.

2.6. Nomenclature

Table 3: List of abbreviations and symbols

Abbreviation/Symbol	Definition	Unit
PhACs	Pharmaceutically active compounds	-
STPs	Sewage treatment plants	-
d_m	Outer diameter of microcapsules	μm
d_{lc}	Diameter of liquid-core of microcapsules	μm
C_{aq}	Pharmaceutical conc. in aqueous phase	mg/l
k_L	Volumetric mass transfer coefficient	m/s
k_La	Overall mass transfer coefficient	1/s
a	Specific interfacial mass transfer area per unit volume	1/m
C_{aq}	Pharmaceutical conc. in aqueous phase	mg/l
C_{aq}^{e}	Equilibrium conc. of pharmaceutical in aqueous phase	-
C_m	Pharmaceutical conc. in microcapsules	mg/l
K	Partition coefficient of pharmaceutical between microcapsules and aqueous phase	-

C_m^e	Equilibrium conc. of pharmaceutical in microcapsules	-
V_{aq}	Volume of aqueous phase	l
C_{aq}^0	Initial conc. of pharmaceutical in aqueous phase	mg/l
V_m	Volume of microcapsules	l
C_m^0	Initial conc. of pharmaceutical in microcapsules	mg/l
C(t)	Conc. of pharmaceutical at time t	mg/l
C(0)	Initial conc. of pharmaceutical	mg/l

2.7. Acknowledgements

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Chapter 3

Capsular Perstraction as a Novel Methodology for the Recovery and Purification of Geldanamycin

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Keywords: Geldanamycin; Capsular perstraction; Liquid-core microcapsules; Alginate and In-situ product recovery.

Publication status: Submitted to Biotechnology Progress (2010) under the above title.

3.1. Abstract

The molecular complex 'Heat shock protein 90' has become a novel target for anti-cancer drugs in recent times on account of its ability to perform as a chaperone towards proteins involved in cancer progression. The ansamycin geldanamycin binds to this complex with high affinity and prevents it from performing correctly, which results in tumor destruction.

The aim of this study was to investigate the feasibility of applying liquid-core microcapsules as a novel technique (termed 'capsular perstraction'), for the recovery and purification of geldanamycin from culture media. Results demonstrated how this procedure was capable of rapidly extracting > 70% of geldanamycin from culture media using a liquid-core volume to medium ratio of only 1%. Optimum conditions for removal, including agitation speed, microcapsule size and membrane thickness were examined and it was shown how the stagnant aqueous film around the microcapsules was the main resistance to mass transfer. A volumetric mass transfer coefficient of 5.66 x 10^{-6} m/s was obtained for the highest agitation speed (400 rpm), which was considerable greater compared to the value of 0.88 x 10^{-6} m/s achieved for the lowest speed of 100 rpm.

Removal of geldanamycin from microcapsules was also examined to fully investigate the potential of such particles for in-situ product recovery, and it was demonstrated how the methodology can be used as a simple mechanism for purifying the compound (> 99%) through solvent extraction and crystallization.

The results of this work demonstrate the novel use of capsular perstraction as a methodology for the recovery and purification of geldanamycin from culture environments.

3.2. Introduction

The benzoquinone ansamycin geldanamycin is a naturally occurring antibiotic produced as a secondary metabolite by the actinomycete *Streptomyces (S.) hygroscopicus* var. *geldanus* [1]. It was discovered in the culture filtrates of a submerged fermentation in 1970 [2] and has a broad spectrum, exhibiting activity against Gram positive and negative bacteria [3], protozoa and fungi [2]. Interest in the molecule was stimulated as a result of the discovery of its antitumor properties [4]. Whitesell and coworkers showed in 1994 that one of the principal cellular targets for geldanamycin is the Heat shock protein 90 (Hsp90) [5], an ATP-dependent chaperone complex found in eukaryotes [6-9], which is expressed at elevated levels (2-10 fold higher) in tumor cells compared to normal tissue [10,11]. This chaperone is necessary for the folding, assembly and activity of multiple mutated and over expressed signaling proteins, which initiate and promote growth and/or survival of tumor cells [12]. Geldanamycin binds with high affinity to the *N*-terminal domain ATP binding site of Hsp90 [13], causing the loss of its chaperone capabilities towards client proteins [14], subsequently leaving them malformed, unstable and readily degradable by the cells proteosome, which results in tumor destruction [13].

Unfortunately, geldanamycin cannot be used clinically for targeted cell-therapy as it has shown significant hepatotoxicity in animal models during clinical trials [15]. However, semi-synthetic derivates of the antibiotic such as, 17-allyamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG) were found to be less potent and toxic compared to the parent molecule whilst retaining similar molecular activities [14,16,17]. These novel antitumor agents are presently in clinical trials with 17-AAG being tested on patients

with advanced-stage cancer [12]. At present, the future clinical success of geldanamycin as a novel chemotherapeutic agent will be dependent on chemically altered derivates of the drug [18] with large quantities of natural geldanamycin required for synthesis to take place.

To date, only limited research has been published on the fate and behavior of geldanamycin during production in submerged fermentations [18-21]. Recently it was shown how the in-situ removal of the compound from cultures of *S. hygroscopicus* cultivated in Bennett's media, resulted in a 3-fold increase in production titers [19]. It has been suggested that this increased quantity was due to removal of the antibiotic from a hostile fermentation environment which caused product degradation [19]. Indeed, geldanamycin concentrations rapidly declined to negligible amounts in cultures, and it has been postulated that this is due to either chemical and/or enzymatic mechanisms [22]. While neither study discovered the defining reasons behind the breakdown during fermentations, they did clearly emphasis the importance of in-situ recovery of the compound from the culture environment.

In recent years a novel and very effective process termed 'capsular perstraction' has been developed and has shown the ability to recover successfully, a large range of compounds from a diverse range of aqueous environments, whilst also being able to provide a protective environment for the extracted component. The procedure involves the envelopment of organic solvents within a porous hydrogel membrane to form liquid-core microcapsules [23]. This novel technique has being applied to a number of areas, including: water treatment [23-25], enzyme technology [26,27], Bioprocessing [28] and cell culture [29,30]. Capsular perstraction has shown many advantages over traditionally used recovery methods, such as liquid-liquid extraction, including the ability to perform rapid extractions at low agitation rates due to the large interfacial

contact area provided by the capsules themselves. This requires the use of less solvent and energy, which is not only environmentally beneficial but also improves the overall economic prospects of the process. Another benefit is the avoidance of direct contact between the aqueous and organic phase, which prevents contamination, as well as averting the formation of stable emulsions, which present a difficult downstream processing challenge [31].

The aim of this study was to examine the possibility of employing liquid-core microcapsules as a novel technique for the recovery and purification of geldanamycin from culture media. Initial experiments involved the selection of a suitable solvent to recover the antibiotic; followed by encapsulation within a porous hydrogel membrane. Capsular perstraction experiments were then undertaken to examine the factors affecting the uptake of geldanamycin into the liquid-core. Finally the recovery and purification (crystallization) of the compound from the microcapsules was investigated.

3.3. Materials and Methods

3.3.1. Chemicals

Geldanamycin microcrystalline powder (> 99% purity) was obtained from LC Laboratories (Massachusetts, USA). Water, methanol, acetonitrile and dichloromethane were of high performance liquid chromatography grade and were all obtained from Sigma-Aldrich (Dublin, Ireland). Dibutyl sebacate, oleic acid, calcium chloride, 3-(N-Morpholino)propanesulfonic acid (MOPS), Tween 80, 1-octanol, hexane, N-Z amine A from bovine milk and glucose monohydrate were all obtained from Sigma-Aldrich (Dublin, Ireland) and were of at least analytical grade. Yeast extract and beef extract were obtained from Oxoid (Basingstoke, UK). Sodium alginate (Keltone LV) was

obtained from Inotech Biotechnologies (Basel, Switzerland). Miglyol 812 N was a kind gift from Sasol (Witten, Germany).

Two previous studies[20,32] had successfully used the following liquid medium composition (termed Bennett's medium) to produce and recover geldanamycin from cultures using the organism *S. hygroscopicus* var. *geldanus*: 1 g/l yeast extract, 1g/l beef extract, 2 g/l N-Z-amine A and 50 g/l glucose monohydrate which was autoclaved separately from the other components. The media had a pre-sterilization pH of 7.0 and was used throughout this study as the aqueous phase to closely mimic the fermentation conditions.

Stock solutions of geldanamycin in Bennett's media and water were prepared using acetonitrile as a co-solvent to facilitate the solubilisation of 10 mg/l of geldanamycin in the aqueous phase. Firstly geldanamycin was dissolved in acetonitrile by agitation on a magnetic stirrer, and added to the media to a final acetonitrile concentration of 5% (v/v) in order to obtain the desired concentration of the compound. Standard solutions of geldanamycin for calibrations were prepared in 100% acetonitrile. Due to the reported photolytic nature of geldanamycin, all solutions and experiments were stored and performed in the dark to maintain maximum stability.

3.3.2. Methods

3.3.2.1. Quantitative determination of geldanamycin

Reverse phase high performance liquid chromatography (RP-HPLC) was employed throughout this work using an Agilent 1100 HPLC series (Agilent Technologies, USA) consisting of a quaternary pump with online degasser, thermostatted auto-sampler, diode array detector (DAD) and thermostatted column compartment. All modules were interfaced to a PC operating with ChemStation software, systems version A.08.03. The

analytes were separated on a reversed-phase C_{18} Luna (2) column, dimensions 150 mm x 4.60 mm x 5 μm, with associated security guard cartridge system (Phenomenex Cheshire, UK). Column temperature was maintained at 25 ^{o}C. Isocratic elution was employed using a mobile phase flow rate of 1 ml/min. For the detection of geldanamycin in dibutyl sebacate, oleic acid and methanol, a mobile phase of 80% methanol:20% water (v/v) was applied. For it's detection in Bennett's medium, water and acetonitrile, a mobile phase of 50% acetonitrile:50% water (v/v) was used. All samples were filtered with a 0.20 μm nylon filter (Sigma, Dublin, Ireland) and degassed prior to HPLC separation and detection. Samples (50 μl) were then injected onto the column after dilution with their respective mobile phase and detected at a fixed wavelength of 308 nm. Internal and external standards were used to ensure accurate results were obtained. The quantification of geldanamycin was based on the ratio of the chromatogram peak areas.

3.3.2.2. Preparation and characterization of liquid-core microcapsules

The full details of the procedure to manufacture and measure the size of the microcapsules used in this study have been described previously [23]. All capsules used in this work were manufactured from solutions contain 2% (w/v) alginate, and these particles can be described as having an average diameter (d_m) consisting of the alginate shell and liquid-core, and an average internal diameter (d_{lc}) consisting solely of the liquid-core (Figure 4a).

3.3.2.3. Liquid-liquid extraction and capsular perstraction

All liquid-liquid extraction and capsular perstraction experiments were carried out in 100 ml Duran bottles containing a constant volume of aqueous phase (100 ml Bennett's

medium containing solubilized geldanamycin) and one of the organic solvents (1 ml), to allow experiments to be compared. The organic solvents were either added directly to the aqueous phase (liquid-liquid extraction) or enclosed within the microcapsules (capsular perstraction). Since capsular perstraction experiments required a number of different sized microcapsules to be added during experiments, the 1 ml volume of organic solvent was kept constant by adding different amounts of capsules using graduated tubes. Before addition, microcapsules were filtered through a porous mesh (pore size 100-200 μm) to remove any water present. Duran bottles were then placed into a thermostatted incubator shaker (Innova 43, Brunswick Scientific, New Jersey, USA) at 25 ºC and a sample taken immediately from the aqueous phase to determine the initial concentration $\left(C_{aq}^{o}\right)$ of the antibiotic. Geldanamycin extraction was determined by removal of samples from the medium at defined intervals using a syringe and 22 G needles with non-coring deflected tip (Foss, Dublin, Ireland). Such a procedure avoided the removal of organic solvent and/or microcapsules. For liquid-liquid extraction experiments, samples were centrifuged at 25 ºC before sample removal to ensure complete separation of the phases. Quantification of the antibiotic was performed using RP-HPLC. Control experiments were carried out to ensure that any decline in concentration was due to removal by the organic solvent and not by degradation.

For liquid-liquid extraction experiments, the partition coefficients $\left(K_{org/aq}\right)$ for the distribution of geldanamycin between one of the organic solvents and the aqueous phase (Bennett's medium) was calculated using equation 1.

$$K_{org/aq} = \frac{C^e_{org}}{C^e_{aq}} \quad (1)$$

where, C^e_{org} is the concentration of geldanamycin in the organic phase and C^e_{aq} is the concentration in the aqueous phase at equilibrium.

3.3.2.4. Determination of mass transfer

Mass transfer of geldanamycin into the microcapsules and their resistance to mass transfer was calculated using a simple global mass transfer equation (equation 2) as previously described [23].

$$\frac{dC_{aq}}{dt} = k_L a\left(C_{aq} - C^e_{aq}\right) \quad (2)$$

In this equation C_{aq} is the geldanamycin concentration in the aqueous phase at time t, k_L is the volumetric mass transfer coefficient, which is calculated by taking into account a, the specific interfacial mass transfer area per unit volume between the two phases.

The concentration of the antibiotic inside the microcapsules at time t was obtained from the following mass balance:

$$V_{aq}\left(C^0_{aq} - C_{aq}\right) = V_m\left(C_m - C^0_m\right) \quad (3)$$

in which V_{aq} is the volume of the aqueous phase (bulk liquid) and is calculated by taking into account its dilution with water contained within the membrane of capsules. C^0_{aq} is

the initial concentration of geldanamycin in the aqueous phase, V_m is the volume of microcapsules, C_m is the concentration of geldanamycin in the microcapsules at time t, whilst C_m^0 is the initial concentration in the capsules.

3.3.2.5. Recovery of geldanamycin from microcapsules

Geldanamycin extracted into dibutyl sebacate or oleic acid liquid-core of microcapsules was recovered (back-extracted) by washing the capsules with acetonitrile, methanol or acetonitrile saturated with oleic acid (back-extraction solutions) at 25 ºC. Saturation of acetonitrile with oleic acid was performed by mixing both liquids together for 24 hr in a thermostatted shaker. After agitation, both phases were allowed to settle for at least 24 hr before use. Firstly capsules were removed from Bennett's media using a porous mesh and washed three times in ultra pure water to remove any remaining media components. Capsules containing the organic solvent and geldanamycin within their core were then added to acetonitrile, methanol or acetonitrile saturated with oleic acid. Solutions were agitated at 300 rpm for 30 min to reach equilibrium. To ensure complete recovery of the compound, additional washing steps were performed until no more geldanamycin could be detected. When back-extracting the antibiotic from the oleic acid liquid-core using acetonitrile saturated with oleic acid, equation 4 was employed to calculate the partition coefficient $K_{acn/oa}$ for the distribution of geldanamycin between the two phases.

$$K_{acn/oa} = \frac{C_{acn}^e}{C_{oa}^e} \qquad (4)$$

where, C_{acn}^e is the concentration of geldanamycin in acetonitrile saturated with oleic

acid at equilibrium and C_{oa}^{e} is the concentration of geldanamycin in the oleic acid phase when equilibrium was obtained.

To examine the effect of back-extraction on capsules, photographs of the particles before and after the washing steps were taken on a camera (model DP30BW, Olympus, Japan) attached to a light microscope (model BX-51, Olympus, Japan) interfaced to a PC operating with Cell^{F} image analysis software (Olympus, Japan).

3.3.2.6. Re-crystallization of geldanamycin

Geldanamycin contained within the oleic acid saturated acetonitrile was re-crystallized using the following procedure. The back-extraction solution was separated from the capsules by passing through a porous mesh and the fatty acid was removed by adding equal volumes of water to the solution. This addition caused the precipitation of oleic acid, which formed an emulsion and was separated by centrifugation at 4000 rpm for 30 min at 25 ºC. The water addition and centrifugation steps were repeated twice, prior to filtration through a 0.20 μm nylon filter to ensure all oleic acid had been removed, as determined by RP-HPLC analysis. The solution was placed in a graduated glass beaker in a water bath set at 82.5 ºC ± 2.5 ºC to enable evaporation of acetonitrile. During evaporation, the solution was agitated slowly to facilitate nucleation and subsequent crystal growth. When no visible change in volume was observed, the temperature was increased to 100 ºC ± 2.5 ºC and evaporation continued until approximately 10% of the original volume was left. After boiling, the solution was immediately cooled to 4 ºC, which further reduced the solubility of geldanamycin in the suspension. Crystals were collected by filtrating under vacuum using 0.2 μm nylon filters and drying overnight at 30 ºC.

3.3.2.7. Purity of re-crystallized geldanamycin

The purity of the crystals was determined using either RP-HPLC or by measuring their melting point and comparing the results to those obtained for high purity commercial geldanamycin. For RP-HPLC, crystals were tested by re-dissolving in acetonitrile. Re-crystallization did not affect the retention time of the antibiotic during the HPLC analysis. The melting point of re-crystallized and pure geldanamycin was established using a procedure outlined by the OECD guidelines for the testing of chemicals [37]. The melting point of the drug was determined by placing the samples in a Gallenkamp melting point apparatus (in capillary tubes) and measuring their melting temperature with a thermocouple thermometer 2022T (Digitron, UK).

