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Tesi sperimentale per il conseguimento del titolo di Dottore di ricerca in Scienze e tecnologie delle produzioni agro-alimentari

Microencapsulation for tailored food using microfluidics in industrial processes

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ABSTRACT

Microfluidics technologies are of great interest on research due to their advantages and the possibility to use it on different industrial areas. For food industry could be an important innovation for the microencapsulation of aromas to improve flavors or mask disgusting flavors improving the palatability of the product or giving an added value to the product. The aim of this work was to develop a microfluidic device to encapsulate essential oil through a flow-focusing technique using mainly sodium alginate as matrix. Different devices were created to test and to compare the possibility of produce microparticles and the important parameters for the production of microparticles production using flow focusing. Due to the versatility of the devices, the diameter size of the droplets generated in different emulsion configuration such as (o-w) (w-o), is possible to reach a size of the droplets generated from 200µm (CV > 9%). A monodispersed droplets generation of proteins and pectin (P/P) using sunflower oil for their formation using an opposing focusing flows in a coaxial capillaries device and varying the flow ratio between P/P inner flow rate and oil and consequently controlling with precision the droplet diameter. Obtained droplets have dimensions from 200 µm diameter, showed good stability over time and good properties to be used as a material for active films production. Moreover, the microfluidic device was modified using 3 capillaries, by the central capillary was injected a first fluid as a core of the droplet (active principle) then, a second fluid recovers the (oily) core part of the disperse phase, a third fluid (sunflower oil) continuum phase is useful to focus the disperse phase generating the droplets.

For the microparticles production using sodium alginate, different geometries configurations were tested, trying to keep the simplest design that allows the scaling up easily for the industrial application. However changing the configuration allows to produce forms of microparticles of diverse kinds such as matrix, mononuclear, or multinuclear for instance. Although for practical uses the most of the tests were conducted in a configuration of the geometry to produce microparticles in a matrix form due to its simplicity. Also there are proposed different uses that can be applied easily, using the same technique and the same devices with a minimum of modifications that can be used in the food industry. Some examples are the mixing of solutions with accurate precision, cell encapsulation and the production of complexes of protein pectin. Another modification of the device was the use of air to improve the microparticle production. In this case, the alginate solidification of microparticles was performed using gellification with direct immersion on calcium chloride at relative high speed.

Finally, an industrial application into a baked-frozen bread was made to conserve the aroma and/or to mask disgusting aromas, preparing an emulsion with alginate, rosemary essential oil and surfactants such as Tween 20 and Span 80. The microparticles were prepared previously and solidified then were added to the bread and packed. Samples were analyzed using a gas chromatograph. The results did not shown any difference between the samples with alginate microparticles containing rosemary essential oil and the control samples prepared in the absence of microparticles. In conclusion, it is of great importance to know the different characteristics involved in this technique such as materials used, solution polarity, configuration of the geometry, emulsionant presence, but the most important consideration for the well functionality of the devices is the concentricity due to the nature axisymmetric of the system.

RIASSUNTO

La microfluidica è un campo di ricerca emergente che suscita un grande interesse. Le tecniche di microfluidica possono essere applicate in diversi settori industriali con notevoli vantaggi. In particolare, nell'industria alimentare puo essere una importante innovasione il suo utilizzo per la microincapsulazione di aromi al fine di migliorare o mascherare sapori poco gradevoli incrementando l'appetibilità del prodotto o dando valore aggiunto per il prodotto. Lo scopo di questo studio è lo sviluppo di un dispositivo di microfluidica per incapsulare oli essenziali mediante una tecnica di *flow-focusing* utilizzando principalmente alginato di sodio come matrice. Diversi dispositivi sono stati creati per testare e confrontare la posobilità di produrre microparticelle e i pui importanti parametri per la produzione di microparticelle (*droplets*) utilizzando *flow-focusing*. Dovuto alla versatilità dei dispositivi le misure dei diametri delle *droplets* è stato studiato mediante diverse configurazione, usando emulsioni come olio in acqua (o-w) e acqua in olio (w-o). I risultati mostravano che era possibile generare delle *droplets* partendo da dimensioni a partire da 200μm (coeficcente di variazione CV > 9%). Successivamente, una soluzione monodispersa di *droplets* a base di proteine/pectina (P/P) usando olio di girasole è stata ottenuta utilizzando *opposing focusing flows* con un dispositivo con capillari coassiali. In questo caso si facevano variare il rapporto tra il flusso interno di P/P e il flusso dell'olio, controllando con precisione il diametro delle *droplets*. Le microparticelle ottenute presentavano diverse dimensioni, a partire da 200μm di diametro e mostravano una buona stabilità nel tempo e delle buone proprietà per poter essere utilizzate come materiale per la produzione di *film* attivi. Al fine di apportare miglioramenti al sistema, il dispositivo di microfluidica è stato modificato utilizzando 3 capillari, nel capillare centrale si iniettava un primo fluido come nucleo del *droplet* (principio attivo), un secondo fluido veniva usato per coprire la parte centrale della fase dispersa, ed in fine un terzo fluido (olio di girasole) rappresentava la fase continua che focalizzava la fase dispersa e generava le *droplets*. Per la produzione di microparticelle di alginato di sodio, sono state provate diverse configurazione della geometria mantenendo il disegno più semplice che permetta lo *scaling up* per una applicazione industriale. Tuttavia, cambiare la configurazione permette la produzione di microparticelle di diversi tipi come matric, mononucleare o mulinucleare. Per praticità, i test sono state condotti usando una configurazione della geometria che produce matrici, questo dovuto alla semplicità della tecnica. Inoltre, in questo lavoro si propongono diversi usi che possono essere aplicate nella industria alimentare in modo semplice usando le stesse tecniche e gli stessi dispositivi con un minimo di modifiche, per esempio una miscellazione di soluzione con precisione, incapsulamento di cellule e la produzione di complessi proteine/pectine. In un secondo momento, veniva presa in considerazione anche un'altra modifica del dispositivo che prevedeva l'utilizzo d'aria per migliorare la produzione delle microparticelle. In particolare la solidificazione delle microparticelle di alginato mediante gelificazione veniva realizzata mediante immersione diretta in cloruro di calcio a velocità relativamente alte. Infine, è stata fatta un'applicazione industriale su un prodotto da forno surgelato al fine di conservare l'aroma e/o per mascherare sapori non gradevoli al consumatore. In questo caso veniva preparata un'emulsione a base di alginato, olio essenziale di rosmarino e diversi tensioattivi come il Tween 20 e Span 80. Le microparticelle solidificate venivano aggiunte al prodotto da forno conservato in buste di PET. I campioni venivano analizzati utilizzando un gascromatografo. I risultati ottenuti non mostravano nessuna differenza tra i campioni preparati con microparticelle di alginato contenenti olio di rosmarino e i campioni controllo preparati in assenza delle microparticelle. In conclusione, gli studi condotti indicano che nelle tecniche di microfluidica è di fondamentale importanza la conoscenza sulle caratteristiche dei materiali utilizzati, sulla polarità della soluzione, sulla configurazione del dispositivo e la presenza di emulsionanti. Tuttavia, per la funzionalità dei dispositivi, l'elemento che riveste maggiore importanza è la concentricità dovuta alla natura assial-simmetrica del sistema.

1. STATE OF THE ART

1.1 INTRODUCTION MICROFLUIDICS

The history of the microfluidics begins thanks to the microelectronic field. Although to the end of 20th century techniques such as gas chromatography that exploits molecular distributions between mobile and stationary phases within a column using theoretical work of Golay on gas chromatography, and van Deemteer on liquid chromatography improved their results reducing open column diameters and packed column particles sizes.

Microfluidics was born when scientist were seeking to reduce their analytical methods, obtaining the unification of bioanalytical and microelectronics sciences. In the Stanford University was created the first silicon-based device called GC air analyzer on a silicon wafer (Terry *et al.*, 1979) as shown in figure 1.

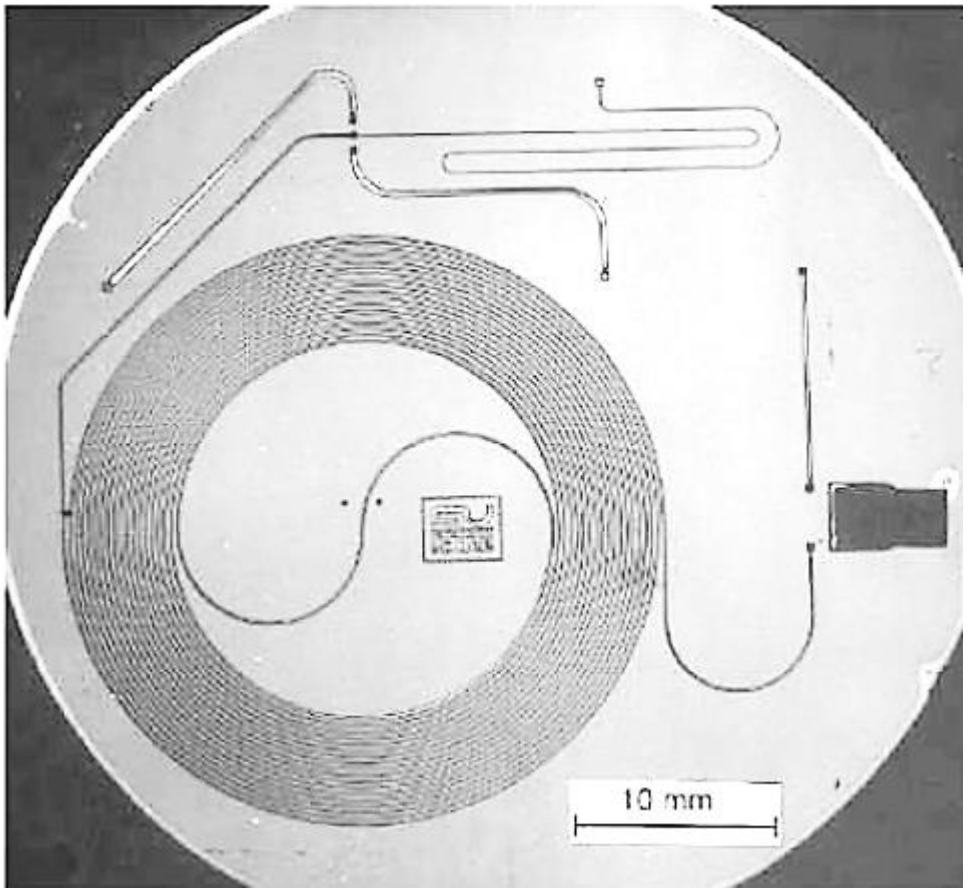

Figure 1. Example of the first silicon-based microfluidic device Gas chromatography.

The real boom of microfluidics was in 1990s pursuing many new concepts, not only separations of substances, establishing new applications with varied vantages over the macroscopic methods (table 1).

Table 1. Advantages obtained using microfluidics over the traditional Methodologies (Tian & Finehout 2008).

Microfluidic Advantage	Description
Less sample and reagent consumption	Microfluidic devices typically require $10^2 - 10^3$ less sample volume than conventional assays.
Enhanced heat transfer	Higher surface area-to-volume ratio of microfluidic channels increases effective thermal dissipation.
Faster separations	Higher E-fields results in faster sample migration.
Laminar flow	Low Reynolds number flows reduce sample dispersion.
Electrokinetic manipulation	Electroosmotic flow enables fluid pumping with flat "plug-like" velocity profiles solely via applied E-fields.
Lower power consumption	Fewer components and enhanced thermal dissipation require less power input.
Parallelization	Several assays can be "multiplexed", or run in parallel on a single chip.
Portability	System integration and reduced power allows for assays to be conducted using portable, hand-held device.
Improved separation efficiency	Efficiency in electrophoretic and chromatographic separations (i.e. number of theoretical plates) proportional to L/d.

1.2 MICROFLUIDICS TECHNICS

There are many microfluidics techniques that have being developed. One of the fields more developed is the MEMS devices sector born on the 1990s that constitutes an important piece of the new growing area of microfluidics. This are micro-electromechanical devices with an average size from 1 to 30μm, and without overlooking that the micro-fabrication are available to fabricate submicron MEM systems. There are many examples and applications fields being from food industries, pharmaceutical, automotive, and chemical, etc.

An example is shown on the figure 2. A gear of several hundred of micron size reached using the modern techniques such as: photolithography, etching, deposition, micro-wetting, or micro-impression. Another important example of development on the MEMS field is the inkjet printer head as shown the figure 3.

Figure 2. MEM device example a several microns gear microfabricate near to an ant.

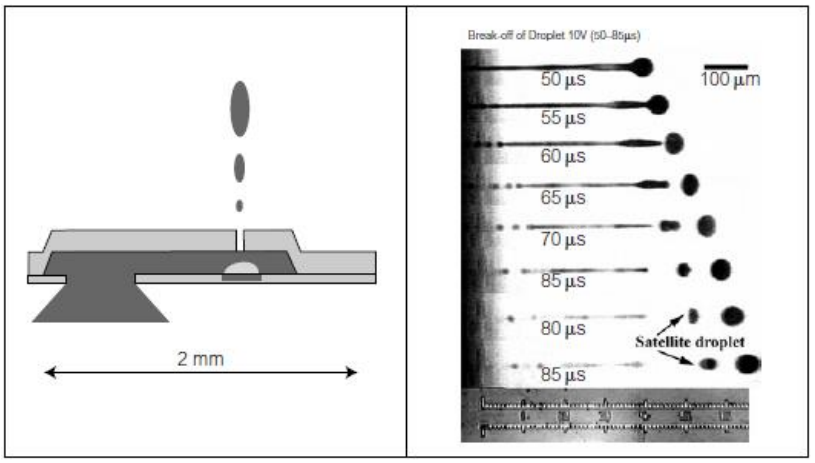

Figure 3. Example of an application of MEM head of Inkjet printer (left), droplets of ink emitted onto an object there are evident the satellite drops very important for the precision of the printing (right).

The lab on a chip devices are integrated to the modern chemical analysis laboratory with a high increase mainly in the biological field. This kind of devices gives the possibility to scale the processes of a traditional laboratory to micro scale having the possibility to integrate valves, pumps, fluid injection, interconnections etc. Due to the high level of control on the flow of micro-scale is possible to design highly complex microsystems adding many tasks over a large number of microchannels, an example of Lab-on-a-chip device is shown on figure 4.

There are some considerations of scaling laws in nature for plants and animal, their importance has already been reported by Galileo (Stone *et al.* 2004) Scaling effects were discussed in the past years, and in the present days these questions are still debated (Tabeling, 2005).

Figure 4. Example of device Lab-on-a-chip in a small size sell by Agilent Technologies for identify specific genetic sequences using just 1μl of sample in few minutes.

As mentioned microfluidic devices works with channels of just microns and they can be designed together to perform diverse functions. They are usually classified in two main classes: to perform chemical and biological assays, and to synthesize microparticles (Tumarkin *et al.* 2009; Dendukuri & Doyle 2009).

1.3 MICROFLUIDIC DEVICES PDMS

One of the techniques for producing microfluidic devices is using a polymer called Polydimethylsiloxane (PDMS), is a polymer used for photolithography that allows to reach the width down to the micron, allowing the flexibility for the design of channel for specific tasks. The concept is to fabricate three-dimensional microfluidic devices, the image is transferred to the PDMS using different tools such as photomask, silicon wafer photoresist as SU8 and applying the appropriate technique that consists in coating the wafer and heat it to evaporate the solvent, then is cooled to solidify the coating. Afterwards is placed the photomask over and the wafer is exposed to ultra-violet light. The photo resist is cross-linked, the regions that the photons penetrate hardens after the wafer is heated for a minutes, then is positioned in solvent to remove the unexposed material. This part is an inverse of the photomask that is a positive is usually called master. Finally more PDMS is poured over the "master" being baked and hardened.

The first drop formation geometry designed with PDMS is called "T-Junction". This device is designed to produce drops injecting a disperse phase from a side channel and injecting the continuous phase from a vertical channel forming the drops in the intersection of both channels, working with a low flow rates the viscous forces are small compared to surface tension low capillary number (Ca), drops are produced for the plugging and squeezing. The inflating tip of the dispersed phase blocks the downstream nozzle constricting the path of the continuous phase which continues to be pumped in, rising the pressure in the continuous phase that in turn, squeezes on the dispersed phase, eventually pinching off a drop.

1.4 CAPILLARY MICROFLUIDIC DEVICES

Another of the most used microfluidic technique is known as glass capillary. This kind of device permits the possibility to design diverse configurations of emulsions, and is used widely. Capillary microfluidics presents a way to control production of drops, one liquid in another immiscible liquid.

This classification of devices are designed in a coaxial configuration assembling glass capillaries. One of the advantages of this devices is their glass construction and the wettability that can be adjusted with accurate precision just modifying the surface using a surface modifier. Moreover using glass material in this device shows chemical resistance, and rigidity.

The process of construction of this devices begins with a circular glass capillary with a fine orifice. This precise capillary is inserted into a square glass capillary and this constitutes a simple microfluidic device is important confirm the coaxial alignment of both capillaries, selecting accurately the outer and inner diameter square flowing one fluid inside the circular capillary while flowing a second fluid through the square capillary in the same direction as is possible observe on the figure 5.

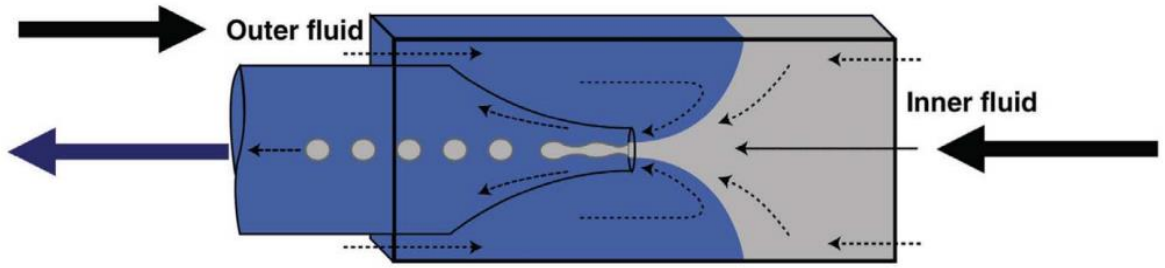

Figure 5. Scheme of glass capillary flow focusing device producing microdroplet in a dripping regime (Shah , *et al*., 2008).

Another characteristic is that if the fluid flows a low rates the production of monodispersed drops intermittently happens at the tip of the capillary orifice the process is called dripping, also if the flow rate increases to critical limits the result is a jet on the inner fluid forming the drops along the end of the inner flow we can observe two different transitions among dripping- jetting varying the flow rates because the different balance of forces over the jetting fluid, dripping is determined by the flow rate of the outer fluid when the outer fluid increase the drops decrease in size until the emerging fluid is stressed into a jet the breakup this dynamics occurs downstream at the end of the thin jet, for the jetting transition is determined by the inner fluid flow rate when it is rise the dripping drop is pushed downstream pinching off from the jet (Abate *et al*. 2007).

An alternative geometry is the flow focusing geometry (Gañan Calvo & Gordillo 2001; Utada *et al.* 2005). The main difference with co-flow both is that fluids are injected from the two ends of the same square capillary from opposite directions where the inner fluid is hydrodynamically focused by the outer fluid through. The narrow orifice of the tapered round capillary on dripping conditions the drop formation is effectuated when the inner fluid enter the circular orifice under jetting regime (Abate *et al*. 2007).

There are years of fabrication of microfluidics devices improving new materials, functionality being fast and cheap. The first prototyping materials were, mainly silicon derivate from microelectronic, using photolithograpy there still used the glass that have vantages over silicon for example the vision of that is happening on the circuit also the polydimethylsiloxane (PDMS) was used in microfluidics (Whitesides *et al*., 1995).

1.5 MICROFLUIDIC TECHNIQUE FOR FOOD INDUSTRY

Microfluidics technologies and applications are increasing each year in many and diverse sectors, essentially scientific and industrial. In food industries is very common to find works discussing about the substitution of the traditional laboratory methodology by modern microfluidic methods to save time, energy, reagents, etc. The figure 6 shown how is the trending on microfluidics on scientific papers also we can see in figure 7 how is the comportment for the publications about microfluidics in food field.

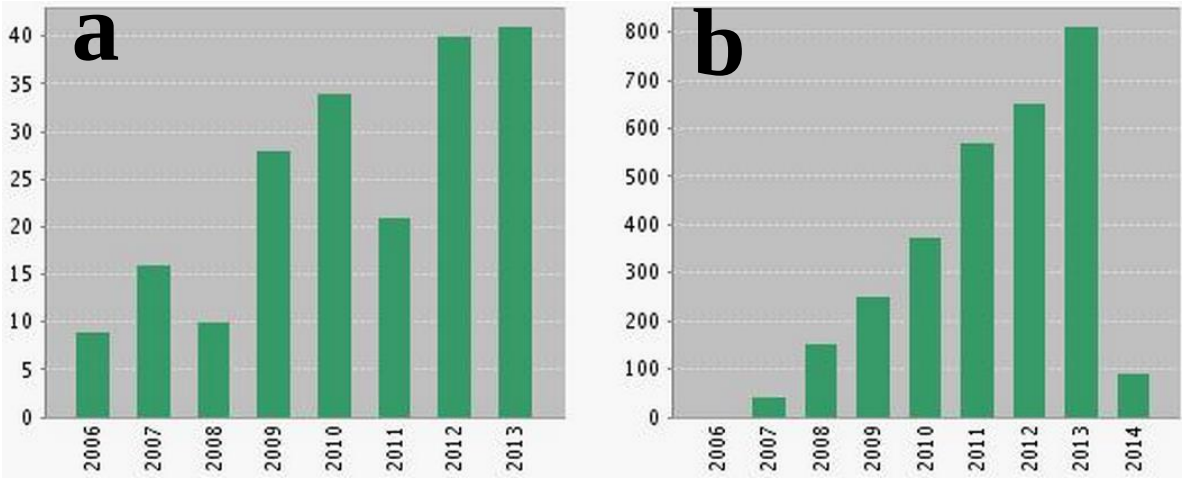

Figure 6. a) elements published by year b) citation by year, both graphics created by web of science of Thomson Reuters Research Science Engine 2014, using the word microfluidics as input for the research. Using the web of science tool is helpful to analyze the scientific interest for the subject, easily we can see the trend of the growth by each year the web address: http://apps.webofknowledge.com/.

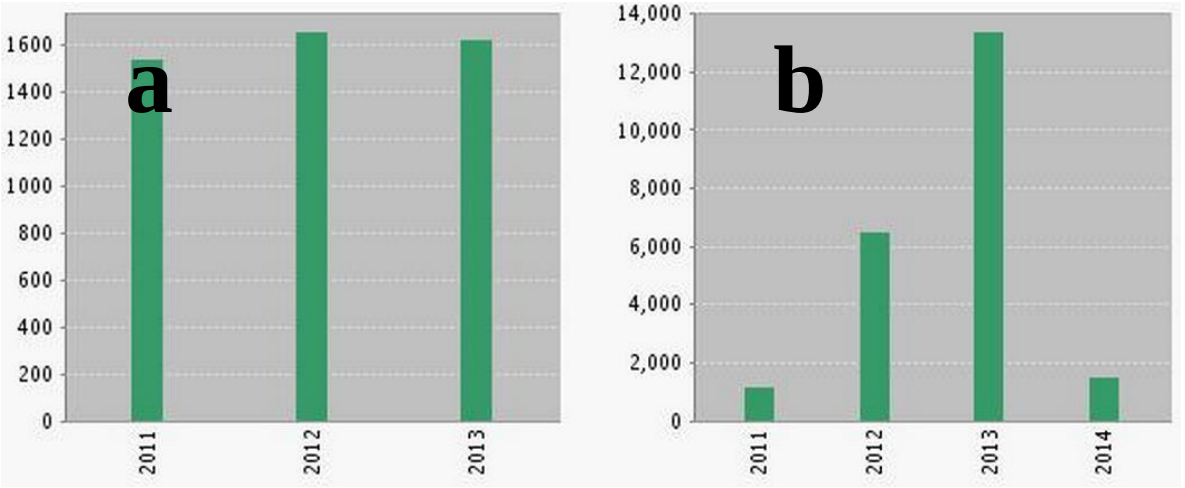

Figure 7. a) Elements published by year b) citation by year, both graphics created by web of science of Thomson Reuters Research Science Engine 2014, using **Microfluidics** and **Food** as input for the research. Using the web of science tool is helpful to analyze the scientific interest for the subject; easily we can see the trend of the growth by each year the web address: http://apps.webofknowledge.com/.

Methodologies adopted in the food sector are divided mainly in detection systems for food security, analytical determination of physical chemical properties such as biosensors, Lab on a chip, etc. Currently is common to use microfluidics circuit fabrication with the polymer polydimethylsiloxane (PDMS). This circuits can include mixers valves and pumps, using the soft lithographic procedures, thanks to this technique of fabrication is possible to try new ideas in less time (Whitesides 2006).

Microfluidics application for food engineering include the designing of innovative micro-structures concentrated at the quality, health and pleasure of consumers, where the unit operations are near to the structural elements for instance 1-100 µm. Flowing in devices with a dimension less than 1mm the laws on this devices not necessary are present the effects of unit operations as in macroscopical systems, the physical laws in the microfluidics are in dependence of the dimensionless numbers also in microdevices the rheological behavior of fluids of transient time is discussed.

All this kind of system can be useful in food processing, and food analysis the most kind of processed food for example frozen produce, emulsions, baked products are dispersed systems multi-components considering the phases that conforms the product (gas, liquid, or solid) distributed within a continuous matrix.

Often, phase dispersions are accomplished applying energy to the mixture, for instance the formation of ice crystals in ice cream (heat transfer to a cooled jacket), and dispersion of an oil phase into fat droplets in mayonnaise (shear action producer by a homogenizer) the size in those cases is larger, and inefficient using energy and is hard controlling the shape, size and distribution in the disperse phase.

Microdevices applications can be for example flow cytometry, multiple reactors, biosensor, genetic analysis, cell analysis, cell migration, drug screening or measuring molecular diffusion coefficient, fluid viscosity, density, pH, and special sensoring in food safety. Other kinds of microdevices are designed to generate emulsions and foams.

The forces that becomes important at microscopically level are the related to surface tension, energy dissipation, fluidic resistance, wetting phenomena all those are very important for the functionality of the devices. By the other hand the real applications on food industries have great availability using microfluidic devices are the emulsions and foams those dispersion of great use in food products including dispersions gas-liquid for example wiped egg white, head on beer.

Food emulsions can be prepared in two fundamental ways oil-water for instance salad dressing, mayonnaise, and water-oil for example butter, margarine. All kind of emulsions made industrially use energy through physical means such as mixers or homogenizer existing limitation about the quantity of power per unit volume.

Instead, manipulation of emulsions using microfluidic devices have the advantages of using efficiently the energy and controlling precisely the flows tailoring the emulsion controlling precisely their size, size distribution and foam properties can be designed also. Different tests demonstrates the feasibility to scale up reporting microdevices with the capacity to produce 3 kg of product by day as a real pilot-plant throughput, food scientists and food technologists to expand high rates that could inspire for practical applications monitoring the costs (Skurtys & Aguilera 2008). Furthermore, some authors have demonstrated the prevention of coalescence producing forming foam adding surfactants (Xu & Nakajima 2004).

Micromicrofluidics as emerging technology is revolutionizing food industries, includes nano and micro particle encapsulation or different substances, for example fish oil for monitoring pathogens or toxins, water supplies, micro-nano filtration improving food quality detection of antibiotics in dairy food. Also is generating innovative food structures thanks to the high demand for research in food field with low cost, safe food, drink, biomaterials turning raw materials in food or improving food quality, quantity, and safety all those challenges in food industry, innovation is needed for new processes, products and tools.

For instance a.k.a. micro total analysis systems (μTAS) and Lab-on-a-Chip (LOC) are the most important technologies because of their economic impact, and how we live or work offering very available applications in the food industry some other characteristics of microfluidics are the principles of operation using laminar flows, large surface-to-volume ratios, surface tension, capillary effects, refining methods for processing and analyzing complexes samples with a lower fabrication cost (Neethirajan *et al*. 2011) and operation.