3.4. Results and Discussion

3.4.1. Solvent Screening: Choice of solvent for liquid-core of microcapsules

Liquid-liquid extraction was carried out to determine the optimal solvent for the liquid-core of microcapsules. For extractions, six different organic solvents were added individually to the media containing the geldanamycin and the two phases were agitated at 25 ºC for 24 hr at a speed of 150 rpm, which ensured equilibrium was obtained and the partition coefficients were determined. A high partition coefficient for geldanamycin extraction between the phases is significant for the success of an extractive fermentation [33], as it results in reduced solvent usage, whilst also enabling high concentrations of the product to accumulate within the organic phase. The solvents chosen were oleic acid, dibutyl sebacate, miglyol, dichloromethane, octanol and hexane. Selection was based on their capabilities to match the following criteria: (1) A relatively high $LogP_{oct}$ value (Table 1). This ensures a negligible diffusion of the solvent through the capsule

wall into the aqueous media phase when equilibrium is obtained. (2) The ability to be encapsulated within an alginate hydrogel membrane using the co-extrusion jet breakup technique. (3) To be stable under fermentation conditions, and (4) the ability to detect geldanamycin in the solvents using RP-HPLC. All solvents had also previously shown the ability to extract compounds with similar $LogP_{oct}$ to geldanamycin ($LogP_{oct}$ of geldanamycin = 2.54[34]). Table 1 displays the $K_{org/aq}$ values for geldanamycin between the media phase and the six pre-selected solvents. From the results it can be seen that both dibutyl sebacate and oleic acid extract the largest amounts of geldanamycin compared to the other solvents under examination, with values of 192.20 ± 2.03% and 212.33 ± 7.08% obtained respectively. This higher amount extracted could be due to the considerably higher $LogP_{oct}$ values possessed by these liquids compared to the other solvents.

Table 1: The partition coefficient values of geldanamycin between Bennett's medium and one of six pre-selected organic solvents.

Organic solvent	**$LogP_{oct}$ of solvent**	$K_{org/aq}$
Miglyol	N.A.	54.88 ± 0.911%
Dichloromethane	1.25[35]	179 ± 1.56%
Octanol	3.07[35]	51 ± 5.68%
Hexane	3.90[35]	15.85 ± 1.95%
Dibutyl sebacate	6.2[33]	192.20 ± 2.03%
Oleic acid	7.7[33]	212.33 ± 7.08%

Solvents are listed according to increasing hydrophobicity ($LogP_{oct}$).

N.A., not available.

3.4.2. Capsular perstraction of geldanamycin: Factors affecting mass transfer

Both dibutyl sebacate and oleic acid liquid-core microcapsules have been previously used for the successful removal of hydrophobic compounds from aqueous environments [23,24,26,28]. Although both solvents showed similar affinities for the antibiotic, dibutyl sebacate was firstly encapsulated within an alginate membrane for capsular perstraction experiments. This selection was based on the higher encapsulation affinity obtained when dibutyl sebacate was used during the encapsulation process in comparison to oleic acid (results not shown). This is attributed to the higher viscosity of the fatty acid, which makes incorporation within a hydrogel membrane more challenging [27].

As discussed previously [23,24,33], the mass transfer of compounds into the liquid-core of microcapsules can be a function of different mass transfer resistances exhibited by the capsules, and these resistances are described as follows: (1) The stagnant aqueous film around the microcapsules. (2) The resistance due to the charged hydrophilic membrane, and (3) the stagnant organic layer within the capsules. In order to establish the effect of these impediments on geldanamycin diffusion, experiments were undertaken in which agitation speed of the bulk liquid, membrane thickness and the size of the microcapsules were varied. The effects on mass transfer were measured by removing samples from the aqueous phase at defined time intervals and calculating the k_L for each varied parameter using equations 2 and 3.

3.4.3. Effect of agitation speed on the extraction rate of geldanamycin

The effect of agitation speed on the extraction rate of geldanamycin into the dibutyl sebacate liquid-core was examined by performing experiments which involved adding equal volumes of capsules with a d_m of 616.92 μm and a d_{lc} of 551.81 into solutions of

Bennett's media containing the antibiotic. The bulk liquid was mixed at agitation rates of 100, 200, 300 and 400 rpm at 25 ^{o}C and the rate of extraction measured. From Figure 1 it is apparent that increased agitation speeds result in higher removal speeds of geldanamycin. Equilibrium between the two phases was reached after 30, 40 and 50 min for speeds of 400, 300 and 200 rpm respectively, compared to > 100 min for an agitation speed of 100 rpm.

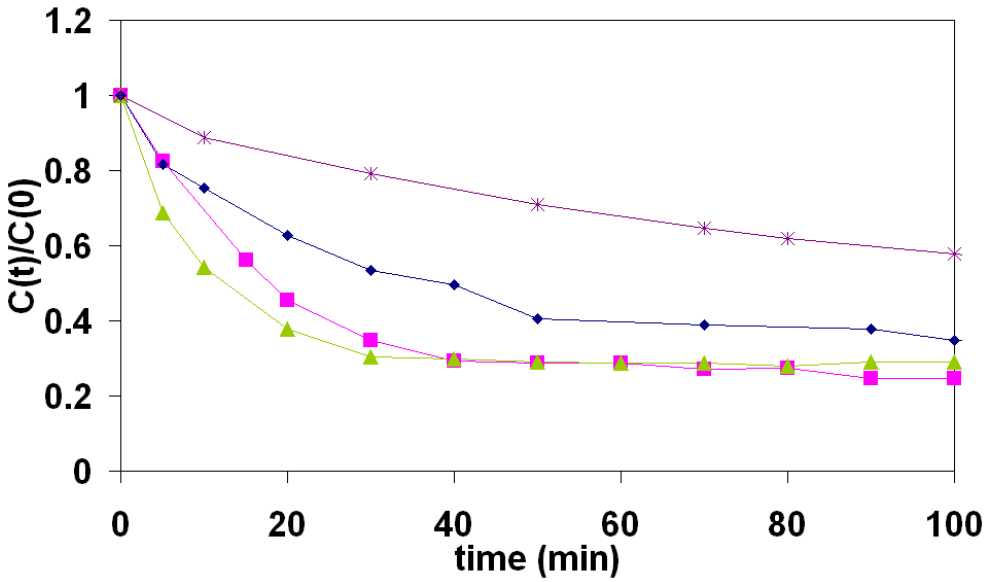

Figure 1: Effect of different agitation speeds on the extraction of geldanamycin from Bennett's media into dibutyl sebacate liquid-core of microcapsules. Symbols: 100 rpm (stars), 200 rpm (diamonds), 300 rpm (squares) and 400 rpm (triangles). C(t)/C(0) represents the ratio of the concentration of geldanamycin at time t to the initial concentration C(0).

3.4.4. Effect of capsule size on the extraction rate of geldanamycin

Previously it has been shown that the diffusion of hydrophobic warfarin [23], phenylethanol [33] and methylparathion [24] into the core was dependent on the size of the microcapsules used in the respective studies. Due to the relatively high viscosity of

dibutyl sebacate and the absence of turbulence within the microcapsules, it can be assumed that movement in the core occurs mainly through convection. To determine the resistance exhibited by the stagnant organic film, equal volumes of capsules with different sizes and a similar membrane thickness were placed into solutions of Bennett's media containing geldanamycin and the bulk liquid agitated at a rate of 300 rpm. The capsules used had a d_m of 484.81 µm, 598.28 µm and 751.125 µm and were termed small, medium and large respectively. Figure 2 shows how equilibrium concentration for all sizes is reached within 50 min of agitation commencing. It can be concluded that the size of the capsules had little or no affect on the extraction rate of the compound into the liquid-core, at least for the range of sizes studied in this work.

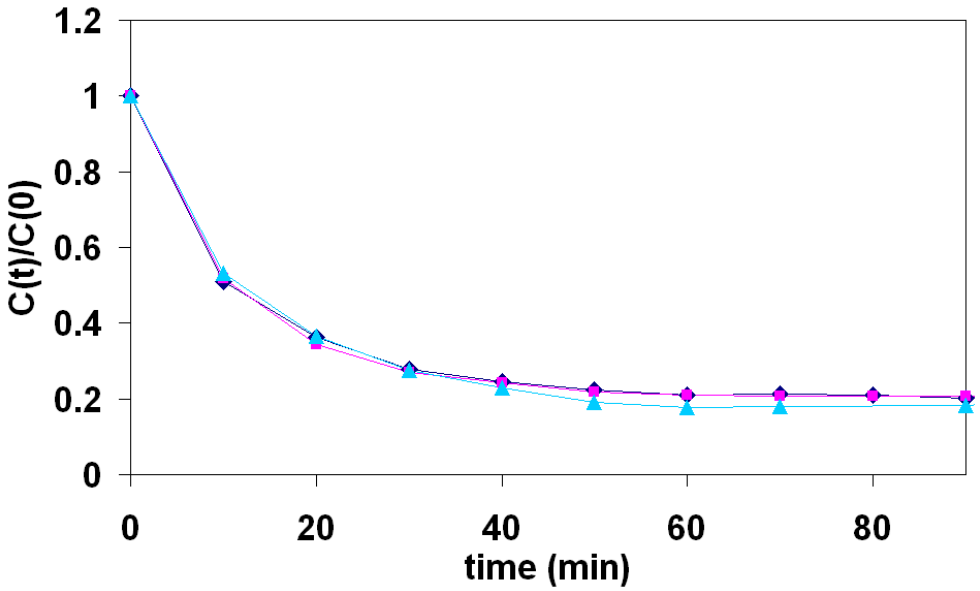

Figure 2: Effect of capsule size on the extraction of geldanamycin from Bennett's media into the dibutyl sebacate liquid-core of microcapsules at an agitation speed of 300 rpm. Symbols: small microcapsules (diamonds); medium sized microcapsules (squares) and large microcapsules (triangles). C(t)/C(0) represents the ratio of the concentration of geldanamycin at time t to the initial concentration C(0).

3.4.5. Effect of alginate membrane on the extraction rate of geldanamycin

To determine the resistance to mass transfer exhibited by the alginate membrane of microcapsules, experiments were performed in which capsules of a similar liquid-core diameter but with varying membrane sizes were placed into the media containing geldanamycin and the bulk liquid agitated at 300 rpm. The capsules tested had three different membrane sizes of 32.55 µm (d_m 616.92 µm, d_{lc} 551.81 µm), 49.143 µm (d_m 648.39 µm, d_{lc} 550.104 µm), and 91.25 µm (d_m 688.88 µm, d_{lc} 506.38 µm) respectively. As seen in Figure 3 the increasing thickness of the membrane appears to only slightly limit the diffusion of the antibiotic into the core. Equilibrium times of around 40, 50 and 60 min were obtained for membrane thickness of 32.55, 49.14 and 91.25 µm respectively. Similar results were observed in two previous studies, which also showed how the hydrophilic membrane had a negligible effect on the diffusion of small hydrophobic compounds into the liquid-core [23,33]. Although the movement of geldanamycin is slightly reduced when moving through thicker membranes, it is still possible to obtain rapid extraction of the compound.

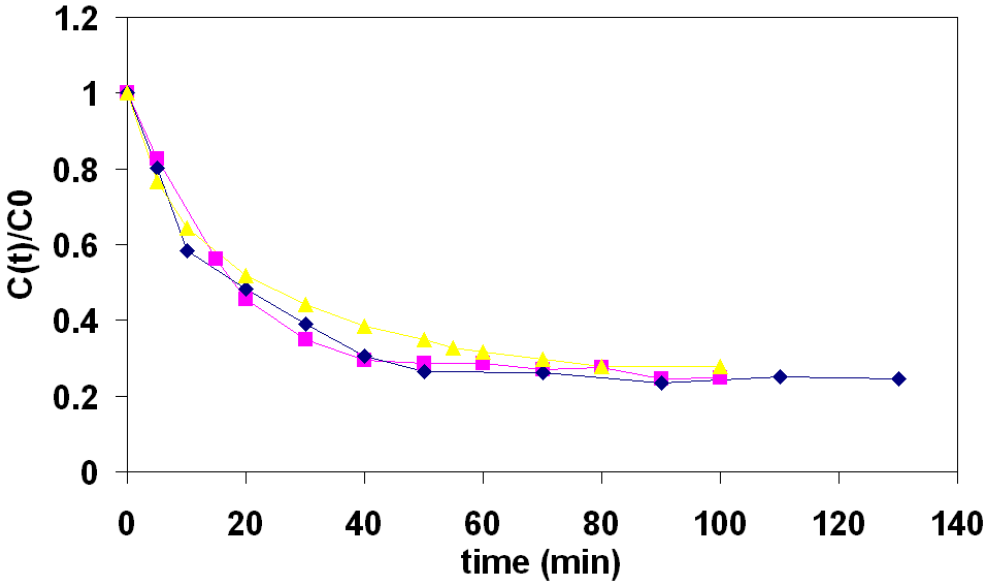

Figure 3: Effect of alginate membrane on the extraction of geldanamycin from Bennett's media into the dibutyl sebacate liquid-core of microcapsules at an agitation speed of 300 rpm. Symbols: membrane thickness 32.55 µm (squares), membrane thickness 49.14 µm (diamonds) and membrane thickness 91.25 µm (triangles). C(t)/C(0) represents the ratio of the concentration of geldanamycin at time t to the initial concentration C(0).

3.4.6. Volumetric mass transfer coefficient

The k_L for the diffusion of geldanamycin into the dibutyl sebacate liquid-core of microcapsules was calculated for each parameter varied during this study and the values obtained are presented in Table 2. As seen in Figure 1, these values demonstrate that the main rate limitation to mass transfer is the resistance within the external bulk liquid phase (agitation speed). There is over a 6-fold difference between the values obtained for the lowest (100 rpm) and highest (400 rpm) agitation speeds, with values for k_L of 0.88 and 5.66 10^{-6} m/s respectively. This reduction could be due to the fact that

diffusion of hydrophobic geldanamycin in the media originally takes place through a stagnant aqueous film, which is found adjacent to the interface between the two different phases. Higher stirring speeds (high turbulence) increase the mass transfer, since eddies will penetrate into the film and reduce its width, hence increasing the extraction rate. Results of agitation speed versus k_L show that both parameters are linearly correlated for the values examined within this work (Figure not shown). It is apparent that the movement of the antibiotic into the liquid-core is also slightly hindered by increases in the thickness of the capsular membrane and decrease in capsule size. Wyss et al. (2004) showed how the alginate membrane can affect the diffusion of hydrophobic compounds into microcapsules when the transfer of methylparathion was examined using a similar type of capsule, and was attributed to the hydrophilic nature of the hydrogel itself. Whilst the membrane can have an impact on the overall mass transfer of geldanamycin, it is nevertheless possible to control this resistance by reducing the membrane size during the microcapsule production process [23].

The results indicate that the external resistance controls the rate of geldanamycin diffusion through the bulk liquid. However, provided this is agitated at sufficiently high rates ≥ 200 rpm (turbulent conditions); rapid extraction rates can still be achieved for geldanamycin recovery from fermentation environments.

Table 2: The k_L values determined for the extraction of geldanamycin from Bennett's media into the dibutyl sebacate liquid-core of microcapsules.

Varied parameter	Value of varied parameter	k_L (10^{-6} m/s)
Agitation speed (rpm)	100	0.88[a]
	200	1.77[a]
	300	4.33[a]
	400	5.66
Membrane thickness (μm)	32.55	4.33
	49.144	4.26
	91.25	3.33
Microcapsule size (d_m μm)	484.815	3.033
	598.279	4.14
	751.25	5.14

Agitation was carried out at 300 rpm, unless specified otherwise.

[a]Size of microcapsules: d_m 616.92 μm and d_{lc} 551.81 μm.

3.4.7. Recovery and purification of geldanamycin contained within liquid-core microcapsules

The successful application of capsular perstraction as a method for the recovery of geldanamycin from culture environments depends not only on the rapid and efficient extraction of the drug, but also on the feasibility to recover large amounts of the compound in a purified stated from the capsules, once extraction is complete. Ideally this technique should be a simple and rapid process, which is not detrimental to the recovered product, and which can be readily integrated into the downstream processing procedure for the compound of interest.

Due to its simplicity, the recovery of geldanamycin from dibutyl sebacate liquid-core microcapsules using solvent extraction was studied. The solvents acetonitrile and methanol were selected based on the following criteria: (1) Both were constituent solvents of the mobile phase used in RP-HPLC for the detection of geldanamycin. This enables the direct quantification of geldanamycin removed from the capsules without the need for a pre-treatment step (i.e. solvent evaporation), which prevents the loss of the product and also reduces process time. (2) Geldanamycin is soluble in both acetonitrile and methanol. (3) The compound is stable when stored in both solvents ≥ -20 ^{o}C for long periods of time (> 1 year). (4) Both solvents are relatively volatile (boiling point of 82 and 64.5 ^{o}C for acetonitrile and methanol respectively), which allows evaporation (distillation) of the solvents to be carried out at low temperatures for final product purification, and (5) the calcium-alginate membrane of the microcapsules is stable when immersed in both liquids. These hydrogels have proven to be very durable in the presence of a range of organic solvents by comparison to other commonly used hydrogels [36].

3.4.8. Recovery of geldanamycin from dibutyl sebacate liquid-core microcapsules

Dibutyl sebacate liquid-core microcapsules with a d_m of 502.63 µm and a d_{lc} of 327.48 µm (Figure 4a) were used to extract geldanamycin from Bennett's medium. Following capsular perstraction, the capsules were removed from the media and washed in acetonitrile or methanol to recover the antibiotic. In both cases, use of this procedure facilitated the retrieval of > 95% of the geldanamycin contained within the capsules. However, due to the core material being very soluble in both solvents, the complete loss of this material from the capsules occurred (Figure 4b and 4c). Whilst this method enabled the recovery of large amounts of antibiotic, the continuous loss of the core

material meant only single usage of capsules was possible. Similar results were observed by Stark et al. (2003) when ethanol was used to recover the aroma compound 2-phenylethanol contained within dibutyl sebacate liquid-core microcapsules.