In this way the technology is growing annually in a rate of 15.5% (Microfluidics Technology http://www.bccresearch.com/report/SMC036B.html) exceeding US$ 3billion in market revenues in 2014 (Emerging Markets for microfluidics aplications, http://www.researchandmarkets.com/reportinfo.asp?report_id=1081630) around 118 university research group worldwide. In the figure 8 and 9, we can see the trending and the forecast of microfluidics market.

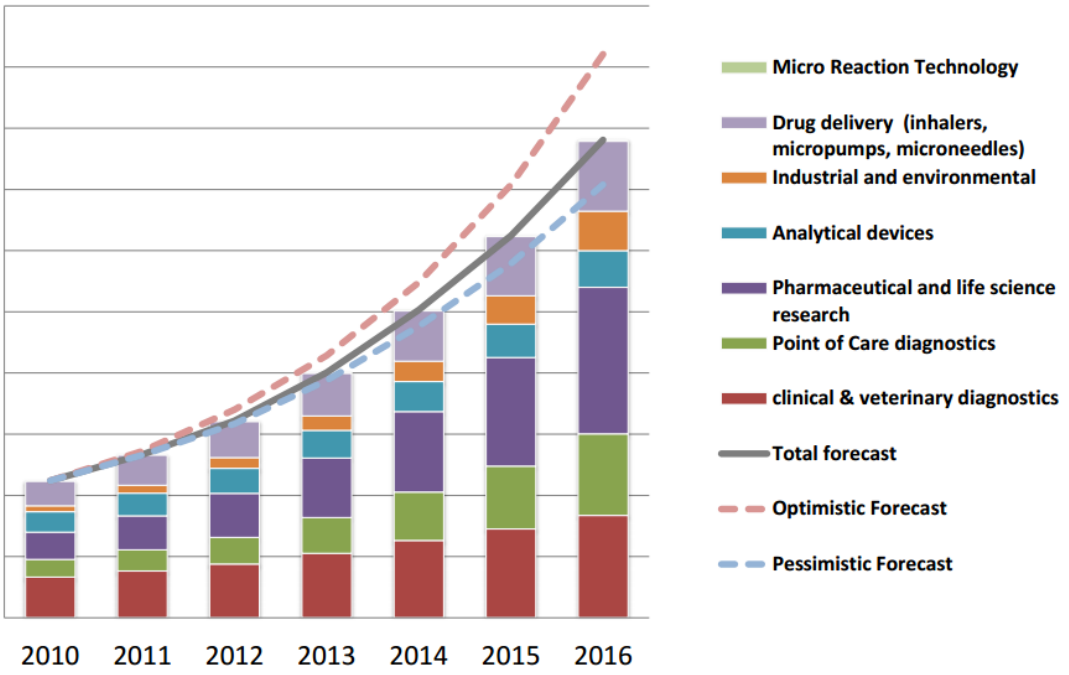

Figure 8. There is shown the classification by sector and their possible trending, interesting the pessimistically and optimistic forecast for the microfluidic devices market obtained from http://www.i-micronews.com/upload/Rapports/Yole_Emerging_Markets_For_Microfluidic_Applications_sample.pdf information obtained from (Yole Dévelomppement EMMA, 2011).

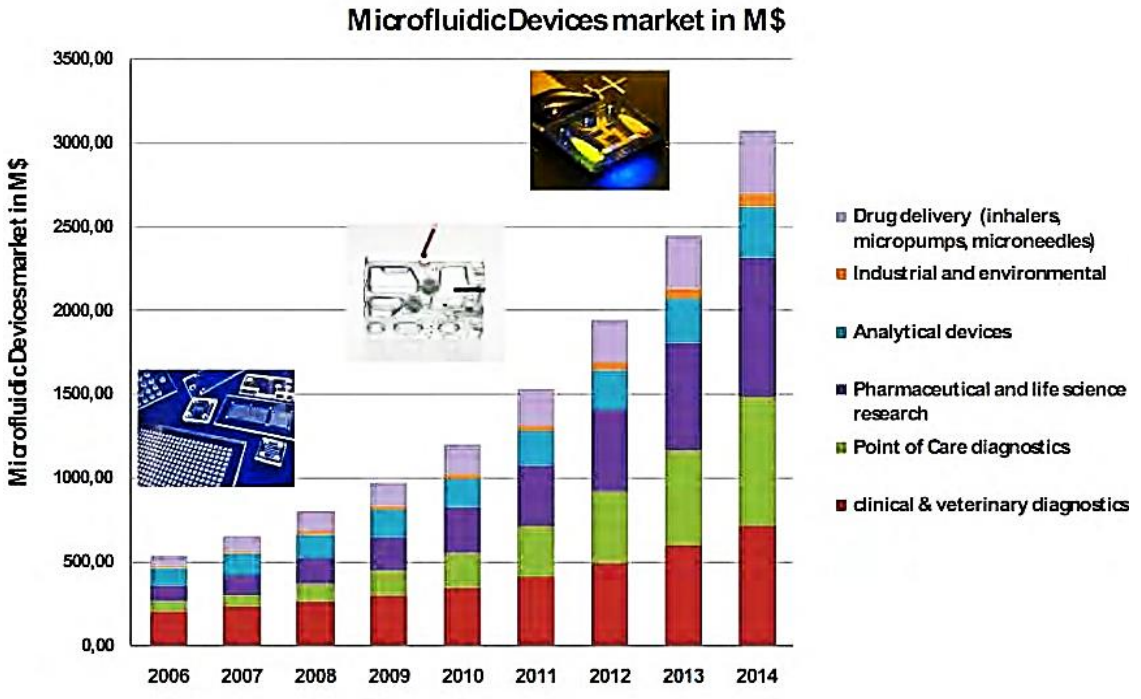

Figure 9. Evident the most growing sector on microfluidic technologies classification by function and their corresponding values estimated world market of about 3 billion dollars in the year 2014 (Argentiere *et al*. 2012). clearly there is a developing of applications and generating a wide range of industries, and research fields for the microfluidic devices market obtained from (http://www.yole.fr, 2012)

This information make evident the interest on microfluidic technologies for food industries, although are recent the applications still growing rapidly demonstrated by the number of publications and patents over the last decades.

Some others examples of applications on food industries are related to the toxin detection the contamination could be deliberate or incidental neuro toxin (BoNT) is a form of bioterrorism and concern to the US Homeland security, university of Wisconsin –Madison have developed a microfluidic device for detection in solution on-site reliable Botulinum Neurotoxin. Other important feature is the reduction of reagent consumption important for industrial processes also in food industries microfluidics has the capacity to generate new products and processes improving the microstructure physical and functional properties on the final product.

An important part in food industry is the dairy where liquids and solids are mixed and blended for several reason including dispersing gums and stabilizers in ice-cream mix or dairy products and dissolving salt and sugar in water to make brines. By the other hand the microfluidics devices working as an effective tool for mixing liquid-liquid, liquid-solids with effectiveness those that can be integrated with food processing equipment also for a large volume production could be more viable design modules instead to design stand-alone systems.

Is possible with microfluidic devices to obtain uniform droplets from 1 µm to 200 µm there are conducted tests using array to produce mass production using a great variety of food grade materials such as vegetable oil, medium chain-triglyceride oil, essential oils, hydrophilic emulsifiers, proteins. There are around 30 companies that work in microfluidic research, have vital importance make evidently the advantages and limitations of the microfluidics systems in food systems an example presented on the table 2 extract from (Neethirajan *et al*. 2011).

Table 2. Microfluidics systems are applied to improve some of the most important operations in the food industry. Food safety and food processing, marking the important limitations on each operation being more the advantages founded.

Application area	Examples	Advantages	Limitations
Food safety	Pathogen and antigen detection	High sensitivity High speed High throughput	Lack of interfaces for bridging microfluidic systems and electronic read-out instruments
	Pathogen sorting	Label-free miniaturization	
	Toxin detection	High sensitivity High speed High throughput	Lack of function integration
	Immunoassays	High sensitivity High throughput Low reagent consumption Ease of operation	
Food processing	Food mixing	High efficiency	Lack of control of infusion and mixing of liquids
	Calcium alginate gels preparation	High throughput Improved homogeneity	
	Microchannel emulsification	Operation Control	Not meeting the large scale production requirement
	Micro-nano-bubbles	Control of bubble generation	

There are more areas where the microfluidics could be applied in food industry processes in order to obtain a strong growth of this technology is necessary the academic and industrial research to adapt the technology in the food industry becoming in a useful technology easy to use and to the consumers reach another interesting topic could be reduce the microfluidic systems costs because the existent technologies are complicated or expensive, the future efforts can be focused on this subjects.

1.6 FLOW FOCUSING

Probably the first system of microfluidic that have shown the capacity for aerosol production and droplets called capillary flow-focusing was developed by Gañan-Calvo (1998). This technique produce monodisperse aerosol of droplets and suspensions in liquid (Gañan-Calvo & Gordillo *et al*., 2001).

This system consists in a continuous production of droplets that can work in different modalities such as mainly dripping and jetting where one fluid current induces a steady-tip streaming in a liquid meniscus attached to a feeding capillary focusing the inner fluid forming a meniscus tip across an orifice emitting a fine stream, the microjet breaks up because the axisymmetric perturbation downstream breaking in droplets (Plateau, 1849; Basaran *et al*., 2002; Gañan-Calvo, 2004; Eggers & Villermaux, 2008). In production of multiple emulsions is helpful this kind of systems works with axi-symmetric modality and low capillary number flows (Utada *et al*., 2005) or capsules (Takeuchi *et al*., 2005). Instead for the planar format of flow focusing reported by Shelley *et al.* (2003) there are different kind of geometries, but always keeping the main principles of the design.

Although the dripping mode of flow focusing is very advantageous for the reason that can produce relatively just low amount of monodispersed microdroplets by each singular device being necessary coupling several devices in serial or parallel to increase the droplet production or depending of the application requirements production.

There are another kind of processes to obtain a high number of microdroplets such as mixing and stirring (Freitas *et al*., 2005), but with this method is possible to obtain very low predictability of droplets size. There is too the jet-disintegration technique where a wide range of droplets sizes are obtained with different distributions depending as always in fluid dynamics of the values of dimensionless numbers in this case specially the Reynolds number that observes the inertia effects and weber number which compares inertia to interfacial tension (Baroud, *et al*., 2010), this important numbers where the jet more specifically laminar jets disintegration or Rayleigh breakup (Rayleigh L, 1878), where the droplets breaks up because there is a capillary instability a very important particularity of the capillary jetting is possibility of production of very small droplets, more than those produced by a dripping mode in similar working settings.

The main advantages of using flow focusing techniques are:

- Sterile conditions
- Accuracy in particle size
- Production temperature 25 °C
- Precise control of microjet diameter

Besides, there are some disadvantages on the use of flow focusing for food applications. As the drop-production rate is low and the droplet diameter scales depends of the diameter of the capillary or outlet, which makes it difficult to produce microparticles for a singular capillary device, but an available alternative to this is adding multiple devices and producing simultaneously the rate of production can be increased to industrial requirements levels making easy the scaling up of the devices.

1.7 MICROENCAPSULATION

Microencapsulation is a process that involves the incorporation of food ingredients or diverse materials in solid, liquid, gas, these materials should be protected from diverse agents that can damage the material properties. Encapsulation can include the entrapment of a pure material or a **mixture** into another material calling this modality in a matrix (Bernard *et al.*, 1999) or the complete entrapping of a core material within a matrix (shell) that can be porous or impermeable. This process can produce particles sized from 1 to 1000 mm. First, microcapsules produced were impermeable and had to be broken by mechanical forces to release their content (Whelehan & Marison 2011). Actually, this technique is applied to several areas such as medicine, agricultural, cosmetic, materials engineering and food industry.

There are several advantages on the use of microcapsules such as:

- Controlled release.
- Better conservation.
- Masking of odor and taste.
- Protection against physico-chemical agents.
- Immobilization of enzymes or microorganism.
- Easy and safety to manipulate.

In literature, it has been reported several microcapsules morphology that can be exploited for it application on different products. Microcapsules can be classified considering their structure (Fig. 10) as follows:

- Matrix: the core material is mixed with the shell material, entrapping the material at the same time that the shell material is hardened in that moment the material encapsulated remain in a matrix form (Bernard F. Gibbs, *et al*., 1999), microbeads or microspheres: particles are entrapped within a solid matrix and there is no a distinctive membrane. This is the most common type of microcapsules (Whelehan & Marison, 2011). They can be also irregular or non-spherical: furthermore can be mono, polynuclear or a solid particle entrapment.

- Mononuclear or single core: the core material is surrounded by a continuous membrane. It is the simplest capsule. Diameter of core can vary in size (Wyss, 2005; Kumar Ghosh 2006).

- Double and/or multi shells: in this case, it is a single capsule surrounded by another shell added to the original capsule. These kinds of microcapsules are used to modify the stability and permeability of the original capsule (Stark 2001).

- Polynuclear or multicore: these microcapsules contain two or more separate cores and it is usually formed in emulsions (Strand *et al*., 2004; Raymond *et al*., 2004).

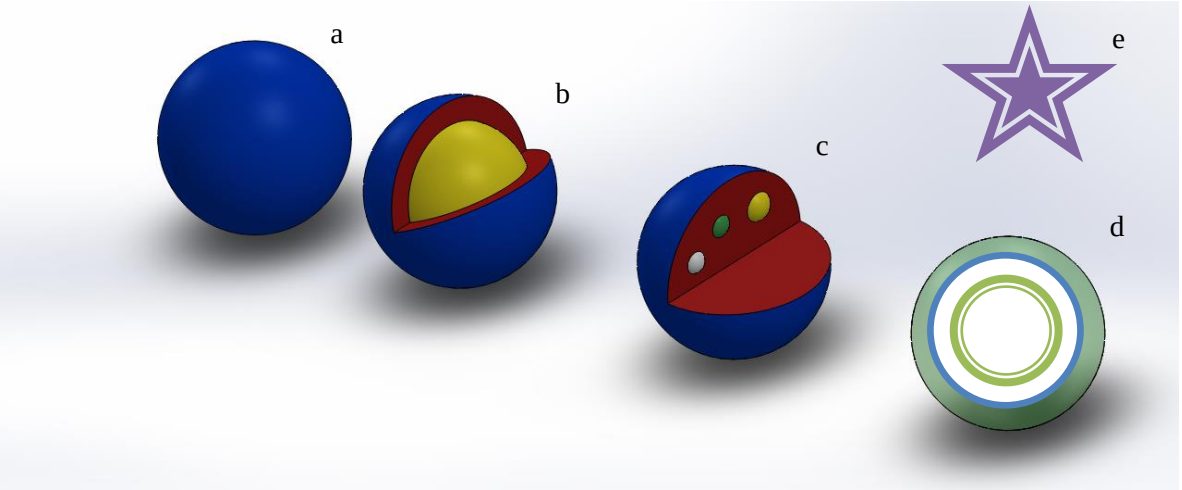

Figure 10. Diverse kind of capsules classified by their form, a) matrix, b) mononuclear, c) multinuclear, d) multi-wall, e) irregular.

Microcapsules can be produce using different methods (table 3) like chemical, physicochemical and mechanical.

Table 3. Different techniques to produce microcapsules (Kumar Ghosh, 2006)

Chemical	**Physicochemical**	**Mechanical**
Suspension	Coacervation	Spray-drying
Dispersion	Layer-by-layer assemblation	Simple dripping
Emulsion polymerization	Sol-gel encapsulation	Spinning disk
	Supercritical CO2-assisted encapsulation	Vibrating gel
		Electrostatic extrusion

The most common methodologies to produce microcapsules are:

a) Emulsification

This method is based on the dispersion of one liquid in a second immiscible liquid; including the core material in the first liquid it is possible to encapsulate a bioactive component. Actually, in industry the encapsulating agent is usually a molecule present in the food composition but can be improve by the presence of a surfactant (Augustin & Hemar, 2009).

b) Coacervation

This technique allows the formation of complexes by mixing the active compound with a matrix molecule of opposite charge. In this case, the complex size and characteristics is influenced by ion concentration, pH and mainly the ratio between the two components. Several authors consider this technique an immobilization rather than an encapsulation (Laneuville *et al*., 2006; Augustin & Hemar, 2009).

c) Spray-drying

It is a methodology easy and cheap it is one of the most common methodologies used to encapsulate. The procedure of this principle is the dissolution of the core on a dispersion of the matrix material; afterwards, the dispersion is atomized in heated air favoring the water/solvent evaporation. Particles obtained are in powder presentation.

d) Fluid bed coating

This is a modification of the spray drying technology. It is based on the suspension of the active food component in air and the matrix compound is sprayed onto the active components to form a capsule (Champagne & Fustier, 2007). In this case, the principle or active compound is always solid.

e) Extrusion technologies

The extrusion methodology involves the passing of solution trough nozzles of droplet generating devices to obtain small droplets. It has the advantage of the large scale microcapsules production (De Vos *et al*.,1997; Kailasapathy, 2002).

1.8 FLAVOR/AROMA ENCAPSULATION

During the last decades, flavor/aroma encapsulation research had an increase due to the correlation with food quality for the consumers. Normally these components are microencapsulated to protect them against chemical and physical degradation, to avoid its volatilization and to facilitate their handling and storage (McClements, 2012).

Flavor microencapsulation is not easy, because it must be consider diverse parameters. For example, it is preferred that molecule chemical composition does not change during the process to maintain it original flavor. Furthermore, it is important to load enough substance to obtain the

desired effect, and this depends on the nature of flavor and the product that will be apply on (Thies, 2007).

1.9 MICROENCAPSULATION WITH FLOW-FOCUSING

There is the possibility to microencapsulate diverse materials using the innovative flow focusing technique. There are two main options, the first uses a microfluidic circuit devices made with techniques of microfabrication using the polydimethylsiloxane (PDMS), the second possibility is the capillary device fabrication using glass capillaries, the droplet based microfluidic viable to produce microparticles from 10-1000µm, with very precise control of size and single or multi-phase core (Shah *et al*., 2008). On figure 11 there are some examples about the wide possibilities using microfluidics.

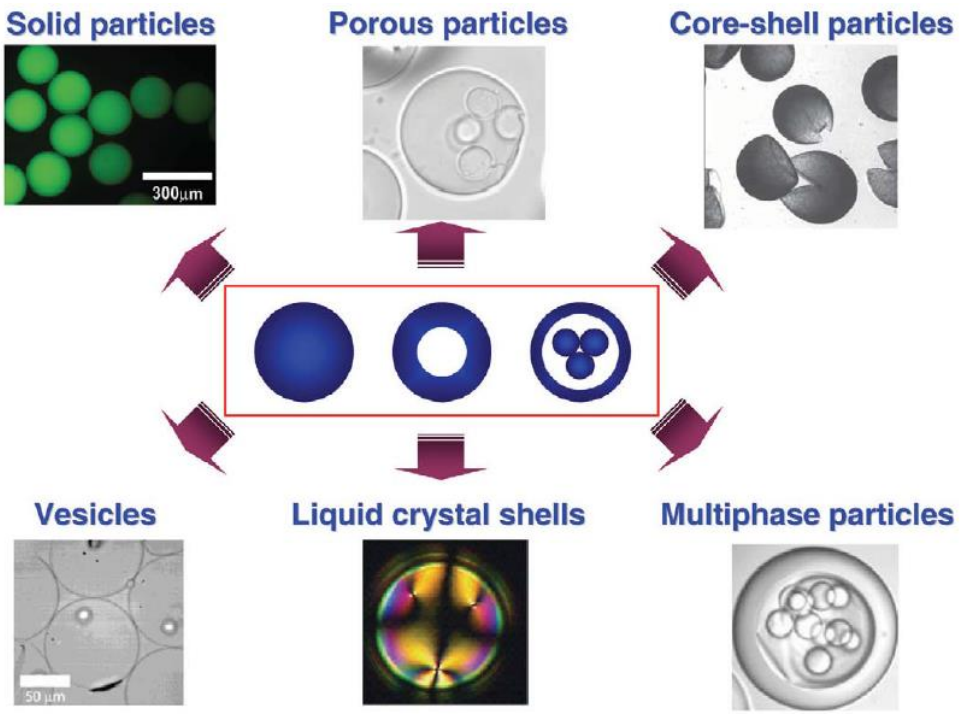

Figure. 11 Several possibilities of flow focusing microencapsulation each kind of particles for each specific use, those microcapsules produced using the flow focusing technique their functions depends of the material properties used as wall and shell (Shah, *et al*., 2008)

For the production of monodispersed droplets is necessary to apply an hydrodynamic or aerodynamic flow focusing to liquids or gases. Flow focusing is a method where two or more immiscible liquid or gas streams are coaxially focused the streams are forced through a small opening or orifice, an outer continuous phase has flow rate higher several times in magnitude than the inner disperse phase a central stream, that could be liquid or gaseous flow, is forced into a thin jet, like stream passing through the orifice, the central stream is forced to break up

into droplets, due to a rapid change in fluid pressure, and the prevailing shear stress of the outer surrounding covering flow. The continuous phase being respectively aerodynamic if there is using gas and hydrodynamic flow focusing if there is using on the system fluids.

Some of the main flow focusing advantages are the opportunity of droplet formation in a gentle mode, encapsulation of labile compounds, are not necessary surfactants use for droplet formation simple one-step additional procedures unnecessary, easy particle size set up adjusting fluid flow velocity phases, microparticles size are not limited by the device (orifice) size, easy scaling up, reduced cost of droplets and microspheres (Martin-Banderas, *et al*., 2005). Flow focusing requires the active components codissolve in the polymer solvent those fluids must to be immiscibly (Schneider *et al*., 2008).

A good manner for realize predictions on the droplet size, jet size, kind of flow inside of the system and other important parameters for the device design and the particle production there exists the possibility when is necessary calculating the dimensionless numbers, and others important parameters. We can observe the main dimensionless numbers, each one of those correlates the forces that are interacting over the system giving the possibility to improve the technique for production of microparticles, it could be used in designing foams or emulsion droplets and microparticles without forget that for each case and type of fluid are more parameters to take in consideration (Schneider *et al*., 2011), (Luque *et al*., 2009).

Reynolds number can indicate the kind of regime is working on the system there are Laminar, transition, or turbulent.

$$\mathrm{Re} = \frac{\rho D v}{\mu},$$

Capillary number help to understand the relationship between viscosity and surface tension.

$$\mathrm{Ca} = \frac{v\mu}{\sigma},$$

Weber number relates the surface tension and the pressure using the jet diameter to make this relation this number must be greater than 1. The figure 9. Show an image of the device highlighting the (dj) jet diameter (Schnider *et al*., 2011).

$$d_j = D\sqrt{\frac{Q_D}{Q_C}},$$

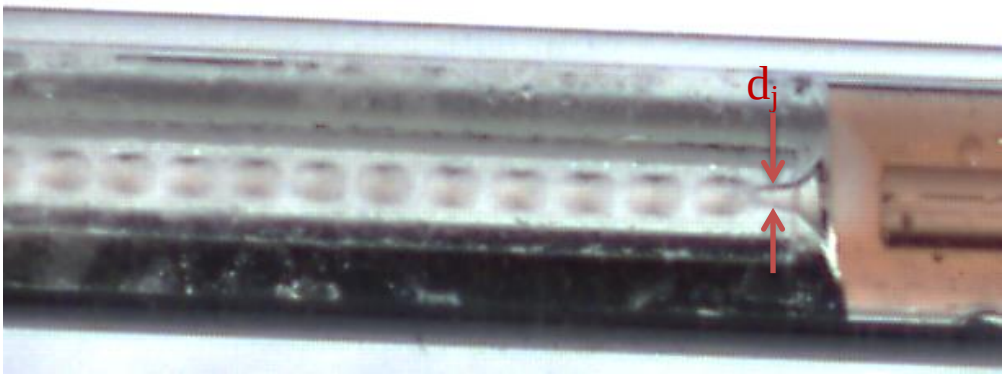

Figure 9. Is possible to observe on the figure the diameter of the jet formed near to the fluids interface this image was made using a CCD camera set up at 100 fps also it is possible to observe the droplet production after the break up near to the jet end.

$$We = \frac{d_j \Delta P_g}{\sigma},$$

Bond number it must be less than 1 it is possible reach this value if the inner capillary (outlet) diameter is less 200μm neglecting the gravitatory forces over diameter jet (dj).

$$Bo = \frac{\rho g D}{\sigma}$$

1.10 ALGINATE

Although the alginates are not appreciated for their nutritional values have other properties that can be very useful in diverse sectors of the food industry.

It can be utilized as an additive in many food products improving their characteristics, for example to regulate the viscosity (thickening), also as stabilizer/emulsifier gel former/binder or being an available option to create interactions with other constituents of the food product, for example proteins, fat, or fiber, can be utilized to stabilize and increase the mechanical properties of some restructured food.

There are reported many possible uses for alginates in food applications like in foods based on gels because it is simple to produce (gelation is independent of temperature) having an increasingly number of applications (Cottrell & Kovacs, 1980; Littlecott, 1982; McHugh 1987; Sime *et al*., 1990).

Restructuring process is based on binding together a deteriorated segmented crushed food to make it resemble the original.

Studies that involves gelling of bovine serum albumin (BSA) and sodium alginate demonstrated a considerable increase in Young's modulus within some ranges of pH and ionic strength (Neiser *et al*., 1997; 1998). The structure and ion-binding properties of alginates can be explained using egg-box model (Grant *et al*., 1973). This model is simple and intuitive as we can observe on figure 12, but has not been confirmed (Mackie *et al*., 1983). Viscosity measurements at different ionic strengths have been applied to approximate the chain rigidity of the alginate molecules (Smidsrød & Haug, 1971).

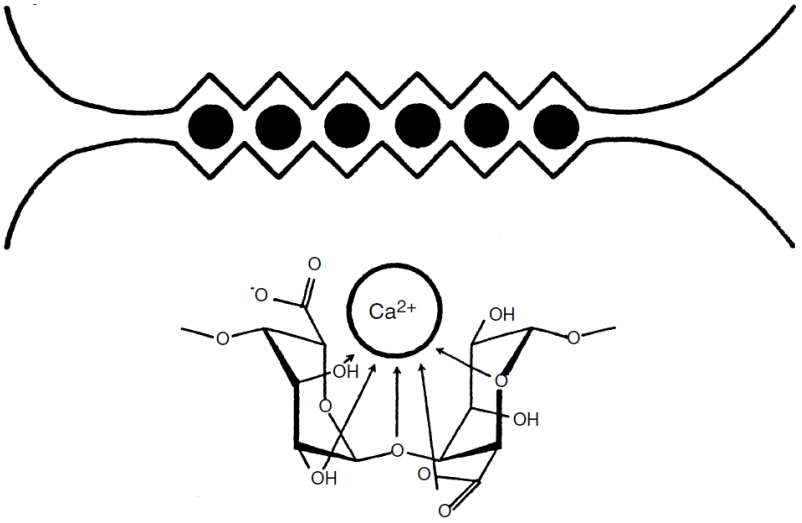

Figure 12. Egg box alginate ion binding

By the other hand gelling properties are guided by ion-binding properties. Alginates show typical ion-binding properties where the affinity for multivalent cation depends on their composition, as demonstrated by Haug (1964). Characteristic affinities were shown to be properties of polyguluronate, while polymannuronate was almost without selectivity. The affinity of alginates for alkaline earth metals increases as follows Mg, Ca < Sr < Ba, in which correspondingly alginates differ from some polyanions but resemble, for example, pectic acid, whose affinity follows the scheme Mg, Ca, Sr < Ba.

The selectivity of alginates for multivalent cations is also dependent on the ionic composition of the alginate gel, as the affinity towards a specific ion increases with increasing content of the ion in the gel "cooperativity" (Smidsrød, 1973). Also an important parameter for alginates is the dissociation constants (pKa) for mannuronic and guluronic acid monomers to be 3.38 and 3.65, respectively the values depends directly of the ionic strength of the solvent, and the alginate concentration (Haug 1964).

There is a importance in variety where were the alginates isolated from for example *A. nodosum* are generally more heterogeneous in polymer sequence; i.e., they contain considerably more of the alternating structure (MG-blocks) than the alginates isolated from *Laminaria* species, which are characterized by more homogeneous block structures (poly-M and poly-G) on the figure 13 is shown the alginate structure considering the differents configurations o M and G blocks.