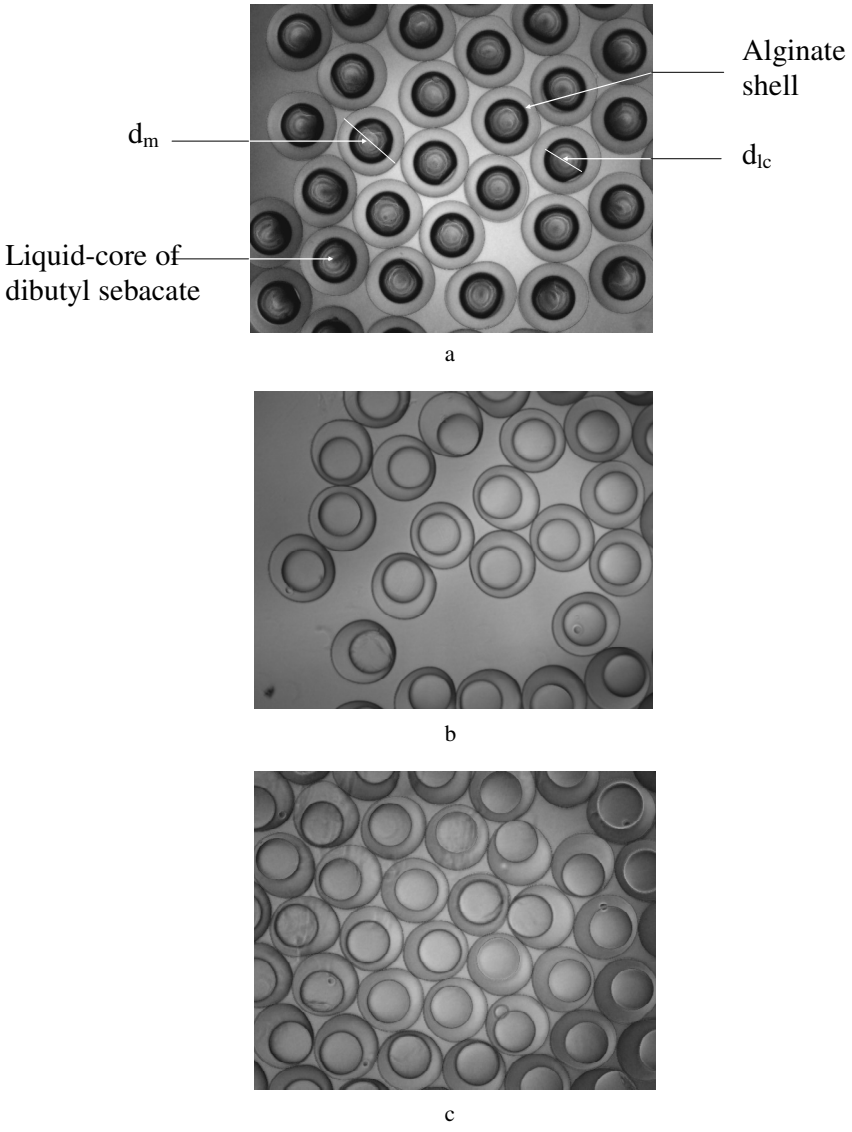

Figure 4: Light microscope image of: (a) Dibutyl sebacate liquid-core microcapsules with a d_m of 502.63 µm and a d_{lc} of 327.48 µm which were used to extract geldanamycin from Bennett's media. (b) Microcapsules after washing with acetonitrile, and (c) after washing with methanol.

3.4.9. Recovery of geldanamycin from oleic acid liquid-core microcapsules

Although large quantities of geldanamycin were recovered from the dibutyl sebacate containing microcapsules, the continuous loss of the core material prevented the recycling of the particles for repetitive use. This implies that a new batch must be manufactured for each extraction and can significantly increase the overall cost of any recovery process. Due to the very low solubility of oleic acid in acetonitrile (result not shown), it was decided to examine the possibility of back-extracting geldanamycin from oleic acid liquid-core microcapsules using this solvent. It was anticipated that close examination of this system could result in the recovery of high concentrations of the drug, whilst maintaining the integrity of the capsule structure.

To begin with, geldanamycin was recovered from Bennett's medium using oleic acid containing microcapsules, which had a d_m of 926.86 μm and a d_{lc} of 734.12 μm (Figure 5a). Capsules were then washed with equal volumes of acetonitrile, which resulted in little or no removal of liquid from the core; however > 25% of the geldanamycin remained inside after one wash. Repeated rinsing and/or increasing volumes of solvent enabled larger quantities of antibiotic to be retrieved, but unfortunately also resulted in the loss of some (Figure 5b) or all the core material (as seen in Figure 4b and c). To prevent this from occurring, the capsules were washed with acetonitrile saturated in oleic acid and the outcome displayed in Figure 5c. The saturation of acetonitrile with oleic acid prevented the loss of the core material, even when large volumes were used, due to the equilibrium concentration of the fatty acid being present in the back-extracting solvent. The $K_{acn/oa}$ for geldanamycin between the two different phases was calculated using equation 4 and equaled 3.3 ± 1.89%. Owing to its higher affinity for geldanamycin compared to oleic acid, the use of acetonitrile reduces the amount of solvent required to recover large amounts of the antibiotic. Also, due to the intact oil-

core and the saturated acetonitrile not affecting the mechanical structure of the capsules (results not shown), it can be foreseen that these could be re-used for many future capsular perstraction applications.

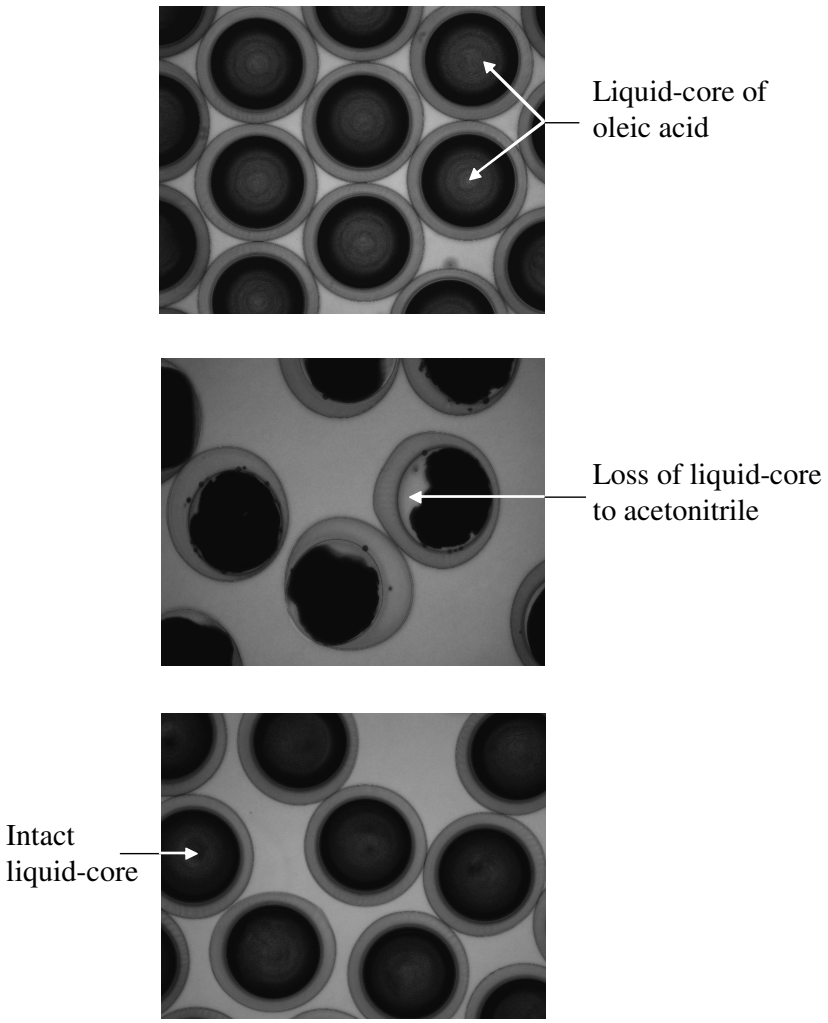

Figure 5: Light microscope image of: (a) Oleic acid liquid-core microcapsules with a d_m of 926.86 µm and a d_{lc} of 734.12 µm, which were used to extract geldanamycin from Bennett's media. (b) Capsules showing the loss of some of the oleic acid liquid-core after three individual acetonitrile washes, and (c) capsules shown no loss of the core material after three separate washes with acetonitrile saturated with oleic acid.

3.4.10. Purification of geldanamycin recovered from microcapsules

Crystallization is one of the oldest and most effective processes for the preparation of solid products and is widely used for the manufacturing of specific active ingredients during final and intermediate stages of purification and separation, as it enables very high product purities and stabilities to be obtained. Its successful attainment is governed by many factors, two of the most important being solubility and purity of the product in the crystallization solution. The back-extraction process resulted in a very pure solution of geldanamycin dissolved in acetonitrile saturated with oleic acid (Figure 6c), with most of the media components removed after extraction (Figure 6b). Due to this high level of purity, it was decided to examine the possibility of recovering the antibiotic in a solid state using crystallization. The presence of oleic acid in the back-extraction solution (Figure 6c) initially prevented crystallization, and to obtain a successful method the fatty acid had to be removed. The addition of water to this solution resulted in the precipitation of oleic acid from the mixture in the form of an emulsion, which was completely removed by centrifugation and filtration. The geldanamycin which remained in the displaced oleic acid was recovered by repeating the back-extraction process. The removal resulted in a very pure solution of acetonitrile and water containing geldanamycin (Figure 6d) and resembled the chromatogram obtained for pure geldanamycin in water (Figure 6a). Heating of the solution resulted in the evaporation of acetonitrile from the solution, and due to the low solubility of the antibiotic in water (< 15 mg/l), the removal of acetonitrile quickly gave rise to the formation of a supersaturated solution. Continuous removal of the acetonitrile initiated nucleation followed by crystal growth, which was aided by the agitation of the liquid. Table 3 and Figure 6a and 6e display the results for the purity of the re-crystallized geldanamycin compared to the pure commercialized geldanamycin. In both cases, the re-crystallized

antibiotic had the same HPLC retention time and melting point as the market product. This proves that the crystals were indeed geldanamycin and that the purity of the recovered compound was not lower than that of the commercial product (the purity of this geldanamycin is > 99% dried material as claimed by LC Labs). This low temperature distillation resulted in the degradation of only 1.3% of the geldanamycin present in the mixture after oleic acid removal.

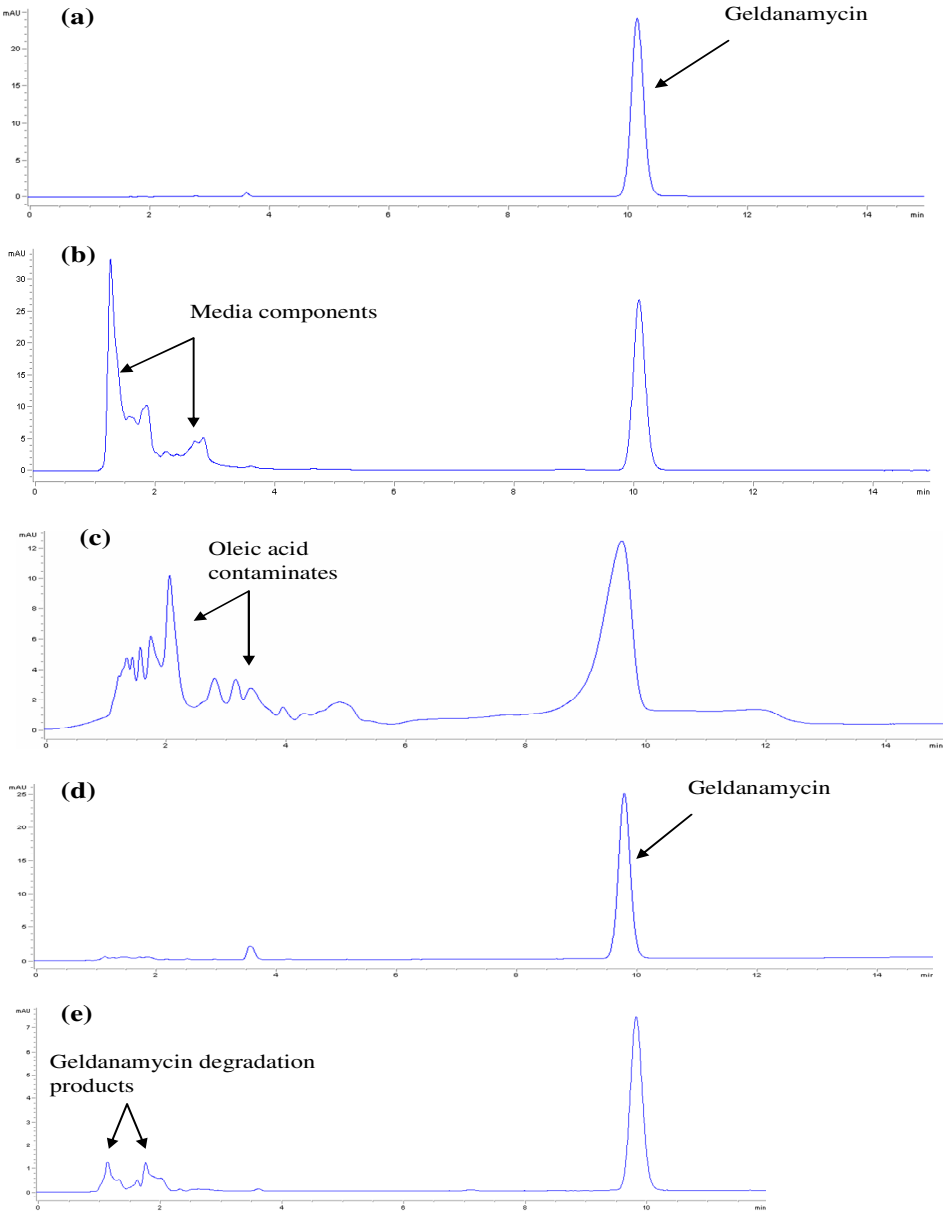

Figure 6: Chromatograms of geldanamycin at different stages of the purification procedure: (a) Commercial geldanamycin in water. (b) Commercial geldanamycin in Bennett's media (c) Geldanamycin in oleic acid saturated acetonitrile (recovered from oleic acid liquid-core microcapsules). (d) Geldanamycin before crystallization in acetonitrile/water mixture (after precipitation and removal of oleic acid), and (e) re-crystallized geldanamycin re-suspended in water. Note: All RP-HPLC runs were performed at a fixed wavelength of 308 nm.

Table 3: Values for the retention time and melting point, determined by RP-HPLC, for commercial and re-crystallized geldanamycin.

	Commercial Geldanamycin	**Re-crystallized Geldanamycin**
Retention time (min)	10.159 ± 1.5%	9.826 ± 1.3%
Boiling point (ºC)	252~254	252~254

3.5. Conclusions

The primary goal of this study was to perform a detailed investigation into the feasibility of using capsular perstraction as a novel methodology to recover and purify geldanamycin from Bennett's media. These results would then be used to determine if the extraction procedure could be feasible for future work involving the recovery of geldanamycin in-situ from cultures.

The results obtained demonstrated how liquid-core microcapsules can be used as a simple methodology to recover large amounts of geldanamycin rapidly from an aqueous environment, whilst also successively providing a platform for the purification (crystallization) of the drug using a very simple procedure. It is envisaged that the application of these particles as a methodology for in-situ product recovery in fermentations producing geldanamycin, will not only increase the overall production titres by stabilizing the product after removal from the detrimental fermentation environment, but most importantly it will lead to significantly increased recovery yields of the antibiotic, mainly due to the successful and simple downstream processing approach offered by the technique. This has the potential to significantly reduce the

overall cost, as downstream processing can account for up to 70-80% of production costs for the manufacturing of pharmaceuticals.

Since many bioprocesses can encounter problems such as product inhibition/degradation and/or low yields of recovery, it seems likely that this novel and simple technique may be readily adapted for use in many such operations. Experiments would simply involve the screening and selection of a solvent, which has a high affinity and selectivity for the target product to be encapsulated within a hydrogel. Most organic solvents can be encapsulated provided a difference exists between the surface tension of the solvent and the polymer.

The results also highlight how different mass transfer resistances can control the rate of geldanamycin diffusion into the microcapsules. Whilst in some instances, these factors can have a significant impact on transfer rates; it is however possible to control the resistances by varying such parameters as, agitation speed or membrane size during capsule production, without affecting critical properties of the microcapsules.

The study has shown the principle and efficiency of capsular perstraction for the recovery of a hydrophobic product from an aqueous environment, by demonstrating how microcapsules can be used as a simple methodology to remove large amounts of geldanamycin rapidly from Bennett's media, whilst also showing the potential of this procedure as a mechanism for the subsequent purification (crystallization) of the antibiotic, using a very simple and straightforward procedure, in which a highly purified product (> 99%) can be obtained.

3.6. Acknowledgements

The authors are very grateful to Agnes Dienes, Patrick Pugeaud and John Casey for assistance in analytical procedures.

3.7. Nomenclature

Table 4: List of abbreviations and symbols.

Abbreviation/Symbol	Definition	Unit
Hsp90	Heat shock protein 90	-
17-AAG	17-allylamino-17-demethoxygeldanamycin	-
17-DMAG	17-dimethylaminoethylamino-17-demethoxygeldanamycin	-
RP-HPLC	Reverse phase high performance liquid chromatography	-
d_m	Diameter of microcapsule	µm
d_{lc}	Diameter of liquid-core of microcapsules	µm
$K_{org/aq}$	Partition coefficient of geldanamycin between an organic solvent and an aqueous phase	-
C^e_{org}	Equilibrium conc. of geldanamycin in an organic solvent	mg/l
C^e_{aq}	Equilibrium conc. of geldanamycin in the aqueous phase	mg/l
C_{aq}	Geldanamycin conc. in the aqueous phase	mg/l

k_L	Volumetric mass transfer coefficient	m/s
a	Total interfacial mass transfer area per unit volume	mg/l
V_{aq}	Volume of the aqueous phase	l
C_{aq}^0	Initial conc. of geldanamycin in aqueous phase	mg/l
V_m	Volume of microcapsules	l
C_m	Geldanamycin conc. in microcapsules	mg/l
C_m^0	Initial conc. of geldanamycin in microcapsules	mg/l
$K_{acn/oa}$	Partition coefficient of geldanamycin between acetonitrile saturated with oleic acid and oleic acid	-
C_{acn}^e	Equilibrium conc. of geldanamycin in acetonitrile saturated with oleic acid	mg/l
C_{oa}^e	Equilibrium conc. of geldanamycin in oleic acid	mg/l
C(t)	Conc. of geldanamycin at time t	mg/l
C(0)	Initial conc. of pharmaceutical	mg/l

3.8. References

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Chapter 4

Successful Application of Capsular Perstraction as a Novel In-Situ Product Recovery Technique: Removal and Purification of Geldanamycin from *Streptomyces Hygroscopicus* Cultures

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Keywords: In-situ product recovery; Liquid-core microcapsules; Capsular perstraction; Geldanamycin and Downstream processing.

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4.1. Abstract

The goal of this study was to increase production, recovery and purification titres of the antibiotic geldanamycin during its synthesis in submerged fermentations, using capsular perstraction as a novel in-situ product recovery (ISPR) methodology. This ISPR-technique involves employing porous particles, known as liquid-core microcapsules, as extraction aides for the recovery of compounds from different environments. Liquid-core microcapsules can be described as miniature sized spherical structures, ranging in size from 1-1000 μm. They consist of a pre-selected liquid-(core) enveloped within a defined membrane of a chosen material. These structures can be manufactured to specific criteria and size distribution using the vibrating nozzle (jet) technique.

The benzoquinone ansamycin geldanamycin is a naturally occurring antibiotic produced as a secondary metabolite by *Streptomyces hygroscopicus* var. *geldanus* in submerged fermentations, and has shown the capability to be very effective in eradicating tumor cells. However, some studies have shown that this compound may be very labile in the culture environment, resulting in low production titres and recovery yields, which have the potential to affect the overall economic viability of the bioprocess.

In this study microcapsules with an outer diameter of 770.86 μm and containing a liquid-core of oleic acid, were added to geldanamycin producing cultures to recover the antibiotic from its detrimental fermentation environment, also to stabilize the compound and to facilitate the subsequent purification. The results showed a rapid and efficient removal of the antibiotic, which enabled an increase in production of 30% compared to control fermentations. It was shown that this immediate and selective in-situ extraction of the product prevented geldanamycin from being degraded (presumably enzymatically) in the culture environment. In addition to increased production levels,

the microcapsules served as a platform for the subsequent purification of the compound. This was achieved through back-extraction and crystallization of the antibiotic using a very simple procedure. This significantly reduced the complexity and number of downstream processing steps required to obtain large quantities of a highly purified product. Application of this innovative procedure allowed the recovery of > 53% of the antibiotic extracted from the fermentation, whilst obtaining a purity > 97%. It is envisaged that this procedure could be applicable as an ISPR-methodology and/or downstream processing procedure to many bioprocesses, which are limited due to product degradation, low recovery yields and/or overly complex purification operations.

4.2. Introduction

Streptomyces (S.) hygroscopicus have attracted great interested due to their well-knowing ability to produce a wide variety of antibiotics and other secondary metabolites, with some of these compounds showing significant importance in the treatment and prevention of many diseases [1,2]. One such antibiotic produced by the bacterium is 'geldanamycin' [Figure 1], a benzoquinone ansamycin first described in 1970 [3]. This compound has received much attention over the last two decades, mainly due to its potential as a novel antitumor agent [4-7]. The antibiotic targets and inhibits the cellular functions of the molecular chaperone complex-Heat shock protein 90 (Hsp90), which can be found at abundant and elevated levels in cancerous mammalian tissue [7,8]. Geldanamycin combines with high affinity to the N-terminal domain ATP binding site of Hsp90, inhibiting its chaperone ability

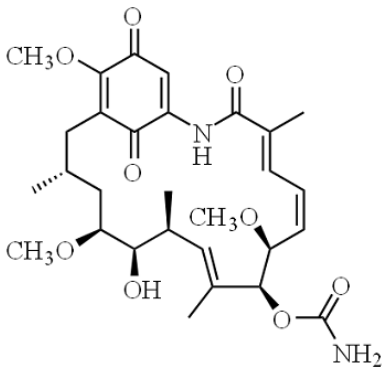

Figure 1: Molecular structure of geldanamycin.

towards its client proteins, which subsequently results in tumor destruction by the cells natural mechanisms [4].

Studies have shown that the antibiotic is produced at relatively low concentrations in submerged fermentations [2,3,9,10]. Whilst little is known on the fate and behavior of geldanamycin during production [4], in their study Dobson (2008) showed that the compound can be sensitive within the culture surroundings, resulting in product degradation [2].

The sensitivity of bio-products within the production environment can be a serious issue for many bioprocessing operations, and can fundamentally lead to very small or negligible production titres. This problem has been overcome using methods, such as strain improvement [11], or media optimization [12] and have been studied and applied for many different bio-products. However, whilst Dobson (2008) showed the capability to increase production levels of geldanamycin by up to 500% using a medium optimization approach, significant antibiotic degradation still occurred, resulting in poor recovery yields [2].

As discussed by Freeman et al. [13], Stark and von Stockar [14] and Lye and Woodley [15] in their very thorough reviews, the problem caused by product accumulation in a culture can be overcome by in-situ product recovery (ISPR). ISPR is a collective term used to describe a technique (or combination of techniques), which can be used as a tool to improve the overall productivity, recovery and/or final purified yield of the desired product in a biotechnological process. It works by continuously removing a produced species from the culture environment as soon as it is formed, and transferring into a stable environment before significant amounts of degradation takes place. ISPR-methodologies are used not only to increase production by preventing degradation, but can also be used to overcome other limitations characteristic of many biological

operations, which can result in low productivities and yields. These include: (1) inhibitory or toxic effects of the product on the producing organism. (2) elaborate and complex downstream processing (DSP) operations and (3) unfavorable reaction equilibrium between the product and substrate.

Selection of an ISPR-methodology is usually based on the physiochemical properties (volatility, size, charge, hydrophobicity and other specific elements) of the product being recovered [16]. This enables the chosen technique to exploit the differences in the molecular properties of the target product, which differ from the background media, hence facilitating removal.

Recently it was shown in [4] how a new technique (developed within the last decade), termed 'capsular perstraction', could be used as an ISPR-methodology for the successful removal of geldanamycin from a culture medium. This technique uses porous particles known as liquid-core microcapsules as extraction aides for the recovery of compounds from their associated environments. Liquid-core microcapsules can be described as miniature sized spherical structures (ranging in size from nm to mm) and consist of a liquid (core) enveloped within a defined membrane [17].

In their study, Whelehan and Marison [4] showed how this innovative technology not only removed geldanamycin rapidly from the production media, but also demonstrated its ability to act as a mechanism to assist DSP. This enabled the purification (crystallization) of the drug using a very simple and straightforward procedure and resulted in a highly purified product. Stark et al. [18] also used the technique to successfully recover 2-phenylethanol from fermentation media. Immediate removal of the compound from the vicinity of the producing cells eliminated the inhibitory effect of the alcohol on the producing yeast, and resulted in a 50% increase in production concentrations.

In this study, liquid-core microcapsules consisting of a porous hydrogel membrane of alginate and a liquid-core of oleic acid were used as a novel ISPR-approach to recover geldanamycin from the fermentation environment. Initially work focused on establishing the impetus behind the loss of produced geldanamycin in bacterial cultures. Subsequently the work undertaken by Whelehan and Marison [4], was used to determine whether liquid-core microcapsules could recover the antibiotic in-situ from the culture broth, and thereby stabilize the product. Finally the back-extraction of geldanamycin from capsules was examined to determine the capability of coupling the extraction process with DSP.

This study endeavors to show the feasibility and efficiency of using capsular perstraction, as a novel ISPR-methodology for the removal and purification of geldanamycin from culture environments.

4.3. Materials and methods

4.3.1. Chemicals

Geldanamycin microcrystalline powder (> 99% purity) was obtained commercially from LC Laboratories (Massachusetts, USA). Water, methanol and acetonitrile were of high performance liquid chromatography grade and were all obtained from Sigma-Aldrich (Dublin, Ireland). Oleic acid, calcium chloride, 3-(N-Morpholino)propanesulfonic acid (MOPS), Tween 80, N-Z amine A from bovine milk, magnesium sulphate heptahydrate and glucose monohydrate (dextrose) were all obtained from Sigma-Aldrich (Dublin, Ireland) and were of at least analytical grade. Yeast extract, technical agar No. 3, bacteriological peptone and 'lab-lemco' beef extract

were obtained from Oxoid (Basingstoke, UK). Sodium alginate (Keltone LV) was obtained from Inotech Biotechnologies (Basel, Switzerland).

Due to the reported photolytic nature of geldanamycin, all solutions and experiments using the antibiotic were stored and performed in the dark to maintain maximum stability.

When oleic acid was used in its free form i.e. not (non)-encapsulated, all experiments were preformed using glass consumables/containers as the fatty acid can react with plastics, and therefore interfere with experiments (i.e. purification procedure).

4.3.2. Methods

4.3.2.1. Strain

The geldanamycin producing microorganism, *S. hygroscopicus* var. *geldanus* strain NRRL 3602 was obtained from ARS Patent Culture Collection (Peoria Illinois, USA) and was used throughout this study.

4.3.2.2. Spore production

S. hygroscopicus spores used for cultures were grown on Bennett's agar consisting of the following components: 20 g/l technical agar No. 3, 1 g/l yeast extract, 1 g/l beef extract, 2 g/l N-Z amine A and 10 g/l glucose monohydrate, which was autoclaved separately from the other components. The agar had a pre-sterilization pH of 7.0. A stock solution of 6 x 10^7 spores/ml was incubated onto the surface of 20 ml of agar in a 250 ml Erlenmeyer flask (No. 4980, stopper No. 6) at 30 ^oC for sixty days. This resulted in the formation of a mycelia lawn containing aerial spores. The spores were removed by washing and re-suspending in a solution containing the following components: 3 g/l yeast extract, 5 g/l bacteriological peptone and 1 g/l magnesium sulphate heptahydrate,

at 4 ^{o}C on an orbital shaker at 100 rpm. Glass beads with a diameter of 5 mm (Sigma-Aldrich, Dublin, Ireland) were added to help break-up the mycelia. The resulting spore suspension was enumerated by placing a diluted spore sample onto Bennett's agar, and incubating at 30 ^{o}C for 5-7 days before counting was performed. When not used, spores were stored in 20% (v/v) glycerol solution at -80 ^{o}C and were employed as the inoculum for cultures after defrosting at room temperature.

4.3.2.3. Antibiotic production

All fermentations were carried out in 250 ml Erlenmeyer flasks containing 100 ml of Bennett's liquid medium, which consisted of the following components: 1 g/l yeast extract, 1 g/l beef extract, 2 g/l N-Z amine A and 50 g/l glucose monohydrate, which was autoclaved separately from the other medium components. The medium had a pre-sterilization pH of 7.0. and was inoculated with a spore suspension (1% v/v) containing 6 x 10^{7} spores/ml. Flasks were subsequently incubated at 30 ^{o}C in a temperature-controlled orbital shaker (model Sheldon SI Series, Medical Supply Company, Dublin, Ireland) at 150 rpm for 20-25 days. The pH was not controlled throughout the fermentation. At each defined sampling point, a single flask was removed from the shaker, the required analysis performed, and the remainder of the culture was discarded.

4.3.2.4. Quantitative determination of Geldanamycin

The full details of the high performance liquid chromatography (HPLC) procedure employed throughout this study to determine the concentration of geldanamycin in different phases can be found in [4]. Standard solutions of geldanamycin for HPLC calibrations were prepared in 100% acetonitrile and methanol.

4.3.2.5. Geldanamycin extraction from cultures

To determine the geldanamycin present within control cultures (containing no capsules), a homogenous 1 ml samples of medium containing the mycelia and cell by-products were removed from the culture, and combined with 4 ml of acetonitrile in a 15 ml plastic universal (Sarstedt, Wexford, Ireland). The suspension was agitated for 15 min using a vortex, followed by rotating the universal through 360^0 about its transverse axis for 1 hr at 25 ^oC. The sample was then centrifuged for 15 min at 4000 rpm and the pellet discarded. The supernatant was diluted in the HPLC mobile phase and filtered using a 0.2 μm nylon filter (Sigma, Dublin, Ireland) and degassed prior to been injected onto the HPLC column to quantify the amount of geldanamycin present. This procedure extracted > 98% of the antibiotic present in the culture environment.

4.3.2.6. Determination of biomass

Biomass concentration (dry cell weight) was determined for control (containing no capsules) and microcapsule containing cultures using the following procedure (for cultures containing capsules, these particles were first removed using a procedure described later). Homogenous samples of whole broth (10 ml) were removed and placed into 50 ml centrifuge tubes (Sigma, Dublin, Ireland). The samples were subjected to three centrifugations at 6000 rpm for 10 min. After the first and second spins the samples were re-suspended and washed in distilled water (20 ml). After the final centrifugation the sample was transferred to a pre-weighed and pre-dried glass universal and incubated at 104 ^oC for 24 hr. Following evaporation of the water the universals were allowed to cool in a desiccator to ensure complete removal of moisture. The universal was re-weighed and the dry cell weight of the biomass determined.

4.3.2.7. Geldanamycin stability

Stability studies were carried out in 250 ml Erlenmeyer flasks with a working volume of 50 ml and performed by examining the breakdown of geldanamycin (fermented and commercial) in culture broth. The broth was obtained by filtering whole fermentation samples using a 0.22 μm sterile filter membrane system (Steritop, Millipore, Cork, Ireland). Experiments were carried-out under sterile conditions to prevent contamination of the utilized media or the re-growth of *S. hygroscopicus*, which may produce more geldanamycin and interfere with results. Commercial geldanamycin was added to the broth using acetonitrile as a co-solvent, which facilitated the solubilisation of the antibiotic [4]. Initially geldanamycin was dissolved in the solvent by agitating on a magnetic stirrer and added to broth to make a final acetonitrile concentration of 5% (v/v) whilst obtaining the desired concentration of the antibiotic (amount depending on experiment being executed). For sampling 0.1 ml was removed from the broth at defined intervals, and was analyzed using HPLC analysis to determine the stability of the geldanamycin under culture conditions.

When required, heat treatment of the broth was performed by heating the filtered broth in an agitated water bath set at 80 °C ± 1 °C (model SBS40, Stuart, Staffordshire, UK) for 90 min. After heating the broth was cooled to 25 °C before use.

4.3.2.8. Preparation and characterization of liquid-core microcapsules

The full details of the co-extrusion/prilling technique employed to manufacture microcapsules used in this study and the procedure used to measure their size has been reported previously [17]. All capsules used in this work were manufactured from sterile solutions. Alginate stock solutions were prepared by dissolving 25 g/l of alginate powder in MOPs buffer [10 mM MOPs, 0.85% (w/v) NaCl pH 7.0]. After solubilisation

the alginate solution was filtered using a 0.45 μm cellulose acetate filter membrane (Whatman, Dassel, Germany) under a pressure of 2-4 bar to remove most of the impurities, which was followed by sterile filtration through a 0.22 μm membrane filter system. The gelling (hardening) solution consisted of 32 g/l $CaCl_2$, 10 mM MOPS pH 7.0 and was filtered sterilized before use. To reduce the surface tension of the hardening solution, 0.1-0.2% (v/v) of Tween 80 was added, followed by heating the solution to 55-60 °C during the capsule production process. Sterile microcapsules were obtained by placing the Encapsulator (model IE-50R, Inotech Biotechnologies, Basel, Switzerland) within a laminar flow safety cabinet (Model TC-48, Gelaire, Melbourne, Australia). After production, capsules were washed extensively with sterile water to remove any un-reacted components using a porous mesh (pore size 200-300 μm). The washing step was unable to sufficiently remove free oleic acid (occurs due to inadequate break-up of the liquid jet, and/or when capsules burst upon enter into the gelling bath, during the production process) from around capsules due its very hydrophobic nature. If the fatty acid was present in a non-encapsulated form in a batch of capsules, they were discarded. Microcapsules were stored under sterile conditions until required.

4.3.2.9. Addition and removal of capsules from cultures

Capsule-assisted fermentations were inoculated with 20 ml of oleic acid contained within the microcapsules. The capsules used in this study had an outer diameter of 770.86 μm ± 2.3% and an inner diameter, consisting solely of the liquid-core, of 667.2 μm ± 1.8%. The hydrogel membrane of microcapsules is mainly composed of water (≥ 97%) and the capsules added to the culture had a total membrane volume of 10.84 ml. The dilution affect of this membrane on geldanamycin and biomass levels in the culture, was taken into consideration when microcapsules were present. Sterile capsules were

added to the fermentation medium five days after inoculation had taken place. The total volume of the capsules was measured using graduated tubes. During harvesting the buoyant nature of the capsules resulted in them floating to the top of the culture, whilst the cells sedimented to the bottom of the flask. The capsules were removed using a pipette and washed with water through a mesh filter to remove any media components. After removal, capsules were immediately treated to recover the extracted geldanamycin.

4.3.2.10. Recovery of geldanamycin from microcapsules

Geldanamycin extracted into the oleic acid liquid-core of microcapsules during cultures was recovered (back-extracted) by one of two methods which have been described previously [4]. When complete quantification (and removal) of the extracted antibiotic in the microcapsules was required, capsules were contacted and washed in equal volumes of methanol and solutions were agitated at 300 rpm for 60 min at 25 °C to establish equilibrium. To ensure complete recovery of all the antibiotic additional washing steps were performed until no more geldanamycin could be detected. The antibiotic required for purification (crystallization) was removed by adding and washing capsules with equal volumes (liquid-core volume, 20 ml) of acetonitrile saturated in oleic acid. Prior to the addition of this solution, geldanamycin containing capsules were placed in an oven overnight at 30 °C to expel the water from the alginate membrane. Saturation of acetonitrile with oleic acid was performed by mixing both liquids at 200 rpm for 24 hr at 25 °C in a temperature-controlled shaker (Innova 43, Brunswick Scientific, New Jersey, USA). After agitation, both phases were allowed to settle and reach equilibrium for at least 24 hr at 25 °C. Capsules were then added to the back-extraction solution and agitated at 300 rpm for 60 min at 25 °C to ensure equilibrium

had been obtained. After back-extraction capsules were removed from the acetonitrile solution by filtration through a porous mesh, and the retained capsules were added to fresh back-extraction solution and the washing step repeated. To ensure that the majority of the antibiotic was removed, three washing steps were undertaken, after which all solutions were pooled and the total quantity recovered determined.