The instability increase at pH values less than 5 and can be attributed to proton-catalyzed hydrolysis, whereas the reaction responsible for the degradation at pH 10 and above is the b-alkoxy-elimination alginate solutions are highly viscous (Haug, 1963).

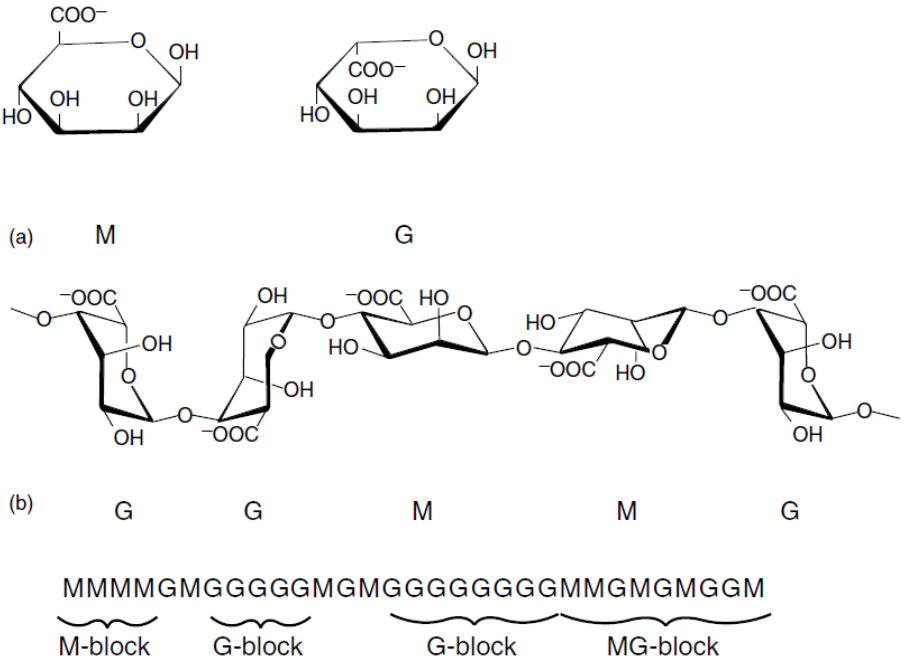

Figure 13. Structure of alginate we can to observe the different kind of configurations of blocks

As an example, a solution of 20 g/l of a low-to medium-viscosity alginate (h 6 dl/g) has a viscosity of about 300 mPa s (300 cp). Since alginate solutions in general are pseudoplastic is quantitatively valid only under the conditions given. For alginates with intrinsic viscosities below approximately 6 dl/g, the pseudoplastic effect in Ubbelohde capillary viscometers no. 2 at concentrations lower than 10 g/l is negligible, and the viscosity measured can be taken as the zero-shear viscosity.

Utilizing internal gelation of alginate has been applied by the food industry (Cotrell, 1980), (Sime, 1990). In general, this method uses an inactive form of the cross-linking ion, either bound by a sequestering agent such as phosphate, citrate, or EDTA (Skjåk-Bræk *et al*., 1986) or as an insoluble salt, for example, calcium sulfate or calcium carbonate (Skjåk-Bræk, 1991). An scheme of the more important reactions shown in figure 3.

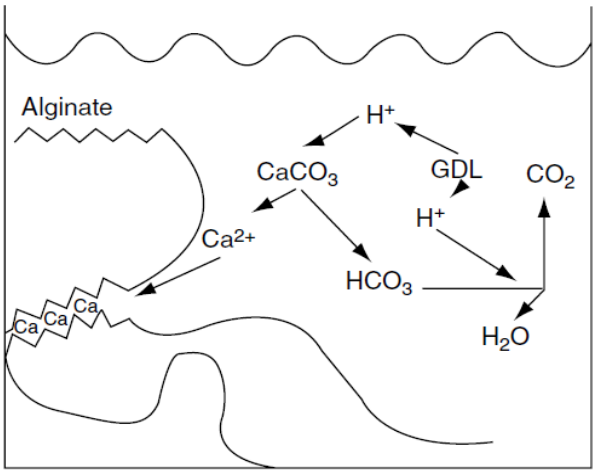

Figure 3. Scheme of alginate gel main reactions using D-glucono-δ-lactone and $CaCo_3$ Internal gelation (Draget K I, 2006).

The versatility of alginates, due to their wide range of chemical composition and their diverse functional properties, successful food formulations will only increase when full account is taken of the firm, basic understanding of the structure±function relationships in alginates.

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2. AIMS OF THE STUDY

The aim of this study was the use of a microfluidic technique that allows the production of microparticles using food grade ingredients, with the called flow focusing technique because of its versatility enables these objectives to obtain microparticles with a uniform shape and good monodispersity. There is possible to use this method to encapsulate different ingredients to improve or to add value to food products.

The main objective of this work was to design a microfluidic device for droplet production specifically a capillary device using flow focusing technique that will serve to encapsulate ingredients for the food industry.

Improve the production of microparticles avoiding coalescence of droplets produced by the capillary flow focusing microfluidic device designed previously.

Using this device directly in the production process of a frozen product, using microcapsules with essential oil, the encapsulation which helps to preserve the aroma even after freezing a food product seeking to maintain the aroma of a fresh product or by adding a value-added, helping to conserve a scent that can be difficult applied in their natural form as in the case of rosemary.

3. RESULTS AND DISCUSSION

3.1 Topic I Alginate Properties

3.1.1 Materials and Methods 1

Reagents. Sodium alginate Sigma and 6021 were a courtesy from Cargill (France), sodium alginate medium viscosity was purchased from Sigma-Aldrich W201502 (UK); sunflower oil was purchased on local market.

Materials. Picnometer blaubrand glass (Germany), pH meter metler-Toledo (Switzerland), Rheometer RFSII Rheometrics fluids spectrometrics (UK).

Surface tension measurement. Samples were analyzed using a tensiometer Sigma 700 (Finland) using the following parameters:

Temperature	20°C
Heavy phase	Water
Light phase	Air
Speed up	5 mm/min
Speed down	20 mm/min
Dwell down	5%

Each sample was processed 10 times. For interfacial tension was used the surface tension method adding into the flask one more phase fluid.

Density: Each sample was processed according with the standard method IUPAC 2.101 (1992).

Viscosity: The liquid is poured into the rheometer geometry (couette), parameters for rheometer test were setting up in a steady mode temperature from 17°C to 25°C, transductor 1 and 2 depending the viscosity shear rate, were programmed a delay before start the test a final frequency selected maximum 500 rad/s. Analysis of data were processed by software TA Orchestrator Ver. 7.2.0.4, TA instruments-Waters LLC, fitting curves of each test was selected for each specific fluid.

pH. After the calibration of the pH meter using the standard solutions pH 4.0 and 7.0 pH, after is placed the sample in solution covering the sensor of the glass potentiometer until value differ less than 0.1 pH.

3.1.2 Results and Discussion 1

For microparticles production was necessary to prepare different solutions of alginate, and is also important to know their physical characteristics to know how to anticipate their comportment. Using this test to improve the microparticles production, it is possible to optimize the functionality of the microfluidic devices.

The pH is one of the most important parameters to know about the sodium alginate solutions, we can find on the Table 1 those values, using the available concentrations of alginates.

Table 1. pH determination for different alginate solutions.

Alginate Type	Concentration (w/v) %	Temp (°C)	Results pH
Sigma	1	25	6.68
Cargill 6021	1	25	6.61
Cargill S505	1	25	6.63
Cargill 3001	1	25	6.60
Sigma	2	25	6.56
Cargill 6021	2	25	6.52
Cargill S505	2	25	6.57
Cargill 3001	2	25	6.54

pH for sodium alginates have a great importance because it is related to the gelation process, which can start with the reduction of the pH value from 7.5 to 6.5 releasing calcium from an insoluble complex for instance (Poncelet *et al*., 1995).

Surface tension is another important parameter in microfluidics for a new device design or fabrication. It is necessary to consider the transport processes, and the device materials or elements to use such as plastic, glass or any synthetic polymer.

Interfacial tension is also of great interest because there are interphases presents in the systems like gas-liquid or liquid-liquid acting in the fluids motion in the small-scale devices mostly times intervenes pressure differences, capillary driving forces due to wetting surface by the fluids. In a free surface flow driven by gradients (Marangoni flows), there is the possibility to be adjusted using the dependence to the surface tension with the mentioned parameters.

Is of crucial importance on the surface tension (Stone and Kim, 2001), to know the effects on the microfluidics devices, there are even studies which have developed microfluidic devices where their working principle is based mostly in surface tension effects (wetting and capillary effects). Just using this effects, exists the possibility to guide the fluids into devices are studied, this technology is call actually surface-tension-confined microfluidics (STCM) (Lam *et al*., 2002).

Our results demonstrate that alginate type and concentration has an effect on surface tension measurements (table 2). In fact, for most of the samples, was less than 60 mN/m, while just Sigma alginate have the different value.

Table 2**.** Alginates surface tension determination.

Alginate	**Concentration (w/v) %**	**Temp (°C)**	**Surface tension (mN/m)**
Sigma	1	25	53.54
Sigma	2	25	55.40
Cargill 6021	1	25	69.56
Cargill 6021	2	25	69.34
Cargill S505	1	25	69.10
Cargill S505	2	25	63.93
Cargill 3001	1	25	62.96
Cargill 3001	2	25	60.92

The interfacial tension is a very importan parameter as mentioned before due to the wetting effects and mainly in this case for the microparticle production device understanding the role that involves the materials and fluids that flows inside the device that intervenes in all the process of microparticles production.

The comparison between different types of sodium alginates are very important because with the parameters we can understand the comportment of the fluids inside of the device. Interfacial measurement was effectuated only for the available sodium alginate solutions that had the best performances on the other tests (Table 3).

Table 3. Interfacial tension determination

Alginate	**Interfacial tension (mN/m)**
Sigma 1%	27.79 ± 0.19
Sigma 2 %	20.34 ± 0.45
Cargill 6021 1%	25.73 ± 0.08
Cargill 6021 2%	29.96 ± 0.96

The microparticle production happens using an interphase system, so it is necessary to know the interphase interaction between the fluids that are forming the system. Thanks to the fluid properties such as immiscibility and solubility, in order to predict setting up the microparticle production, is convenient to characterize the fluids, and the interfacial tension is one of the fundamental parameter to determine.

We can observe a clear example in the electrowetting a microfluidic technique were an electrostatic modulation of the interfacial tension between a solid electrode and conducting liquid phase that could happen using modulated electrical fields with a high accuracy. Can be used in optical application varying the shape of a liquid lenses, viability was also demonstrated in capillary filling of a matrix of column and porous material was controlled applying voltage, the micro pumping of fluids in miniaturized closed channel systems because with the possibility of decreasing the effective solid-liquid interfacial energy (Pollack *et al*. 2002).

The importance in microfluidics devices of the density is fundamental to develop a new device or for improve them, there are density examples of applications in microfluidics even there are sensors or devices that do *in situ* density analysis and has been developed for example a new principle that is called piklinophoresis working with a different kind of fluids using a theoretical estimation (Kang *et al*., 2009). Also the chemical concentration or others attributes can be measured using microfluidics (Sparks *et al*., 2003) because the density is a main parameter

when there are circuits that works with fluids, furthermore, there was a natural increase of density parameters when alginate concentration was increased as shown in (Table 4).

Table 4. Alginates density determination

Alginate	**Concentration (w/v) %**	**Temp (°C)**	**Result (g/cm^3)**
Sigma	1	25	0.9122
Sigma	2	25	0.9814
Cargill 6021	1	25	0.9218
Cargill 6021	2	25	1.008
Cargill S505	1	25	0.9758
Cargill S505	2	25	1.0157
Cargill 3001	1	25	0.9414
Cargill 3001	2	25	0.9691

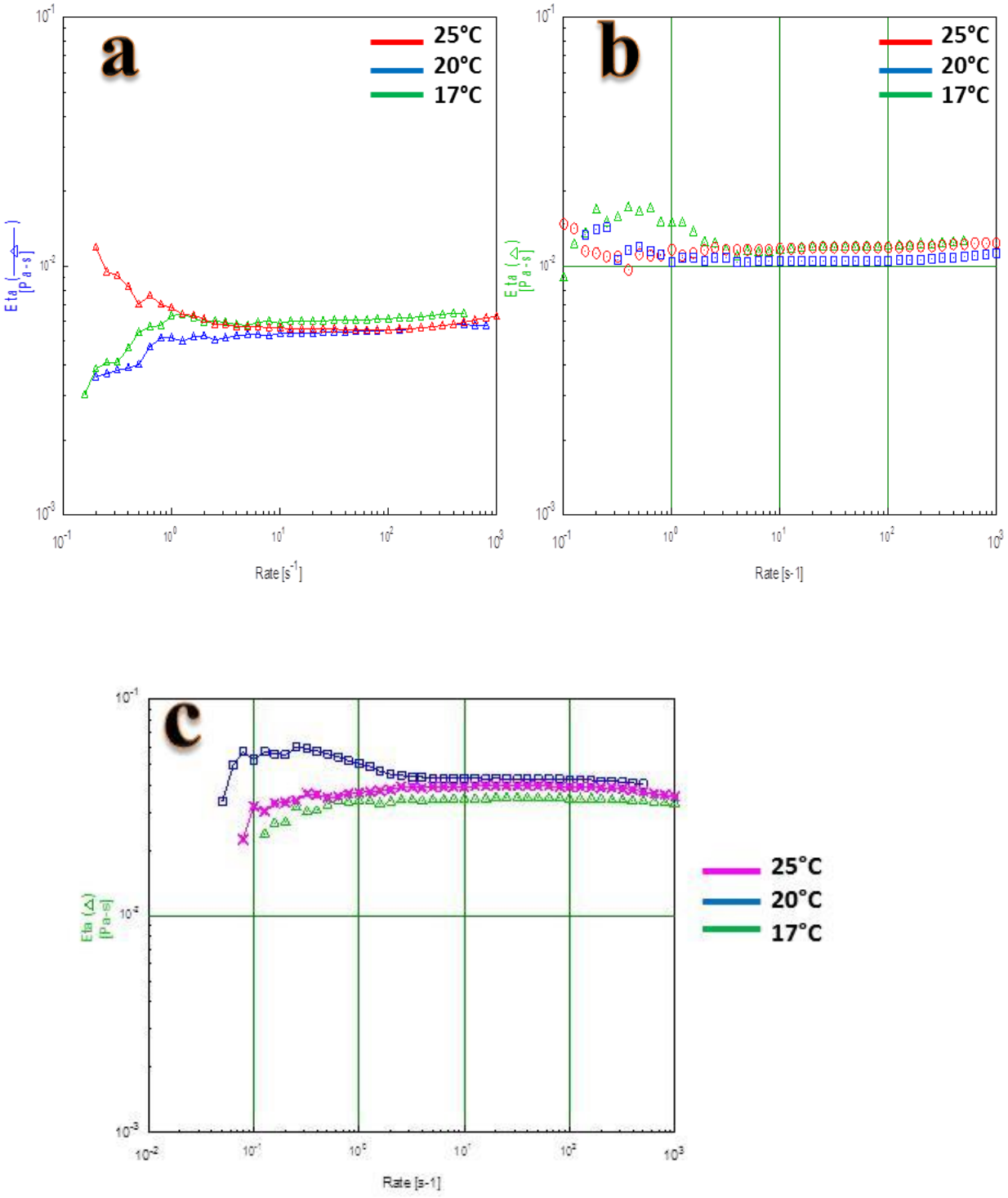

Figure 1. Alginate viscosity comportment of sodium alginate Sigma Aldrich varying the concentration of the solutions a) 0.5% (w/v), b) 1% (w/v), c) 2% (w/v).