4.3.2.11. Purification and crystallization of geldanamycin recovered from capsules

The geldanamycin-containing acetonitrile saturated with oleic acid, was purified prior crystallization by removing the fatty acid, through the addition of equal volumes of water (60 ml) to the solution. This resulted in the displacement (precipitation) of the oleic acid. This precipitate was then separated from the suspension by centrifugation at 4000 rpm for 35 min at 25 °C. Equation 1 was used to calculate the partition ratio $K_{oa/aw}$ for the distribution of geldanamycin between the oleic acid and acetonitrile:water phases.

$$K_{oa/aw} = \frac{C_{oa}^{e}}{C_{aw}^{e}} \qquad (1)$$

where C_{oa}^{e} is the concentration of geldanamycin in the displaced oleic acid at equilibrium, and C_{aw}^{e} is the concentration of the antibiotic in the acetonitrile:water phase when equilibrium was obtained.

The remaining solution was filtered using hydrophilic 0.22 μm Filtropur membranes (Sarstedt, Wexford, Ireland) to remove any unrecovered oleic acid, which remained suspended in the aqueous phase. The water addition and filtration steps were repeated

twice more, and prior to the final filtration the solution was cooled to 4 °C (to precipitate any remaining oleic acid if present). After removal the purified acetonitrile and water solution containing geldanamycin was added into a graduated glass beaker and placed into a water bath set at 82.5 °C ± 2.5 °C to enable evaporation of the acetonitrile. During evaporation, the solution was slowly agitated to facilitate nucleation and subsequent crystal growth. When most of the acetonitrile been removed (no visible change in the volume of the solution), the temperature was increased to 100 °C ± 2.5 °C. Evaporation of the remaining solution was continued until < 5% of the original volume was left. The solution was immediately cooled to 4 °C to further reduce the solubility of the antibiotic in the suspension. Geldanamycin crystals were retrieved by filtering the crystal-containing solution through a 0.2 μm nylon filter under vacuum. Crystals were dried at 30 °C for at least 48 hr and subsequently placed in a dessicator before weighing to determine the amount of antibiotic recovered. The purity of the crystals was determined by re-dissolving in acetonitrile and HPLC analysis. These results were compared to those generated for commercial geldanamycin solutions prepared and analyzed in the same way.

4.4. Results and discussion

4.4.1. Production of geldanamycin

The growth of the bacterium *S. hygroscopicus* followed by the production of geldanamycin in submerged fermentation can be seen in Figure 2. From the results antibiotic accumulation appears from the late exponential growth phase (6-7 days after inoculation) with the majority formed during a 5 day period (between day 8 and 13). During this time the geldanamycin concentration reached a maximum net concentration

of 110 mg/l, with a corresponding biomass concentration of 7.2 g/l. Although the biomass concentration appeared to remain relatively constant during the stationary phase, the concentration of geldanamycin fell rapidly after 15 days, until day 23, where no geldanamycin could be detected.

In previous work Casey [9] and Song et al. [10] had not observed significant reductions in geldanamycin titres during the course of batch cultures. However Song et al. (2008) used a different *Streptomyces* subspecies, whilst Casey (2008) inoculated the culture with an inoculum containing a smaller concentration of *S. hygroscopicus* spores. As reported in numerous publications [19-22], the inoculum size has the potential to affect the morphology of a filamentous organism during growth in shake-flasks, which itself may influence product stability [23]. Dobson [2] reported similar results to those portrayed in Figure 2 when comparable fermentation conditions were employed. In this study it was postulated that the steep decline in geldanamycin concentrations observed at day 15 in this study, could be due to enzymatic and/or chemical breakdown in the culture [2]. Higher net maximum concentrations of geldanamycin were obtained before degradation occurred in Dobson's work [2], probably due to the addition of extra components to the culture medium and cell lysis prior to analysis. This suggests that feed-back inhibition was not responsible for the cessation of antibiotic production in this work.

Other limited research studies published on the fate and behavior of geldanamycin in cultures have proposed that it may be sensitive to certain elements within the production environment [2,3,9]. Indeed, Casey (2006) reported that the use of adsorbent resins resulted in a 3-fold increase in overall production concentrations [9].

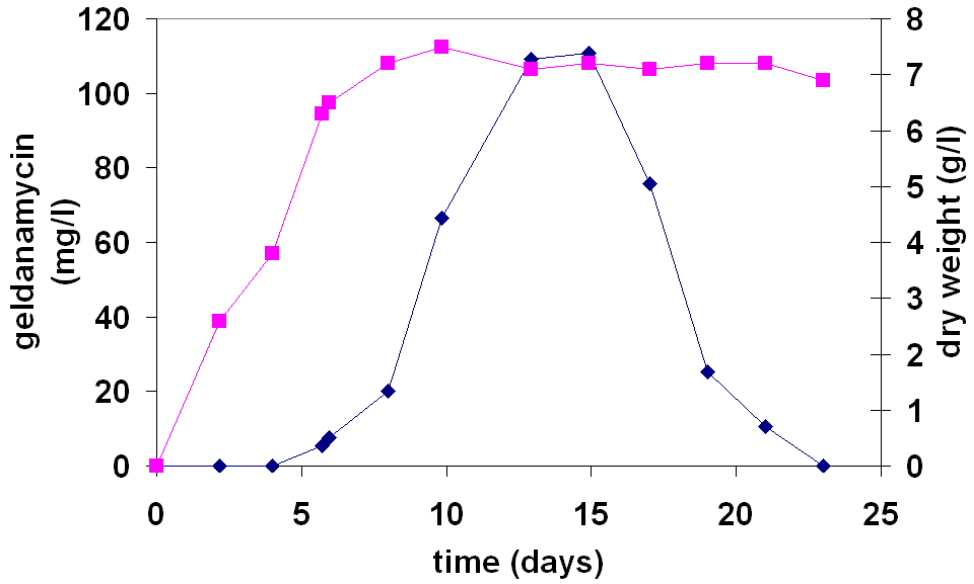

Figure 2: Growth (dry cell weight) of *S. hygroscopicus* and overall net production of geldanamycin in submerged cultures. Legend: dry cell weight (squares) and geldanamycin concentration (diamonds).

4.4.2. Geldanamycin stability

Establishing a successful manufacturing procedure for any bio-product requires a stability profile of the molecule in the production environment and this was the goal of this part of the study. Initially experiments were conducted to examine the effects of different parameters (excluding cells) associated with the culture environment on the stability of geldanamycin such as, agitation speed, temperature, media components and pH. From this work (results not shown) it was deduced that none of these parameters could be responsible for the degradation seen in Figure 2. As a result the stability was assessed by addition of known amounts of geldanamycin (commercial and fermented) to the culture broth produced by *S. hygroscopicus* in Bennett's medium, after the bacterial cells had being removed. The following were used: (a) culture broth from Day 8 of fermentation (Figure 1), which contains geldanamycin (16.95 mg/l) produced by

the organism and (b) culture broth (Day 5.7 of fermentation). This solution contained geldanamycin at 2.3 mg/l, which was increased to the level in solution a (16.95 mg/l) by the addition of commercial geldanamycin. Both samples were incubated under the same conditions as those used in the batch culture experiments and the stability of geldanamycin determined.

The results (Figure 3) indicate that at this stage of the fermentation both fermented and commercial geldanamycin are relatively stable with only an 8 and 15% loss of product occurring respectively over a 15 day period. Such a decrease in geldanamycin does not account for the loss in real-time fermentations, and suggests that considerable breakdown of geldanamycin is not occurring during the production phase. To further understand what maybe responsible for the loss of stability of the antibiotic, two additional experiments were performed using: (c) culture broth from day 15 of the fermentation, where overall geldanamycin concentration (18.65 mg/l) began to fall ('falloff point') and (d) culture broth from day 23 of the fermentation, where no geldanamycin appeared to be present due to complete degradation. To the latter solution, 18.65 mg/l of geldanamycin was added to bring the concentration similar to the level in sample (c).

The stability of geldanamycin in the two solutions was determined under fermentation conditions and the results displayed in Figure 3. The results show the geldanamycin present in both types of solution is completely degraded after 5-6 days. The increased rate of degradation in experiments (c) and (d) compared to that determined in the culture (Figure 2) is almost certainly due to the large difference in concentration of the drug in each environment. The results suggest that geldanamycin is relatively stable in the fermentation environment until a certain critical stage (falloff point) is reached, at which

time the degradation of geldanamycin begins due to the presence of a component in the broth, which is probably released by the cells.

Dobson (2008) postulated that enzymatic degradation of the antibiotic might occur to replace a limiting substrate, as growth enters a decline or death phase [2]. In a similar way Mayer and Decker (1996) speculated that the degradation of clavulanic acid could be related to an enzyme [24]. In this case the biocatalyst sets about modifying the structure of the antibiotic, enabling the organism to consume it and produce more secondary metabolites. It has also been reported that a cell associated enzyme is responsible for the breakdown of the secondary metabolite pristinamycin following the production phase [25].

To determine if a cell associated product such as an enzyme, is responsible for degradation in the culture broth, both solutions (c) and (d) were heated to 80 °C for 90 min before again being tested under fermentation conditions. The heating step was performed to enable the destruction of any proteins, which may be present in the broth and which could be affecting the antibiotic. As reported previously these relatively high temperatures have little or no affect on the antibiotic [4], although other reports suggest that geldanamycin may be thermo-labile [3,26].

From Figure 3 it can be seen that both sources of the drug are more stable in the heat-treated broth compared to the untreated version, in which the antibiotic degrades after 16 days by 13 and 29% for the commercial and fermented geldanamycin respectively, and these values are similar to those obtained for broth samples (a) and (b). On the basis of the available experimental data it can be suggested that the greater stability of the compound in heat treated broth, is the result of certain components (e.g. enzymes) being produced, which are either secreted or released due to cell lysis occurring in the stationary phase and subsequently attack the compound. These substances appear to be

soluble and remain in the cell-free broth after microfiltration. In the presence of cells the rate of breakdown could be greater due to the additional metabolism of geldanamycin by the cells themselves.

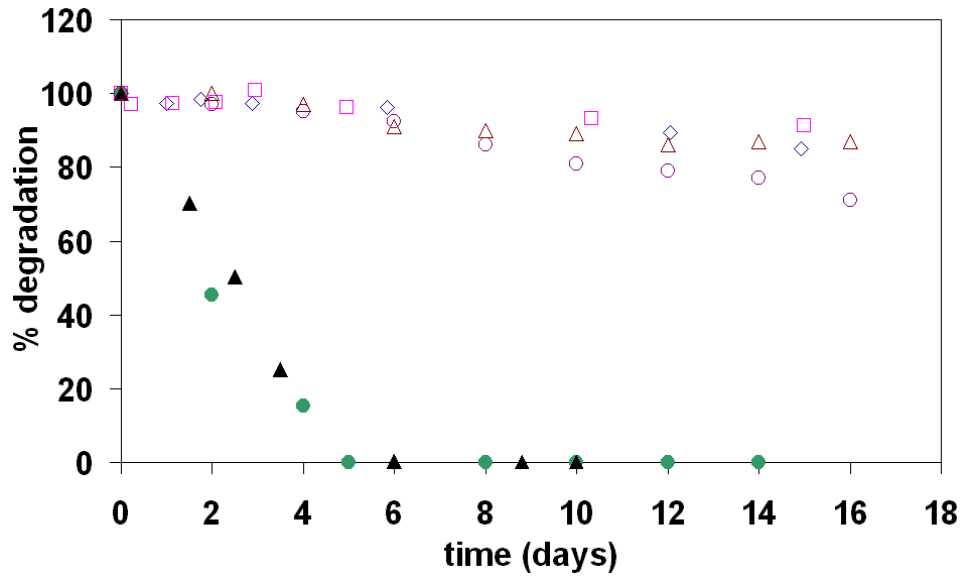

Figure 3: Stability profile of fermented and commercial geldanamycin in different culture solutions. Symbols: solution (a) open diamonds; solution (b) open squares; solution (c) closed circles; solution (d) closed triangles; heat treated solution (c) open circles and heat treated solution (d) open triangles.

4.4.3. In-situ product recovery using capsular perstraction

Due to the apparent degradation of geldanamycin in cultures, particularly during the stationary phase, it was decided to try to alleviate this problem by removing the compound in-situ from the producing environment. An earlier study showed how capsular perstraction could be used to extract commercial geldanamycin from Bennett's medium [4]. This work discovered how the main rate-limiting step to the mass transfer of the antibiotic into the liquid-core, was the stagnant aqueous layer around the capsules. Whilst it was not possible to control this resistance (maximum agitation speed

for cultures was at 150 rpm), it was however feasible to control the other resistances (size and membrane thickness) during the capsule production process [17], resulting in capsules with an outer diameter of 770.86 μm and an inner diameter of 667.2 μm. Such capsules enabled rapid removal of geldanamycin (< 60 min, results not shown); the membrane thickness also guaranteed mechanical integrity of the particles. The core material also ensured that capsules would float to the surface of the culture, thereby facilitating their removal for product recovery. In the aforementioned study two different solvents (dibutyl sebacate and oleic acid) were examined for the ability to extract geldanamycin. In the present work, oleic acid was selected due to its higher affinity for the antibiotic with a partition co-efficient of 212 [4]. Additionally, geldanamycin could be removed from capsules for detection and purification (crystallization), using a back-extraction method which was not detrimental to the capsules, thus allowing them to be re-cycled for future work [4]. Previously it had been stated that incorporation of an oleic acid liquid-core into an alginate hydrogel membrane was technically more challenging compared to incorporation of dibutyl sebacate [4,27]. This difficulty was overcome by adding a surfactant and heating the gelling solution, to reduce the surface tension of the mixture. This decreased the impact force of capsules at the interface between the gelling solution and the surrounding environment, thus preventing capsules bursting and liberating the core material, which further interferes with the production process, and is also very difficult to remove. A quantity of microcapsules containing 20 ml oleic acid was added to the culture on day 5 of growth of *S. hygroscopicus* cells. This volume was chosen as it had displayed the capability to recover > 1 g/l of geldanamycin from the culture. Capsules were not added during inoculation, since initial germination of the spores resulted in growth of hyphae around the microcapsules. This resulted in them becoming entrapped in biomass and this

affected sorption capacity. Post-inoculation addition after 5 days, allowed for the formation of mycelial pellets which did not adhere to the microcapsules. The low density of capsules enabled them to rise to the surface of the medium when agitating was reduced, while the pelted cells tend to sediment. This separation of the microcapsules and biomass significantly facilitates the recovery of capsules.

Figure 4 shows the effect of microcapsule addition on the overall net concentration of geldanamycin produced and recovered during batch growth of the culture. The results of the control fermentation (Figure 2) are also displayed in Figure 4 to simplify the comparison of results. From the Figure 4 it can be seen that the microcapsule assisted-fermentations resulted in a 30% higher maximum net concentration of geldanamycin compared to the control fermentation (143 mg/l to 110 mg/l). More important, the immediate in-situ extraction of the antibiotic resulted in the recovered material being stable in the culture environment over 24 days. It appears that the cell-secreted material(s) responsible for the breakdown of geldanamycin, were not extracted into the core of the microcapsules, thereby indicating selectivity of the capsules for geldanamycin. The addition of microcapsules to the cultures resulted in no great effect on the overall cell density obtained.

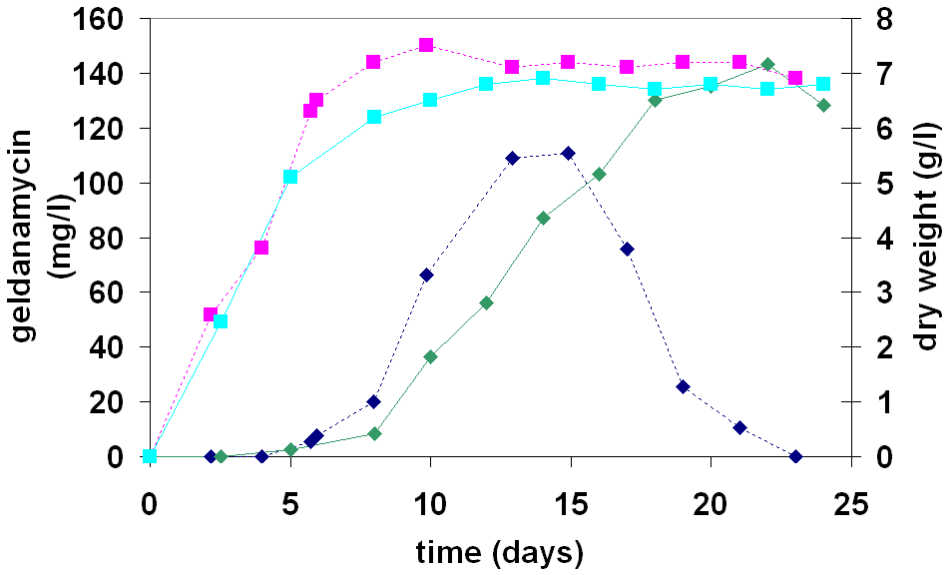

Figure 4: Comparison of the overall net production of geldanamycin and growth of *S. hygroscopicus* between cultures containing oleic acid liquid-core microcapsules (lines) and cultures containing no capsules (dashed lines). Symbols: cell dry weight (squares) and geldanamycin concentration (diamonds).