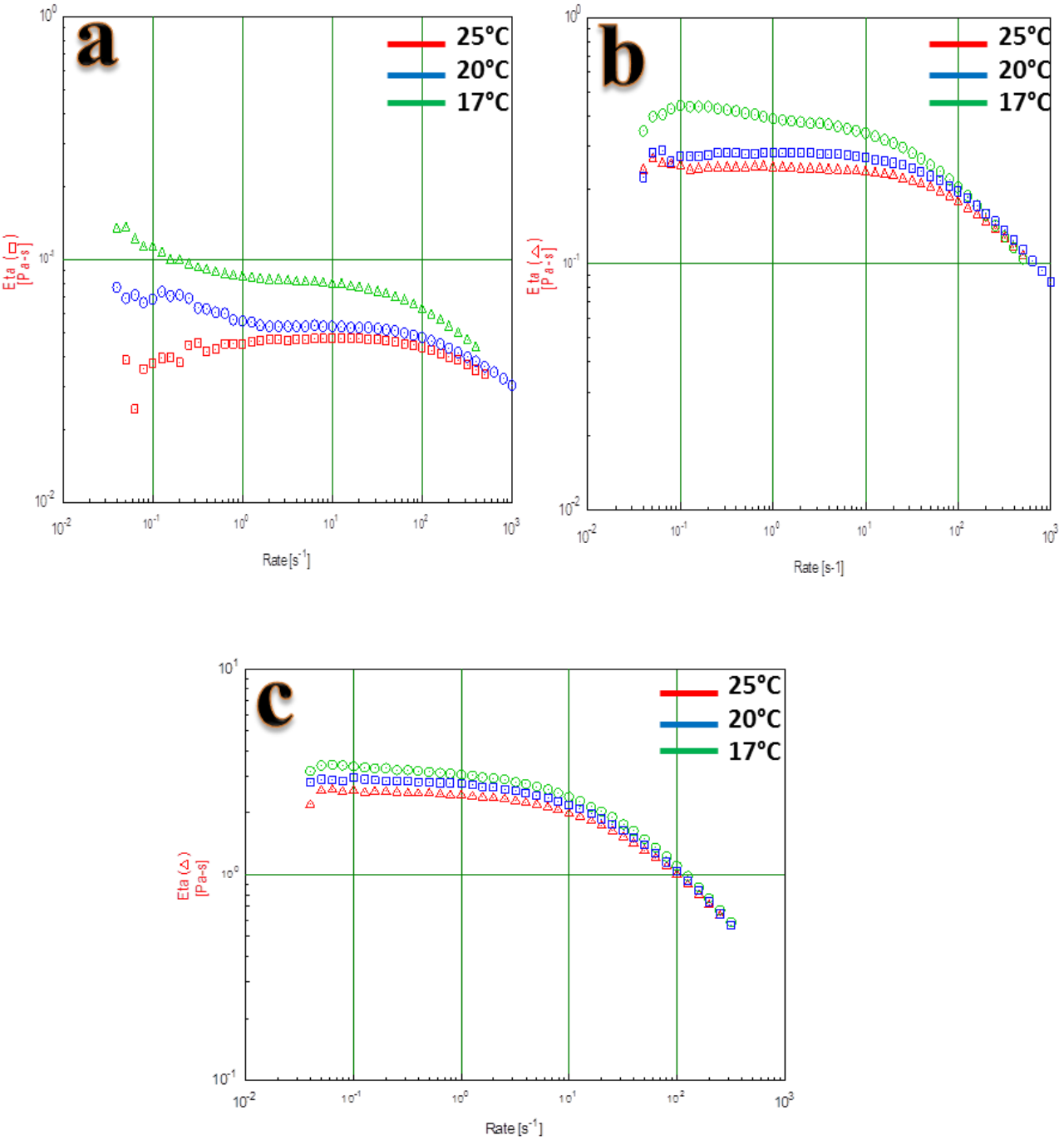

Figure 2. Comportment of sodium alginate Cargill 6021 with diverse concentration of the solutions a) 0.5% (w/v), b) 1% (w/v), c) 2% (w/v). The tests of each curve are the averages of triplicate tests of different batches.

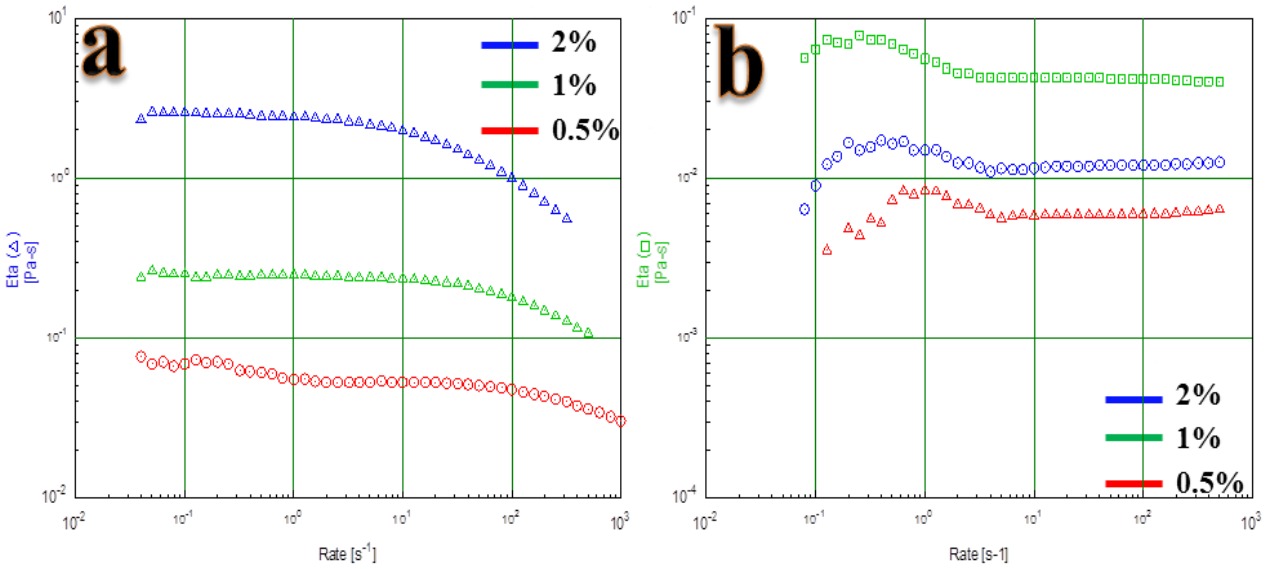

Figure 3. Viscosity comportment keeping the temperature constant at 25 °C and changing the solution concentration of sodium alginate: a) Cargill 6021; b) Sigma.

The chemical alteration can decrease the alginate mechanical properties in solution of sodium alginate. There are a undesirable effects in presence of ions (Na) acting on the kinetics of the dissolution, bringing a reduction of the water chemical potential difference, between the alginate particle and the fluid near to them. An important consideration is that higher salt concentration limits the alginate solubility important factor in the usage of sodium alginate relative to the pH acting directly to the solubility of the polysaccharides also the alginate have due to the carboxylic groups for each block respectively the mannuronic acid value approximate of pKa 3.38 instead guluronic acid has a pKa 3.65.

This is very significant if the value is lower from those ranges a phase separation or the gelation occurs studies reveals that the pKa of the polymeric chain do not differs much from the monomers values separately, but the ionic gels that are in equilibrium as reported studies because they are stabilized by hydrogen bonds. Also alginates are classified as secure additive for EU and USA studies and legislation realized after demonstrate that have not adverse effects for the health (Rehm, 2009).

3.1.3 Conclusion

During the last years, it has been an increase on the use of natural polymers, like alginate, on food industry, generating also interest in different research areas.

Alginate is widely used for microcapsules production because of its capacity to gelificate. However, it is important to characterize the alginate before its use to understand the behavior under the process conditions.

The main physicochemical factors that affect the gelification of this polymer are the temperature viscosity, pH, and concentration. Also, alginate solutions are able to reduce the water surface tension.

Moreover, the guluronic (G) and mannuronic (M) acid content, GM configuration and ratio influences the alginate structure affecting its properties during the hydrogel formation.

3.1.4 References

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3.2 Topic II Microparticle Formation

3.2.1 Materials and Methods 2

Reagents. Sodium alginate 6021 were a courtesy from Cargill (France), sodium alginate medium viscosity (UK), low methoxyl pectin (LM-Pec) from citrus fruits, dichlorodimethylsilane solution, and sucrose were purchased from Sigma (Steinheim, Germany); commercial whey protein isolate was obtained by BioLine (New Zealand); calcium chloride was obtained from AppliChem GmbH (Germany) and sunflower oil was purchased on local market.

Materials. Epoxy resin Henkel (Greece), glass pre-pulled capillaries, tips and microsyringe pump Aladdin 220 (USA), "L","T","X","I" plastic and teflon connectors, fittings and tubing were purchased from Exacta Optech (Italy). Plastic syringes were purchased from Plastipak (UK), plastic pipette tips from Gilson Diamond UK, Master Sizer 3000 (UK).

Different devices were created according to the objective to achieve:

Device I: The main function of this manually assembling glass capillary device is to produce microdroplets of two immiscible fluids in co-flow focusing. The device configuration was performed to create an (w-o) emulsion droplet, taking advantage of the principle that a thin stream of a Newtonian fluid under certain conditions will be unsteady, the flow will be broken in small droplets, almost all of them with the same size.

There are mainly two important types of instabilities called dripping, and jetting, the alteration of the flow stream allows the drop pinch-off modifying the dripping or getting values. The drop pinch-off could be controlled changing the separation and the frequency of the droplet generation this effect is well known and it is called Rayleigh-Plateau instability (Utada *et al*., 2008).

In our experimental set up, it was used a tip-capillary with internal diameter 80μm positioned inside of a bigger capillary with an inner diameter of 2.0 mm, and outer diameter of 2.4 mm, using a 5 mm "L" plastic connector. Next, a perforation that was made near to the curve part, where a bigger capillary must be placed through the plastic piece and must be fixed to the plastic "L" connector using epoxy resin.

The device was tested to confirm the absence of leakages. Afterwards, the device was connected to a syringe of 60 mL, using a 2.0 mm plastic tube. The tubes were attached to the specific type Luer plastic connector to a syringe that it is placed over the microsyringe pump 1 (Water), previously the flow rate was adjusted. Finally the other syringe were placed over the microsyringe pump 2 (Oil), and flow rate is adjusted and connected to the input on the device as shown the figure 1.

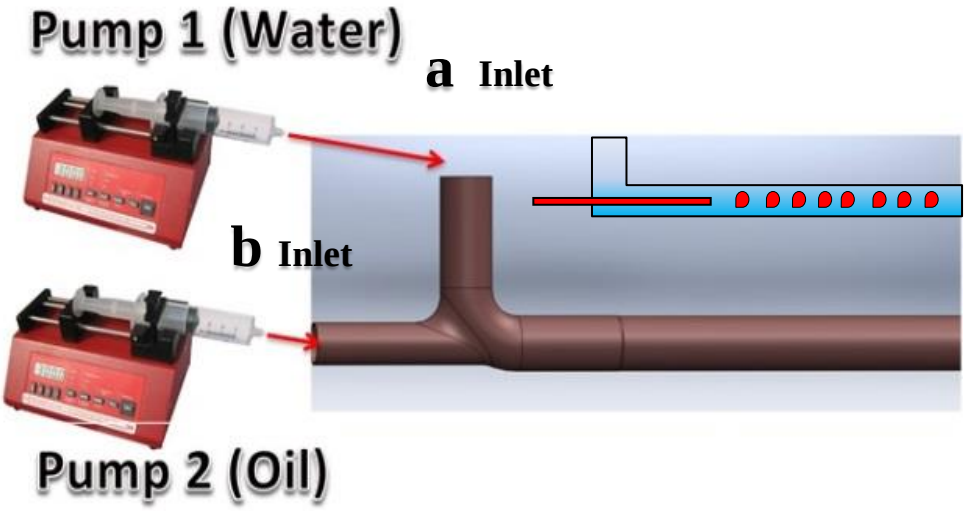

Figure 1. Experimental setup Injecting with the microsyringe pump 1 the continuous phase (water), then Injecting with the microsyringe pump 2 the dispersed phase (oil) to achieve the droplet generation obtaining an emulsion (o-w), exist the possibility to switch the fluids in order to obtain an emulsion (w-o).

Device II: Using microfluidic focusing flow technology for protein/pectin microdroplets generation, it was necessary to prepare a device considering the approaches of the **Device I** with a particular configuration, and geometry. That should help in designing emulsified films by microdroplets. In this case, the inlet **a** flow was sunflower oil, and on the inlet **b** there was the protein/pectin (P/P) complexes solution. It was necessary to adjust the flows previously to get ideal regime called **dripping regime,** in this way it is possible the droplet production as figure 2. Once the dripping regime is reached should be fixed, and the droplets production started and the samples were poured in a flask for conservation.