4.4.4. Recovery and purification of geldanamycin from microcapsules

Exiting streams from bioprocessing operations are usually characterized by relatively low product concentrations of high value products such as antibiotics, antibodies and enzymes, present in large volumes of a complex environment, consisting of medium, cell by-products and biomass [28]. Separation and purification of these compounds to the desired level often involves long, laborious and complex bio-separation schemes. This can severely affect the overall process economics, often accounting for 70-80% of total production costs [29-31]. In some cases such problems have slowed down large-scale manufacture and commercialization of medically important drugs, an example being Embrel [32]. In light of this problem, over the last number of decades there has been a keen interest and desire in developing efficient, simplified and cost-effective

downstream processing techniques, which require little or no change to presently used production equipment, whilst still producing highly purified biomolecules [13,14,29,33-36].

The aim of this part of the work was to develop a method for the recovery of the microcapsules from the fermentation medium, back-extraction of geldanamycin from the capsule-core, and purification of the recovered antibiotic in a crystalline state using straightforward techniques. Figure 5 shows schematically the proposed bio-separation procedure for the purification of geldanamycin used in this work, and is divided into five main steps.

Step 1: Separation of microcapsules from the culture: The incubation of capsules into the fermentation resulted in the total recovery (extraction) of 13.4 mg of geldanamycin (134 mg/l in the culture), contained within 20 ml of oleic acid, which was entrapped within the alginate membrane. Since the buoyancy of microcapsules has been shown to be controllable by the parameters used in the production process, it was possible to recover them by flotation with an efficiency > 98%. The recovered particles were washed with deionized water to remove any media components and cell by-products.

Step 2: Recovery of geldanamycin from microcapsules: The antibiotic contained within the oleic acid liquid-core was recovered by contacting and washing the microcapsules with acetonitrile saturated with oleic acid. The higher affinity of the acetonitrile for the compound compared to the core material [4] resulted in removal of 75% of the antibiotic from the microcapsules after just one wash. A further two washes with fresh acetonitrile solution resulted in a further 18 and 4% recovery, of the original geldanamycin, without affecting the structure of the microcapsules. The extracts were pooled to yield 60 ml of acetonitrile saturated with oleic acid containing > 94% of the antibiotic recovered from the culture.

Step 3: Recycling of microcapsules: For any downstream processing procedure, complete regeneration of the purification material(s) to enable re-use is an important consideration in reducing the overall cost of the process. As a result microcapsules from step 2, from which > 94% of the geldanamycin had been removed, were washed with small amounts of pure acetonitrile. This washing was carried out in ≤ 1 min in order to avoid extraction of the oleic acid core [4], while enabling removal of any fatty acid adhering to the exterior surface of the microcapsule membrane. Capsules were subsequently washed in deionized water to remove acetonitrile and to re-hydrate the membrane.

In order for the microcapsules to be used for future ISPR-cycles, sterilization is required. In an earlier study the capsules were heat sterilized at 121 °C for 20 min, which caused the shrinkage and weakening of the membrane [18]. To avoid these deleterious effects, the particles were sterilized using UV irradiation at 254 nm for 2-3 hr, before addition to fresh culture medium. This procedure resulted in no contamination being detected in the medium, whilst the microcapsules also retained their size distribution and mechanical strength (results not shown).

Step 4: Purification of geldanamycin in back-extraction solution: The geldanamycin back-extracting solvent from step 2 is composed of acetonitrile with traces of oleic acid, which needs to be removed for successful purification and crystallization of the antibiotic [4]. This was achieved by the addition of water to precipitate the oleic acid, which formed a separate phase, aided by centrifugation. From the 60 ml of back-extraction solution, a total of 4.8 ml of oleic acid precipitated. The addition of water to the acetonitrile reduced its affinity for the antibiotic. The $K_{oa/aw}$ value for geldanamycin between the acetonitrile:water phase and the oleic acid was calculated to be 148 using equation 1. Although the oleic acid has a significantly higher affinity for the antibiotic

compared to the acetonitrile:water solution, the majority of the antibiotic (> 81.5%) remained in the latter due to the considerably larger volume. Further water addition, filtration and cooling, performed to remove any residual oleic acid, caused further loses and resulted in 79.46% of the extracted drug being recovered. No effort was made at this stage to improve on the recovery, although suitable choice of conditions should result in higher recovery yields.

Step 5: Crystallization of geldanamycin: Low temperature distillation as performed in [4], was used in this stage and resulted in the crystallization of 71.3% of the antibiotic present after step 4, where < 1% of the antibiotic present at the time was degraded by the process. The recovered and weighed crystals were diluted in acetonitrile and HPLC analysis confirmed quantity and purity of the produced geldanamycin, with values being equivalent to commercially available material. Comparison of weight and concentration of crystals with commercial material, indicated that crystals were of a purity > 97%.

In conclusion, the utilization of this simple DSP methodology resulted in 53.28% (7.14 mg) of the geldanamycin extracted from the cultures (using capsular perstraction), being crystallized at high levels of purity.

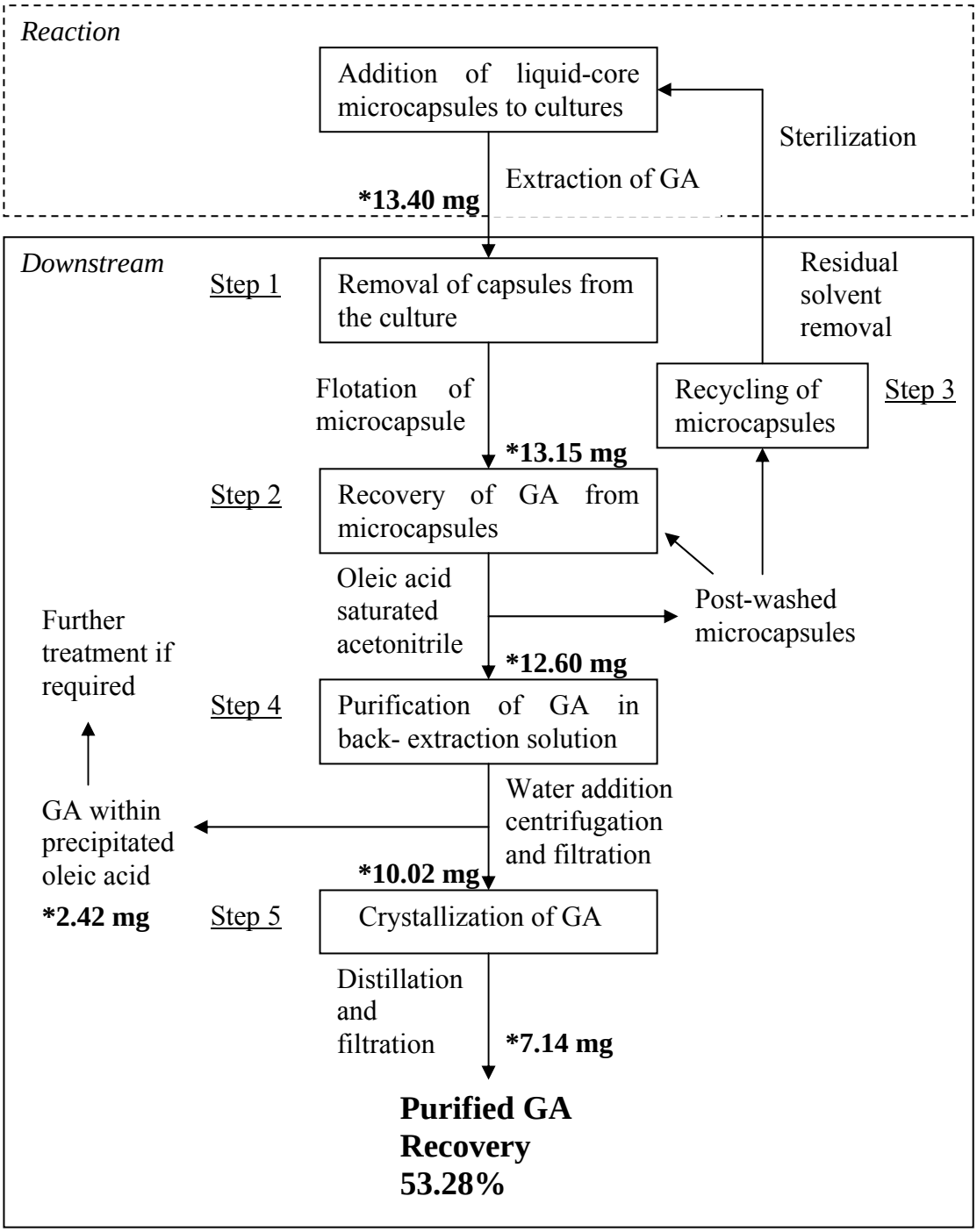

Figure 5: Schematic representation of the downstream processing operation used to purify geldanamycin recovered from cultures using capsular perstraction. The total amount of the antibiotic present after each step is indicated with an asterisk. Symbols: GA (geldanamycin).

4.5. Conclusions

Relatively low concentrations in production streams and/or complex and costly purification procedures have being a major problem for the production of biomolecules. Recent progress in the area of molecular biology and improved cell cultures techniques has resulted in production capacity increasing too many grams per liter. Unfortunately however, these advances still do not address the problems of product accumulation, inhibition and stability in the fermentation, or the requirement for expensive and complex purification methodologies. Indeed the recent advances in production capacities have somewhat further increased the problems for many DSP procedures, as these methods were not designed for the quantities now being manufactured. These complications are also amplified further by reluctance within industry to replace long-established techniques. The outcome is a production bottleneck that is shifting the cost further towards an already overly expensive DSP operation.

Since the first implementation of ISPR-based-methodologies in the early 1980s, it has long being anticipated that these techniques would help to overcome the problems of low productivities and expensive purification techniques, particularly for the manufacture of high valued products such as antibiotics, flavors and fragrances etc. However, over the last two-three decades this premature hope has not transpired into reality, and is often attributed to the ISPR-procedure, which in order for it to be successful can require complex and highly expensive modifications to existing production facilities.

This study demonstrated the feasibility and efficiency of capsular perstraction as a novel and innovative approach, to selectively extract large quantities of the high value product geldanamycin from cultures of *S. hygroscopicus*, whilst subsequently providing a stable

environment. Incorporation of oleic acid-containing microcapsules improved net production by 30% compared to control fermentations. These was achieved by removing the antibiotic from its hostile production environment, which was causing degradation and severely affecting recovery yields. The ability to subsequently use the ISPR-methodology as a DSP mechanism for the purification of the extracted geldanamycin using straightforward techniques was also shown and resulted in high recoveries (53.28%) of the antibiotic at high levels of purity (≥ 97%).

Although this work has concentrated on developing the concept at laboratory scale, the simplicity of the process and the relatively low cost of the encapsulation, extraction, back-extraction[1] and purification materials, as well as the low energy requirements, should make this process economically feasible for large-scale extraction and purification of biomolecules, using scale-up techniques. Nonetheless, further work is required to define the process economics. Applying microcapsules should not require any major equipment changes and in most cases could significantly reduce the volume of DSP equipment required for a successful production process. Addition of capsules to cultures will increase the working volumes of bioprocessing vessels, however this can be kept to a minimal by using an oil core which has a very high capacity for the compound of interest and/or placing the microcapsules in a recycle loop.

Due to the ease of operation, this procedure should be applicable as an ISPR-methodology to many similar processes, which are limited due to product degradation and/or low recovery yields. This process simply involves identifying a suitable organic solvent with a high affinity for the compound requiring extraction; thermodynamic tools

[1] Provided the price of acetonitrile continues to fall considerably, from the peak prices occurring in 2009 to pre-inflated levels of 2008.

such as Unifac and Uniquac could be used to facilitate this. This solvent can then be encapsulated within a porous membrane of choice, under sterile conditions if required.

4.6. Acknowledgements

The authors are very grateful to Dr. Vojislav Vojinovic, Dr. Lynne Dobson, Dr. John Casey, David Cunningham, Brian Freeland and Dr. Barry Byrne for assistance in analytical procedures and cultivation experiments.

4.7. Nomenclature

Table 1: List of abbreviations and symbols, ranked in order in which they appear.

Abbreviation/Symbol	Definition	Unit
ISPR	In-situ product recovery	-
S	*Streptomyces*	-
Hsp90	Heat shock protein 90	-
DSP	Downstream processing	-
HPLC	High performance liquid chromatography	-
$K_{oa/aw}$	Partition ratio for the distribution of geldanamycin between the oleic acid and acetonitrile:water phases	-
C_{oa}^{e}	Concentration of geldanamycin in the displaced oleic acid at equilibrium	mg/l
C_{aw}^{e}	Concentration of the antibiotic in the acetonitrile:water phase when equilibrium was obtained.	mg/l

GA	Geldanamycin	-

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Chapter 5

General Discussion, Conclusions and Perspectives

5.1. Vibrating-jet technique

Spheres/capsules in the micron size range have played a very important role in a vast variety of industries such as, pharmaceutical, cosmetics, agricultural etc., since the early to mid-twentieth century, with the food industry being one of the biggest benefactors. Such has being the success of this employment that in the 1970s scientists set-about in implementing the versatile technology to medical and biotechnological fields. Originally it was anticipated that similar achievement could be obtained in comparison to the aforementioned industries, when it came to developing new products, improving existing ones, or in some situations completely re-defining the role of a commodity.

Unfortunately in this period of time the technology has not reached what many would perceive as its full potential when being applied to these areas. This point is exemplified by the very few medical and biotechnological products available commercially which make use of the science. The problem has being mainly attributed to the slow development of methodologies needed to manufacture the particles at a large scale-level and bearing the required properties (Section 1.3.1.1.). This has culminated in many circumstances whereby a potentially successful product incorporating encapsulation technology has being developed, but no method is available to carry out production.

In recent times mechanical based techniques have been extensively researched on their capabilities to produce microspheres/microcapsules for biotechnology and medical applications. These procedures enable the production of particles with the desired shape and size, under a simple and relatively controllable operation, and do not require the use of toxic chemicals. A limit number of these procedures exist and include the following: spray-drying, jet cutting, spinning disk, electrostatic extrusion, coaxial air-flow and vibrating jet technique. Unfortunately, these methods possess the characteristic problems of being unable to produce small (< 100 μm) and homogenous shaped

microspheres/microcapsules at a large scale level using highly viscous (up to several thousand mPas) materials.

Of all six techniques discussed in Section 1.3.2., the vibrating jet technique seems the most capable of overcoming these obstacles[1]. The problem of extruding highly viscous solutions through the nozzle(s) and ensuring their optimal break-up when subjected to vibrational frequencies can be achieved by heating the polymer (using a heating nozzle/pulsating-head device as discussed in Section 1.5.4.2.) solutions to temperature ≥ 60 °C, and can significantly reduce their viscosity. This decrease enables the extrusion of the highly viscous solution through the nozzle, subsequently forming a jet, which can be broken up into droplets of the desired size.

Whilst elevated temperatures (≥ 45 °C) can be detrimental to certain animal cells, aroma compounds and bioactives, it most be noted that use of these temperatures at the short period of time required for the process to be successful, should not affect most materials. For heat sensitive encapsulants temperatures of 35-45 °C can be employed. This heat should be adequate to reduce the viscosity to the necessary level to obtain extrusion and adequate jet breakup, whilst not damaging the encapsulated product.

Use of the heated nozzle apparatus should provide an optimal method to encapsulate a large range of materials within the desired membranes, especially when viscous materials like gelatin's and gums are required.

As discussed in detail in Section 1.5.4.1., large scale production can be achieved by increasing the number of nozzles on the head plate of the encapsulator. For this to be successful, each nozzle must obtain identical production conditions, such as frequency, amplitude, etc., which can be accomplished in a relatively simple manner. However, obtaining similar liquid flow rates is somewhat more problematic. Fortunately this can

[1] A very good case could also be made for the jet cutting technique, provided cutting losses are kept to a minimal [1].

be overcome by employing a concentric split, which enables an equal flow to be distributed to all nozzles. Provided that all orifice diameters are identical, the relative flow difference between each nozzle should be negligible, if the polymer is pumped using a system incorporating air pressure (Section 1.5.2.1.). This delivery mechanism also enables large volumes of material to be extruded through the nozzle in any given run and reduces or eliminates clogging of the orifice, hence eliminating the need for the nozzle to be cleaned when operating.

It is envisaged that future use of the multi-nozzle system will enable the production of required quantities of microspheres/microcapsules for industrial use.

5.2. Removal of pharmaceutically active compounds from water

Pharmaceuticals are used extensively in the western world for the treatment of human and veterinary diseases with EU members alone using over 3000 different types [2]. Each year thousands of tons of these drugs are excreted from humans as un-metabolized or active forms into sewage water [3], passing onto sewage treatment plants (STPs), which are unable to adequately remove them [4,5]. Research performed globally has shown that the effluent water from STPs is one of the main point source of entry of these pollutants into the environment [4,6-12]. This along with improper disposal of medications contributes to the continuous contamination of receiving waters downstream from STPs.

The presence of pharmaceutically active compounds (PhACs) in different environmental water spheres is now seen as a real-time problem by many environmental scientists and agencies. This is due to the known high level of toxicity possessed by some of these compounds [13,14]. Also, continuous release of large quantities of these

substances increases the possibility of synergistic effects with other pharmaceuticals and/or chemicals released and/or present in the environment [13].

In chapter 2 a novel extraction methodology termed 'capsular perstraction' was proposed, and showed its ability to remove seven commonly found PhACs from water, by the simultaneous use of dibutyl sebacate and oleic acid liquid-core microcapsules. Results indicated that this innovative approach is capable of removing the compounds rapidly from water (> 50 min), with a variable efficiency (15-100%), even at relatively low agitation speeds.

The main hindrance to mass transfer was found to be the stagnant organic phase within the capsules themselves. Mass transfer coefficients were measured for the diffusion of warfarin into different sized capsules (with similar membrane thickness), and it was determined that an inversely proportional relationship existed between different capsule sizes and the extraction speed for the sizes examined in this work. Fortunately the size of capsules can be controlled during the production process (Section 1.5.3.1.), allowing the resistance to be managed. Even with this obstruction, the extracting particles are still capable of the rapid removal of the tested pharmaceuticals ($0.6 \times 10^{-6} – 2.28 \times 10^{-6}$ m/s).

Overall this process offers many advantages over conventional treatment methodologies like liquid-liquid extraction such as: high interfacial surface area for rapid mass transfer, reduction of stable emulsion formation, and most significantly they prevent further contamination of water by impeding the direct contact between the organic and aqueous phases.

Future work will involve screening for a solvent(s) which has the capability to recover a larger range of compounds at higher quantities in contrast to the solvents used in this study. This will reduce the cost of the capsule production process as smaller quantities will be required to achieve the desired removal of the pollutant.

For large scale implementation it is envisaged that capsules should be applied directly to the STPs as a tertiary treatment process, whereby they will remove unwanted pollutants from the effluent before release into the receiving waters. When applied, the purpose of the tertiary treatment process is to provide a final treatment stage in order to raise the effluent quality before being discharged. After primary and secondary treatments the water is usually stored in large lagoons containing thousands of cubic liters of water, and it is anticipated at this point the capsules can be added to extract the pharmaceutically active pollutants. Due to the high mass transfer area offered by the capsules; little agitation is required to obtain a suitable mass transfer, which significantly reduces the energy demand and cost on the system. After removal the pollutant containing particles can easily be recovered by sedimentation or flotation and the treated water can be released into the environment.

5.2.1. Future perspectives: Treatment of the extracted pollutants

Once the pharmaceutical containing microcapsules have being removed from the water the scenario arises of how to remove the pollutant(s) completely or to a recommended level. Ideally this technique should be a simple and rapid methodology, not damaging to the microcapsule structure, which will enable the extractive structure to be used repeatedly.

Wyss at al. (2006a) recovered the carcinogenic pesticide atrazine using liquid-core microcapsules. These were then used as a reservoir for the slow and controlled delivery of the atrazine as the sole nitrogen source to a *Pseudomonas* culture, enabling its growth by keeping the compound below toxic concentrations to the organism [15]. This allowed the *Pseudomonas* to utilize high quantities of the pollutant; subsequently converting (mineralized) it into carbon dioxide and water.

Whilst this process was effective it does suffer from some limitations. Significant amounts of the pesticide can remain within the capsules after the biodegradation process, with the problem being proportional to the initial concentration of the drug within the capsules at the start of the fermentation. When the microcapsules are re-used the pollutant left inside could affect the sorption capacity of the extractive phase during the next course of water treatment. Also disposal steps to discard the biomass produced by the *Pseudomonas* during the degradation process add additional steps (increasing complexity) and costs.

As seen in chapter 3 and 4 it should be possible to back-extract the removed pollutant into a solvent possessing a higher affinity for the compound compared to the core material. This can also help maintain the structural integrity of the capsules and a chemical treatment could be used to completely degrade the pollutant. However this approach could be problematic due to large quantities of exhausted waste reaction solution which could be left after the breakdown of the pharmaceutical, which must be disposed of correctly. This can again increase cost and complexity as well as leading to a heavy payload for the environment.

A possible solution to this disposal problem is to create a reactive capsular perstraction system which combines product extraction with simultaneous mineralization of the pollutant. This proposition could be achieved by incorporating a photo-catalytic mechanism such as titanium dioxide (TiO_2) within the capsule structure (Figure 1).

Photochemical oxidation processes involve using ultraviolet light (UV)-initiated oxidation-reduction reactions for the degradation of many different compounds [16]. The most widely used photocatalysts is TiO_2, mainly due to its desirable properties such as: (1) high chemical stability [17], (2) high catalytic activity under UV light [17], (3) relatively low cost [17], (4) low biological toxicity [17] and (5) its ability to be

implemented in reactions without the need for temperature control [17]. The method is based on the generation of a hydroxyl radical (OH^-) and other oxidant species at concentrations high enough to allow the oxidation of very stable organic compounds and pollutants.

The proposed systems could operate as follows: From Figure 1: liquid-core microcapsules containing TiO_2 embedded in the membrane could be manufactured using the vibrating jet technique and can be subsequently added as a tertiary treatment to remove organic pollutants from water as described previously. During extraction the capsules could be exposed to a UV light source, which passes through the membrane and interacts with the implanted TiO_2. This will result in the release of OH^- radicals, which mineralize the recovered pharmaceutical into non-toxic compounds within the core of the capsules.

It is envisaged that it could be possible to establish a system in such a manner that the core extractant has a higher affinity (partition coefficient) for the extracted hydrophobic pharmaceutical, but not the non-toxic and harmless degradation product(s), which would be released back into the water phase. The continuous breakdown of the pharmaceutical within the capsules will allow much high concentrations of the drug to be removed (controlled by the partition coefficient of the compound between the two phases). It could also enable similar levels of removal using a smaller number of extracting particles in comparison to a capsular perstraction process whereby no degradation of the pollutant occurs.

After complete or adequate mineralization, the capsules can be simple recovered from the treated wastewater containing the non-toxic mineralization products and safe-levels of the original pollutant, which is then released into the aquatic environment.

The successful removal of pharmaceutically active compounds (Chapter 2) and pesticides/herbicides [18] has being clearly demonstrated. It is envisaged that this technique should be capable of removing other persistent organic pollutants from effluent and/or environmental waters. Such pollutants detected regularly in the environment include hormones and steroids, such as 17β-Estradiol and 17α-Ethinylestradiol, as well as industrial dyes (Azo dyes), and the most common pollutants of all; synthetic musk fragrances and examples include Tonalide and Galaxolide. These compounds are found in personal care products, like cosmetics, perfumes, shampoos, detergents etc. The affects of these highly stable compounds on the environment is becoming an important matter given the large quantities of this substances that are being used[2], and because they are generally released directly into the environment without undergoing any major structural or chemical changes [19].

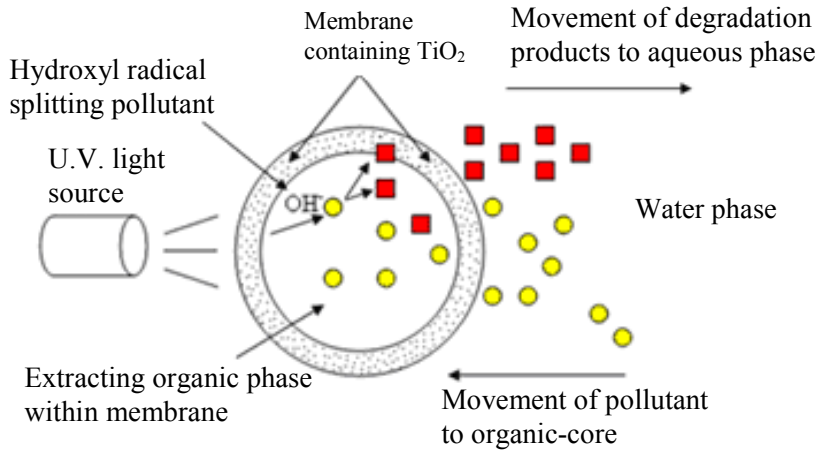

Figure 1: Schematic of the proposed reactive capsular perstraction system. The process uses liquid-core microcapsules incorporating TiO_2 to simultaneously extract and degrade pollutants from water. Symbols: pharmaceutical pollutant (circles) and degradation product (squares).

[2] In 1998 > 2000 ton were produced in the EU alone [19] .

5.3. In-situ product recovery of geldanamycin

The use of microorganisms to transform biological materials for the production of fermented foods has its origins in antiquity. Since then bioprocessing has being developed for an enormous range of commercial products; from relatively cheap chemicals such as industrial alcohols and organic solvents, to expensive specialty materials like antibiotics, therapeutic proteins and vaccines. The process is of tremendous economic importance which is evident in the US alone, whereby the predicted sales forecast for these products for 2010 is expected to be nearly $50 billion [20].

As discussed in chapter 3 and 4 bioprocessing operations are unfortunately adherent to many potential disadvantages during their workings, and is primarily caused by the buildup of the produced bio-product in the system [21]. On such example is the accumulation of geldanamycin. The polyketide antibiotic is derived from the shikimate pathway [22] and is produced by the filamentous organism *Streptomyces hygroscopicus* var. *geldanus* in submerged fermentations, where it is subjected to degradation to negligible amounts. The aim of this section was to develop and implement an in-situ product recovery (ISPR) technique, based on capsular perstraction, to help increase the productivity and recovery of the antibiotic from cultures.

The results of this study showed how capsular perstraction can be executed as an ISPR-technology for improving the productivity and yield of geldanamycin in fermentations. When dissolved in Bennett's media the sensitive product was removed rapidly (between 0.88×10^{-6} to $5.66\ 10^{-6}$ m/s), at high quantities (partition coefficient of 212), with a good specificity, whilst also been concentrated in the extractive phase at a substantially superior degree compared to the fermentation medium (> 1g/l). A similar extraction during geldanamycin producing cultures enabled the batch fermentations to be

extended, as the geldanamycin was rapidly removed from the vicinity of the producing environment which was causing its breakdown. Consequently a 30% increase in antibiotic production was achieved. The controllable buoyancy properties of the capsules also enabled easy recovery of the extractive particles by flotation, helping to improve handling properties of the operation.

More importantly, the selective removal and concentration of the geldanamycin within the capsules enabled the antibiotic to be purified (crystallized) to a very high degree using a very simple and effective downstream processing (DSP) operation. Application of this innovative procedure enabled the crystallization of > 53% of the antibiotic extracted from the fermentation at a purity > 97%, which has the potential to significantly reduce the overall manufacturing costs.

Higher recovery yields from the DSP operation could be achieved by treating the geldanamycin contained within the oleic acid after it is displayed from the acetonitrile during step 4 of the DSP procedure (Section 4.4.4.). Recently it has been discovered that this stage can be simplified even further by removing the water addition, and instead replacing it with a cooling step (results not shown). This procedure involves reducing the temperature of the oleic acid saturated acetonitrile to 2 °C, causing the fatty acid to completely precipitate from the solution. Centrifugation ensures the complete separation of this mixture into two distinct phases and enables them to be withdrawn independently. This has the following advantages to the existing step 4 and is as follows: (1) the absence of water means the acetonitrile solution has a higher affinity for the geldanamycin compared to the precipitated oleic acid (as seen in Section 3.4.9.) and enables higher levels of antibiotic recovery. (2) Oleic acid is denser compared to the acetonitrile. This causes the fatty acid to sink to the bottom of the holding vessel and allows easier removal of the geldanamycin rich acetonitrile phase,

which floats to the top. (3) Replacing the water addition greatly reduces the liquid volume to be displaced by evaporation. This significantly reduces the amount of energy required for distillation and also lessens the handling requirements.

There are several limitations which are blocking the incorporation of ISPR-methodologies as techniques in improving existing bioprocesses. In many cases the successful introduction requires the addition of extra equipment to the operations e.g. vacuum pumps, adsorption columns and membrane modules etc. This inclusion can very much increase the cost, making the process financial unviable and/or too complex. The inherent changes to incorporate the ISPR-technique in the bioreactor can also lead to the whole operation been only suitable for the production of one particular type of product.

In this study capsular perstraction has shown the capability to overcome these considerable limitations, whilst still transpiring as a simple, relatively cheap and effective technology. The procedure itself only requires a small increase in the reactor volume (which shouldn't affect other production operations) for enhanced results and this slight volume change could be reduced further by selecting a solvent which has a very high capacity for the extracted compound, hence reducing the volume even more. A clear role for the application of capsular perstraction as a facile, inexpensive and reproducible ISPR-technology for improving the productivity and yield of geldanamycin in fermentations has been shown in this work. It should be easily implemented at a lab-scale or industrial-level for the in-situ recovery of the antibiotic, but more importantly it could be used for the removal of many other compounds, which suffer from similar or other production limitations, from the vicinity of their culture environments.

5.4. Nomenclature

Table 1: List of abbreviations.

Abbreviation/Symbol	Definition	Unit
STPs	Sewage treatment plants	-
PhACs	Pharmaceutically active compounds	-
TiO_2	Titanium dioxide	-
OH^-	hydroxyl radicals	-
ISPR	In-situ product recovery	-
DSP	Downstream processing	-

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Chapter 6

Appendices

6.1. Appendix A

Determination of pharmaceuticals in water using RP-HPLC (Section 2.3.2.2.).

Compounds: Sulfamethoxazole, Metoprolol, Furosemide, Warfarin, Clofibric acid, Carbamazepine and Diclofenac.

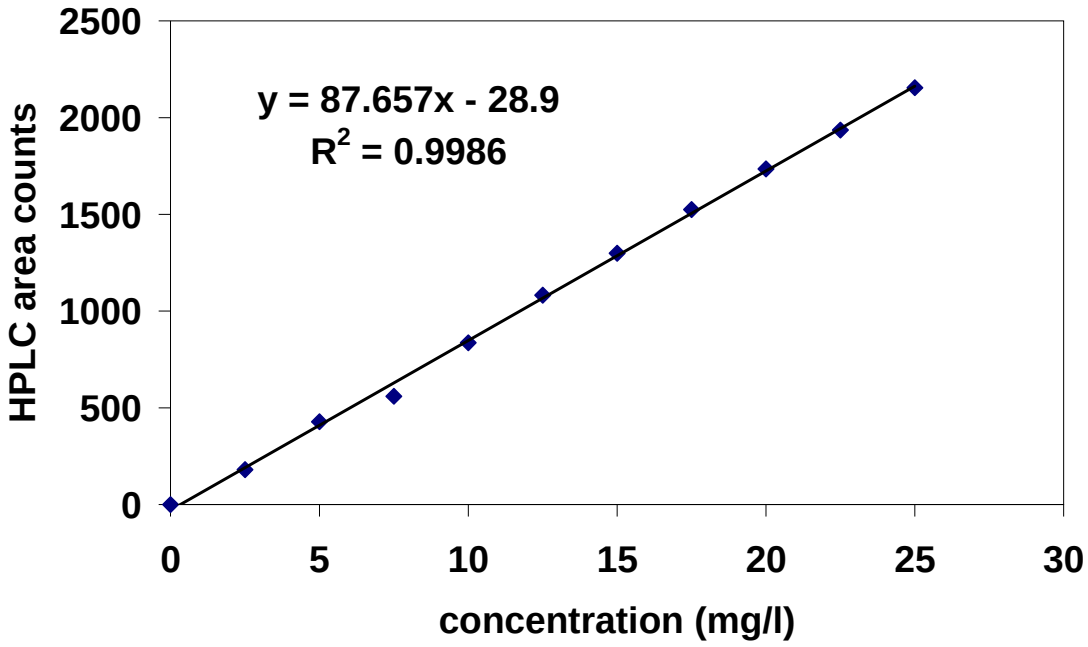

Figure 1: Standard curve for the quantification of sulfamethoxazole.

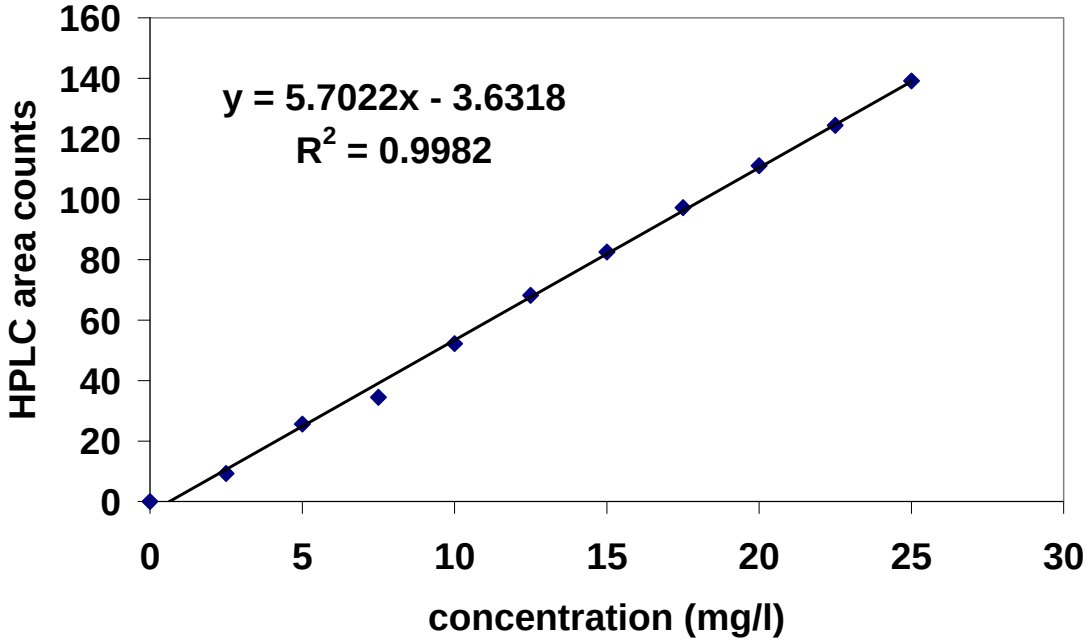

Figure 2: Standard curve for the quantification of metoprolol.