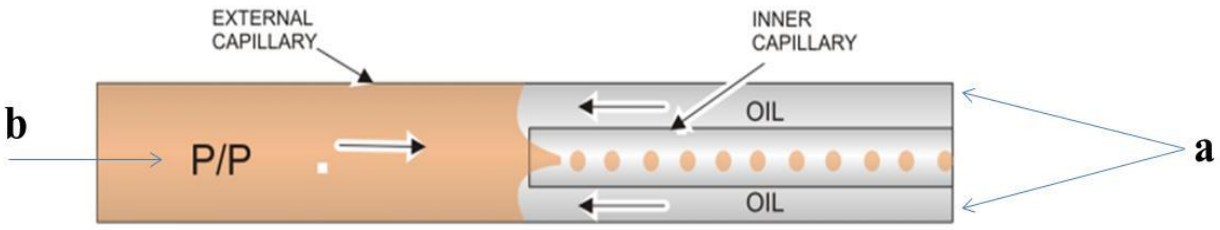

Figure 2. Device I. Inlets of water and oil

Device III: Using the aforementioned glass capillary assembling procedure, with a modification for inlets **a** and **b**, coupling a plastic 1.6 mm "Y" connector with a cooper static mixer attached near to the "Y" connector output figure 3. For proteins 1 g of powder dissolved in 50 ml of distillate water, then was heated at 80°C for 25 min. For pectin, 0.4 g of powder was dissolved in 50 ml, heated at 80 °C for 2 min.

Microfluidics device set up for complexes production was designed as follows (Figure 1): 30 cm Teflon tubing diameter 0.50 mm, "Y" 1.60 mm diameter plastic fitting connector, capillary of 0.80 mm inner diameter and glass pipette 2.0 mm inner diameter and Luer plastic fitting 1.50 mm external diameter.

A previously prepared sample of pectin and proteins has being injected separately by each one of the "Y" inlet by the other side of the device sucrose 66% w/v using a flow rate previously studied to get the best ratio for the complex formation (data no shown). Then, particle size was measured.

Particle size: Solution were analysed using a light scattering Mastersizer 3000 (Malvern, England, UK) adding 500ml distilled water as a dispersant medium using a refraction index of 1.330, then added the required amount of sample until obtain a laser obscuration minimum of 15%.

Protein and pectin solutions were prepared as reported by Di Pierro *et al*. (2013). Pectin solution was flowed through the inlet **a** and the protein solution through inlet **b** preheated in a plastic tubing route for about 30 minutes the time was calculated being the time necessary to get prepared the protein for the mixing process without precipitate both solutions get at the precise flow rate by each inlet. Finally the buffer solution (sucrose) was injected through the inlet **c**. The flow rates can be adjusted accurately by each microsyringe pump, seeking for the necessary mixing rate.

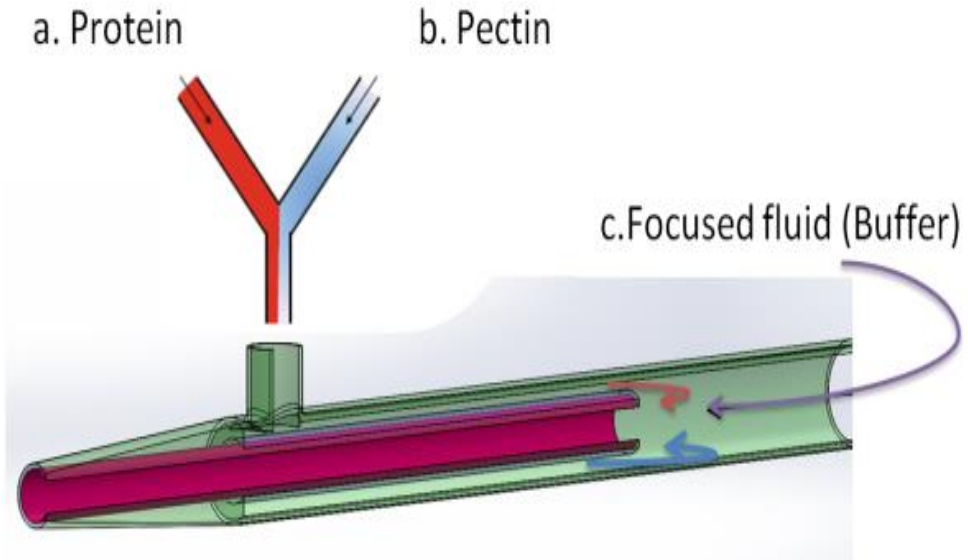

Figure 3. a) Protein inlet; b) Pectine mixed using a cupper static mixer in "Y" connector; c) focused fluid.

Device IV: The device was previously prepared with silanized glass capillaries, using a solution of sodium alginate 2% w/v, and sunflower oil as a vehicle, both fluids are injected using a microsyringe pump setting respectively the flow rate for each fluid, adjusting the microfluidic device in a dripping regime at the tip of the internal capillary that allow the microparticle formation the measures, and geometry configuration is shown in figures 4 and 5.

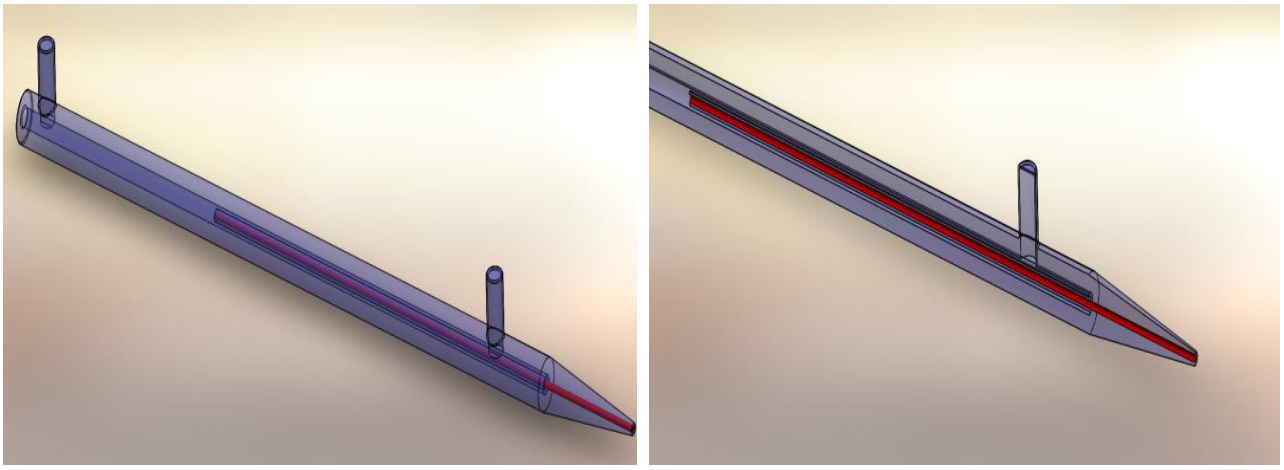

Figure 4. Device IV left and sectional view right.

After microparticle formation both phases continuous (sunflower oil), and discontinuous (alginate) were poured into a flask with calcium chloride ($CaCl_2$) 2% w/v, where the solidification begins. Samples were kept until the complete gelation approximately 45minutes after. Subsequently, the microparticles are rinsed several times using deionized water to eliminate residues of oil and calcium chloride for their future characterization.

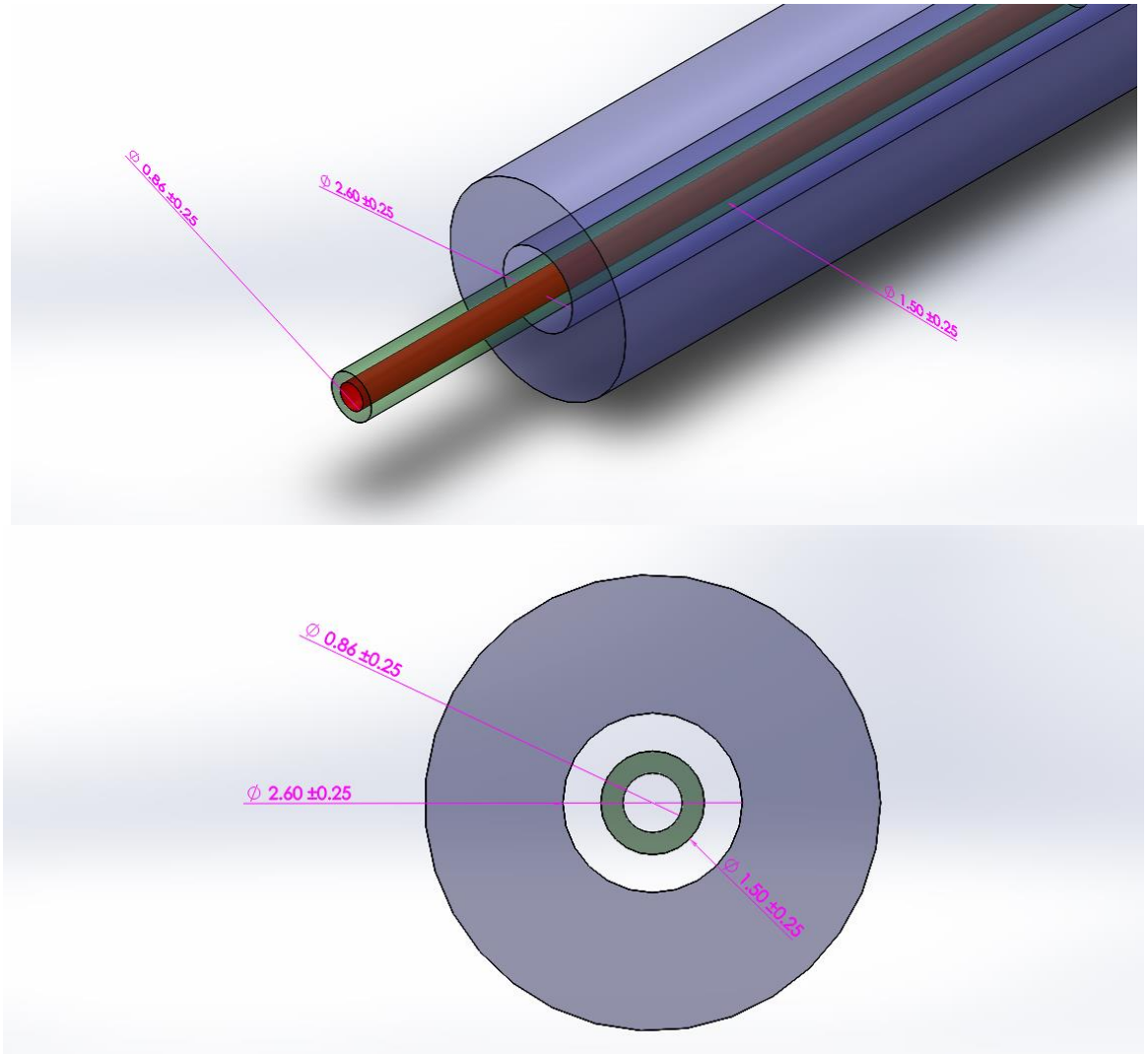

Figure 5. Device IV prepared using glass capillaries concentrically prepared with or w/o silanization process to change the glass wettability properties. Measures are reported in millimeters.

Device V: The geometries **Device IV** and **Device V** were created to compare the differences of the microparticles produced using co-flow focusing configuration evaluating two different glass geometry. In this case, device dimensions were smaller than **Device IV** as shown on figure 6.

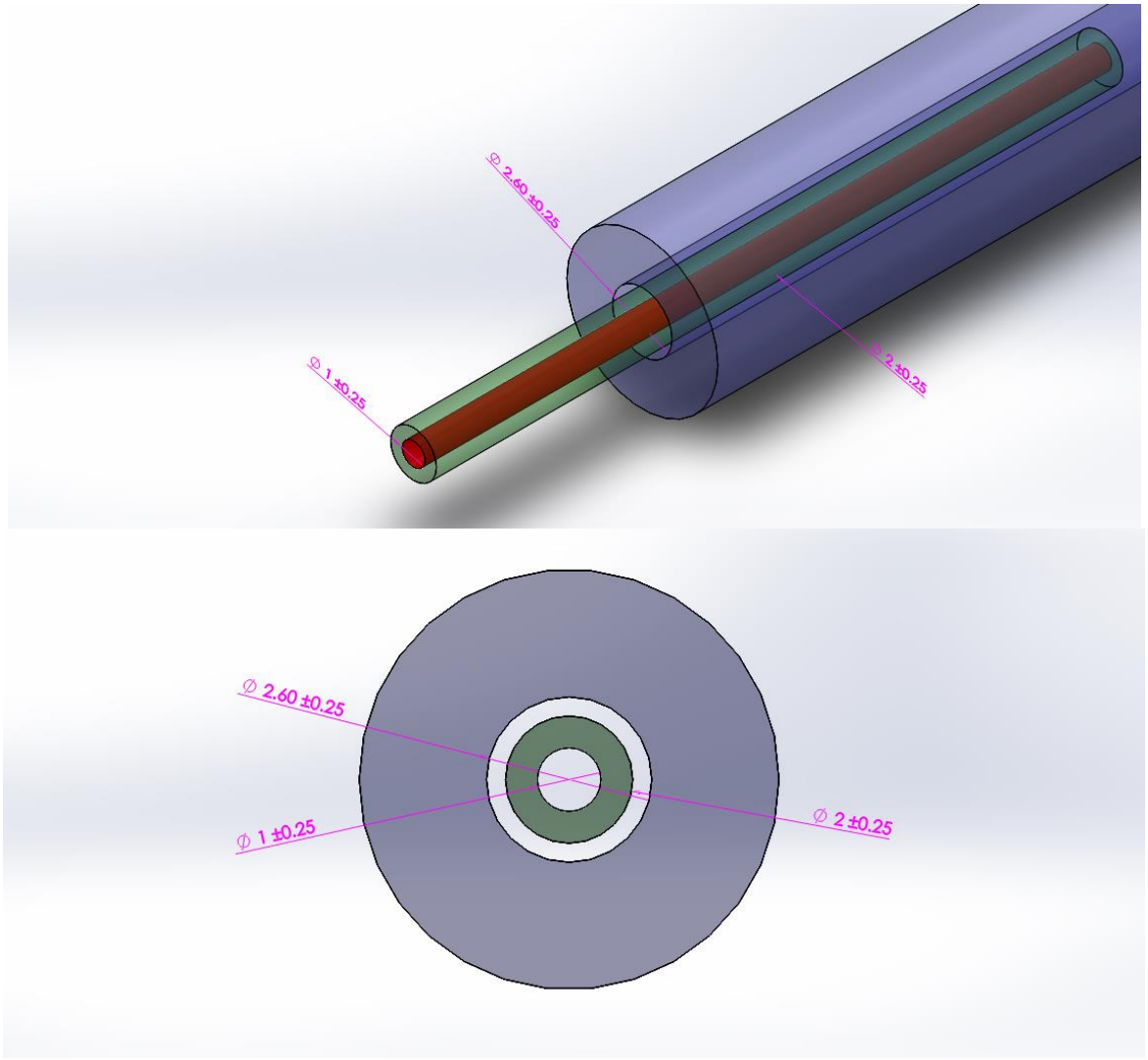

Figure 6. Reducing device measures and comparing with Device IV, prepared with or w/o silanization process to change the glass wettability properties. Measures are reported in millimeters.