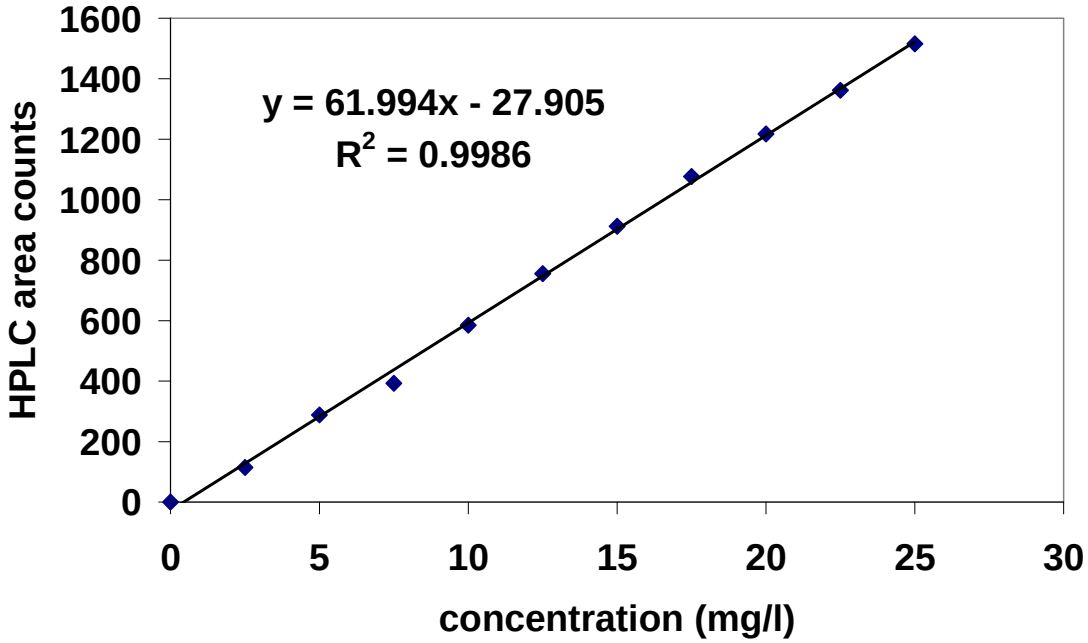

Figure 3: Standard curve for the quantification of Furosemide.

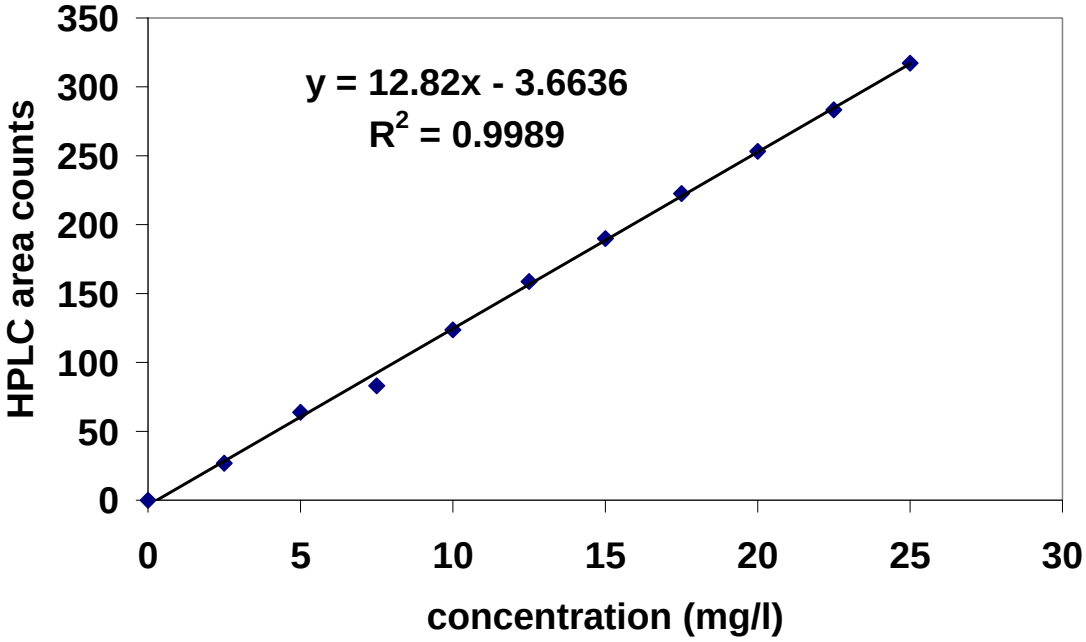

Figure 4: Standard curve for the quantification of warfarin.

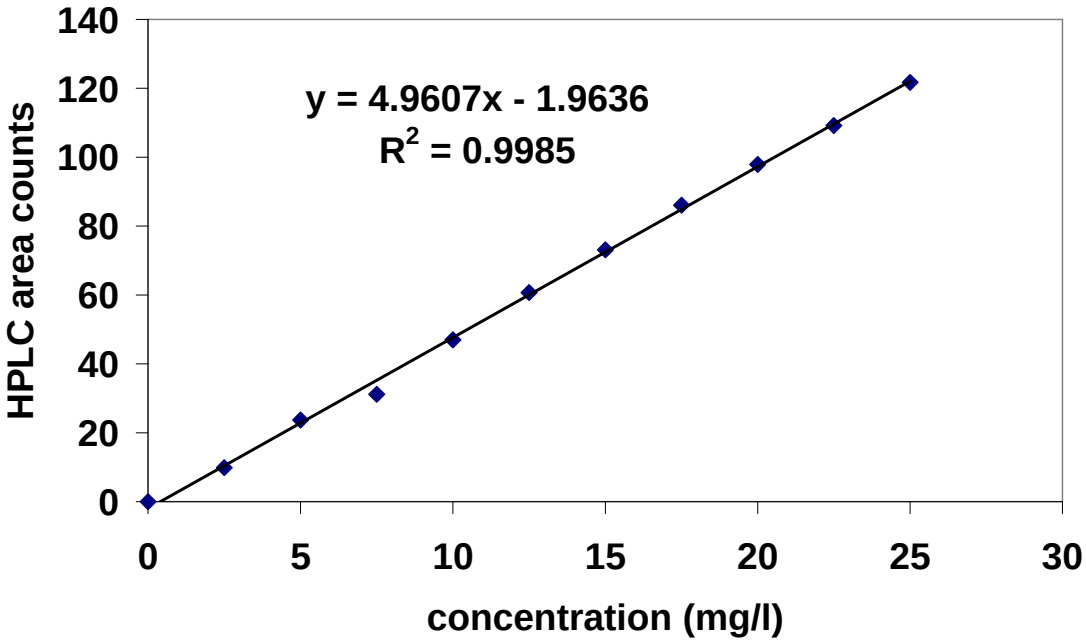

Figure 5: Standard curve for the quantification of Clofibric acid.

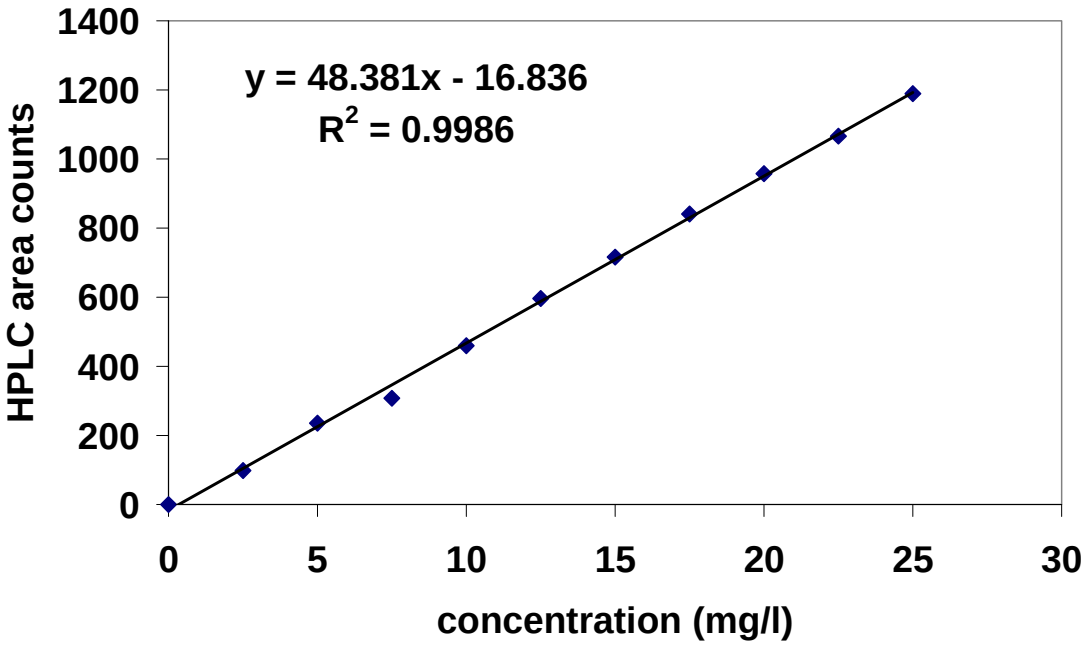

Figure 6: Standard curve for the quantification of carbamazepine.

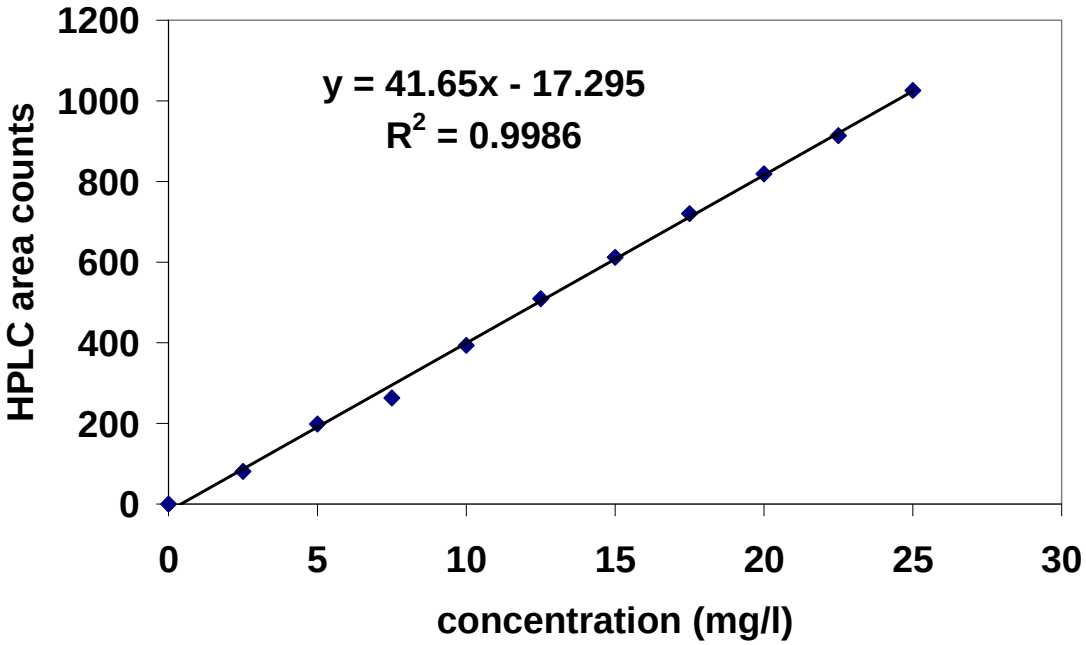

Figure 7: Standard curve for the quantification of diclofenac.

6.2. Appendix B

Determination of geldanamycin in different phases using RP-HPLC (Section 3.3.2.1.)

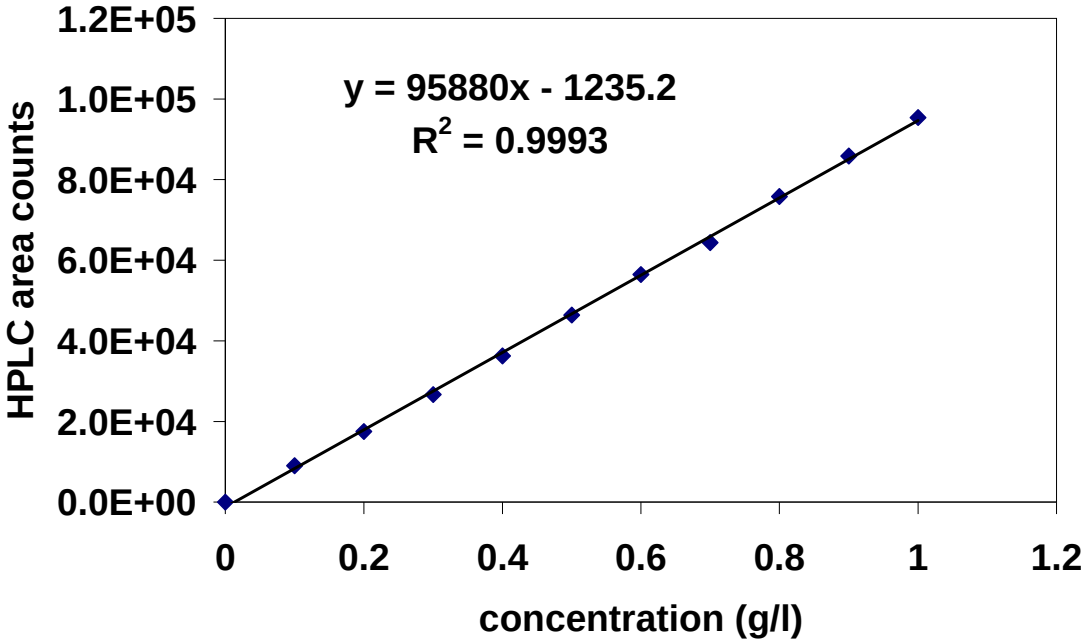

Figure 7: Standard curve for the quantification of geldanamycin in dibutyl sebacate. HPLC mobile phase: 80% methanol:20% water (v/v).

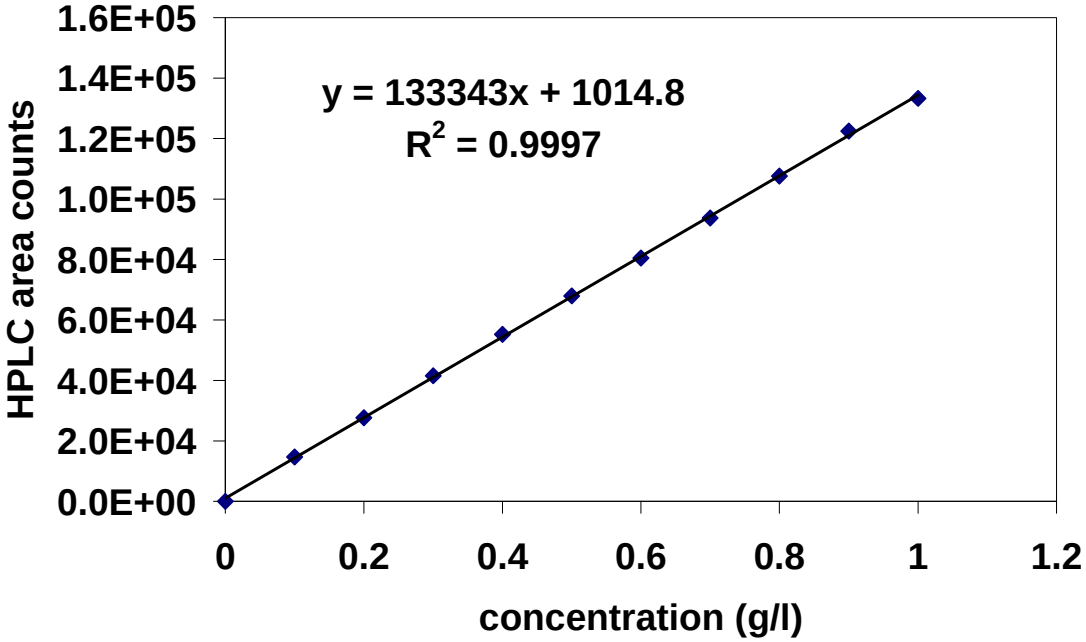

Figure 8: Standard curve for the quantification of geldanamycin in oleic acid. HPLC mobile phase: 80% methanol:20% water (v/v).

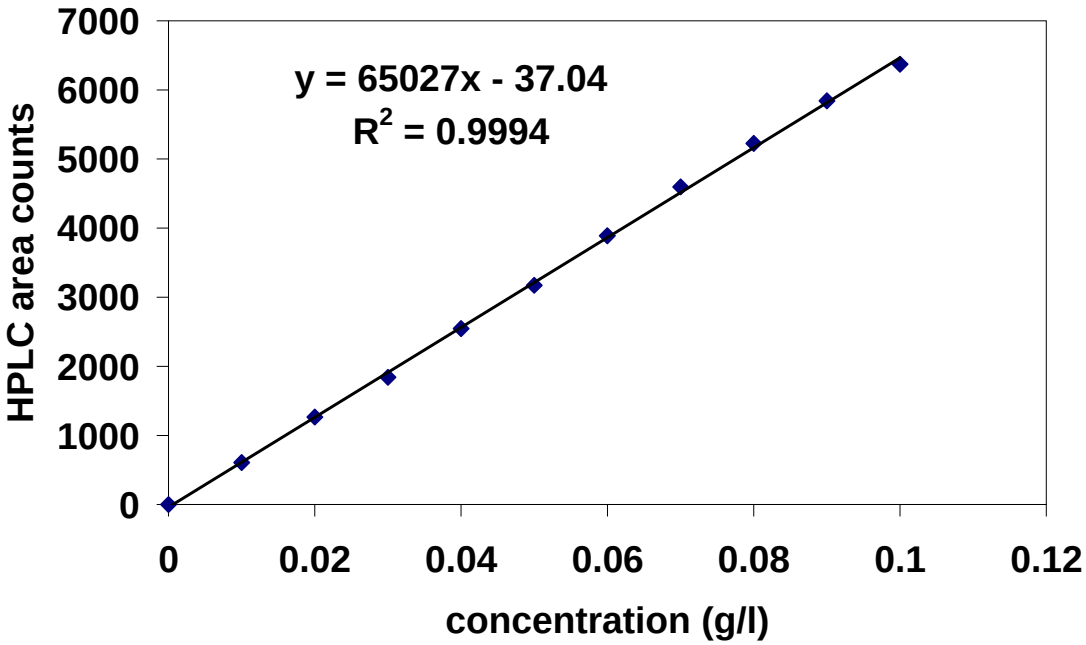

Figure 9: Standard curve for the quantification of geldanamycin in Bennett's medium and water. HPLC mobile phase: 50% acetonitrile:50% water (v/v).