Device VI: The manually assembled glass capillary device is prepared with a different geometry configuration. The most important changes are the inlets: adjusting three flow rates, and the number of capillaries (Fig. 7). In figure 8 we can see the conditions to set up the device and in figure 9 are explained the flows.

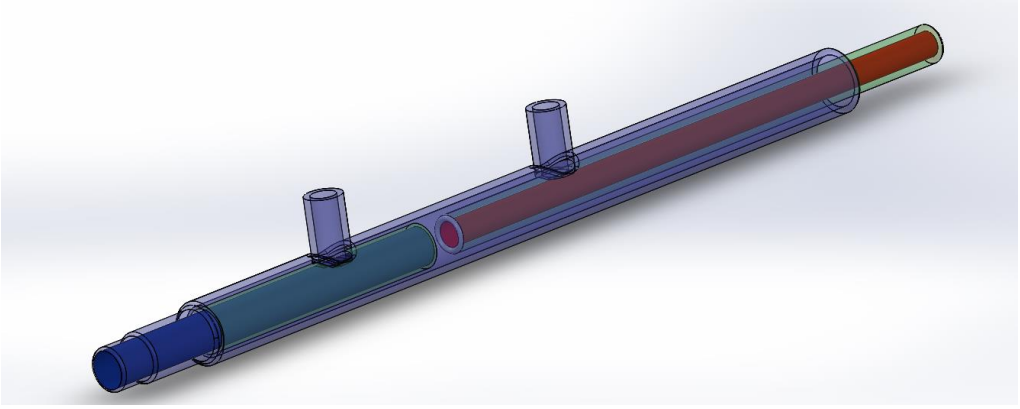

Figure 7. Adding one more capillary to the configuration is another possibility to produce microparticles.

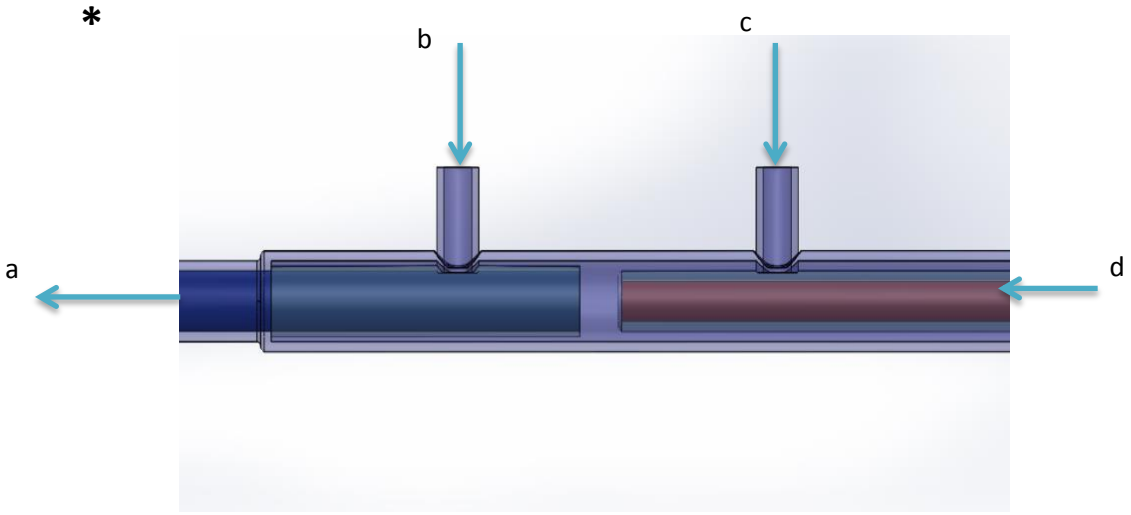

Figure 8. a) device output; b) focusing fluid (vehicle); c) shell fluid (focused fluid); d) core.
* Diagram of device VI; ** Dynamics of injected flows inside of the device.

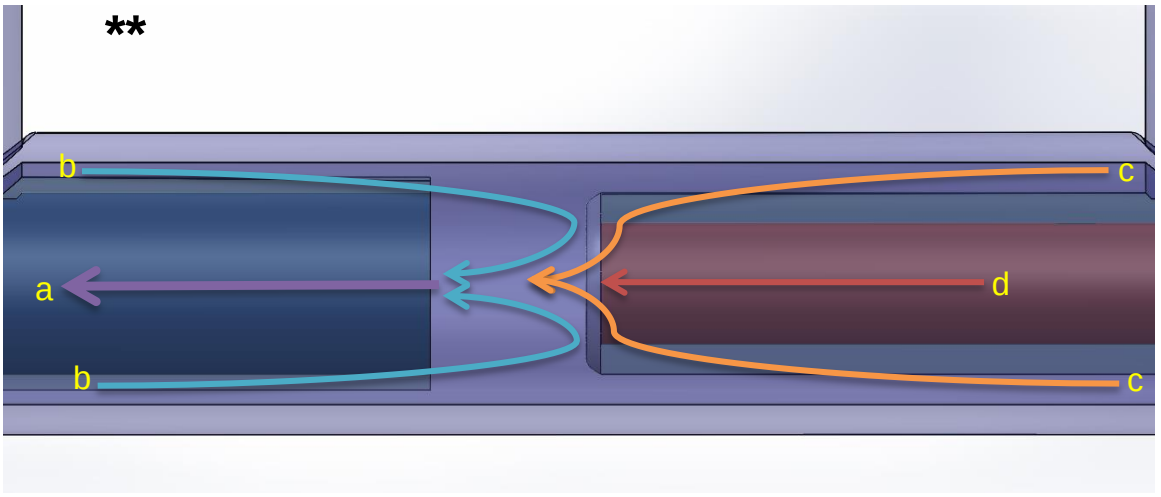

Figure 9. a) device output; b) focusing fluid (vehicle); c) shell fluid (focused fluid); d) core.
* Diagram of device VI; ** Dynamics of injected flows inside of the device.

Device VII: The aim of this device is to produce microparticles in mono and multi-nuclear structures, encapsulating the desired active compound, using same precedent techniques of device preparation, and exalting an important characteristic such as the gelation beginning *in situ* using $CaCl_2$ as vehicle. This device was prepared using a glass pipette (previously treated with the silanization procedure as outer wall and putting inside three different diameter capillaries one inside the other. Through the smallest must flow the inner fluid (essential oil), the medium diameter capillary is a channel for both fluids inner and middle fluid (alginate). Finally, the last one capillary conducts inner, middle, and outer fluid that works as vehicle, and it could be a low concentration of $CaCl_2$ (0.5% w/v) in this way the pre-gelation starts *in situ* and it continues once all fluids are reached the output as shown in figure 10, the microparticles are poured into a flask containing more concentrated solution of $CaCl_2$.

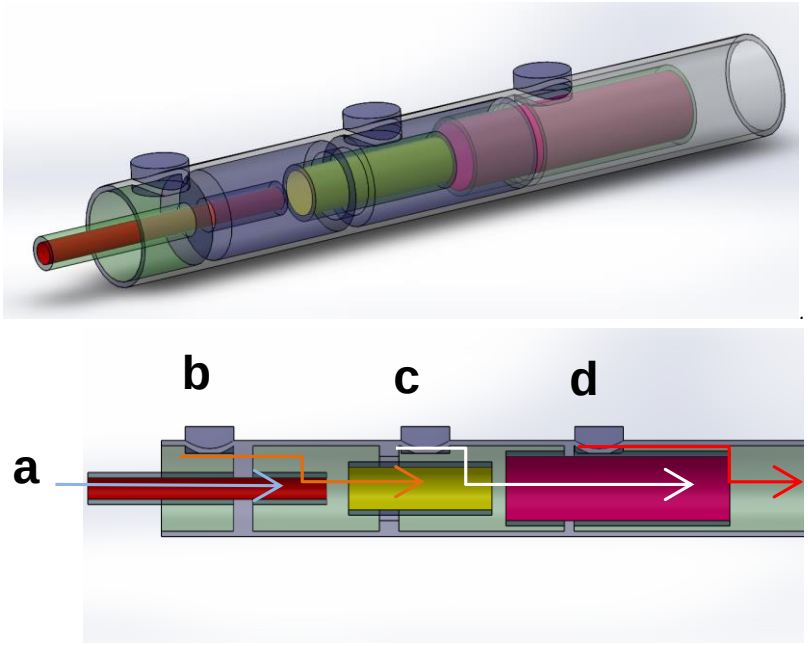

Figure 10. Scheme of device for nuclear an multinuclear microparticle production.
a) core (oil); b) focusing fluid (vehicle) sodium alginate; c) sunflower oil (focused fluid); d) calcium chloride ($CaCl_2$).

Device VIII: Using the **Device II** just changing the fluids that flows through the device adding a solution containing microorganisms (cell immobilization), encapsulated in matrix of sodium alginate.

Device IX: Changing the configuration using **Gas** as focusing fluid and **Liquid** as focused fluid (gas-liquid) to produce sodium alginate microparticles (fig. 11). The device was manually prepared using plastic tips, attaching concentrically two or more plastic tips fixing and sealing the junctions using an epoxy resin, testing that the tips are perfectly centered, and perfectly sealed. The system must be closed, inlets are designed depending of the configuration using

plastic pipette tips positioning, over previously perforated holes, and sealing again with the epoxy resin testing the leakages.

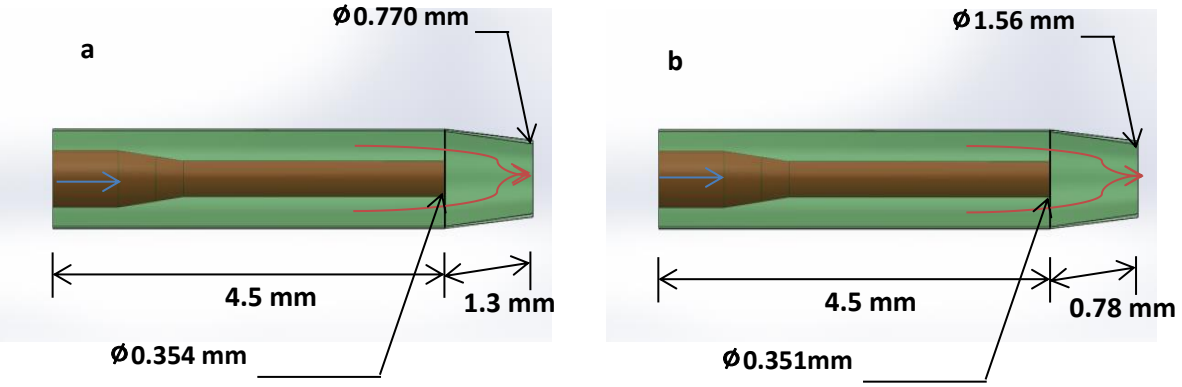

Figure 11. Measures and flows inside the device **a**; measures and flows inside the device **b**.

Microparticles analysis. The sample preparation for image analysis was prepared withdrawing 10 mL of dilute in deionize water sample 1:10 using a 1 mL plastic pipette before sedimentation, the sample is situate in a black matte surface using a concentrated white light source under a stereoscope Olympus SZ-40 Germany, and a photographic camera Olympus. Once prepared the photograph using the threshold tool converting to binary setting the scale, adjusting the image using proceeding to analyze the sample.

SEM. The sample was placed over the stub using a plastic covering but allowing the air pass to reach the evaporation of exceeding solution the sample must be totally dried. Once the sample is dried next step is the metalizing procedure using gold, sputtering the surface of the sample. Finally the observation of the sample in the SEM EVO-40 Zeiss. Some samples are previously prepared fixing the sample with glutaraldehyde 4%.

Particle size determination. Solutions were analyzed using a light scattering Mastersizer 3000 (Malvern, England, UK) adding 500 ml distilled water as a dispersant medium and using a refraction index of 1.3. Sample was added until obtain a minimum laser obscuration of 15%. Experimental test were repeated three times for each sample, and the solution was characterized with particle adsorption index equal to 1.51 and particle refractive index equal to 1.33. Small variation of optical indexes did not show significant variation of results. Although the non-spherical option was used, a certain amount of size distribution spread is related to the relative orientation of the particles passing by in the detection area respect to laser light scattering detection sensors. Results were averaged using at least 10 independent measures.

3.2.2 Results and Discussion 2

Different devices were created, as described above, to test and to compare the importance of materials and diameters on the spheres production using flow focusing. For **Device I**, glass capillary microfluidic devices were very suitable for production of core/shell drops of controllable shell thickness, and multiple emulsions with a controllable number of inner droplets and/or inner droplets of two or more distinct phases with a production of monodispersed droplets, and droplet throughputs of up to 100 l h-1 (fig. 12). According to Vladisavljevi *et al*., (2012), due to the versatility of the devices, the diameter size of the droplets generated in different emulsion configuration such as (o-w) (w-o), is possible to reach a size of the droplets generated from <100mm (CV < 3%).

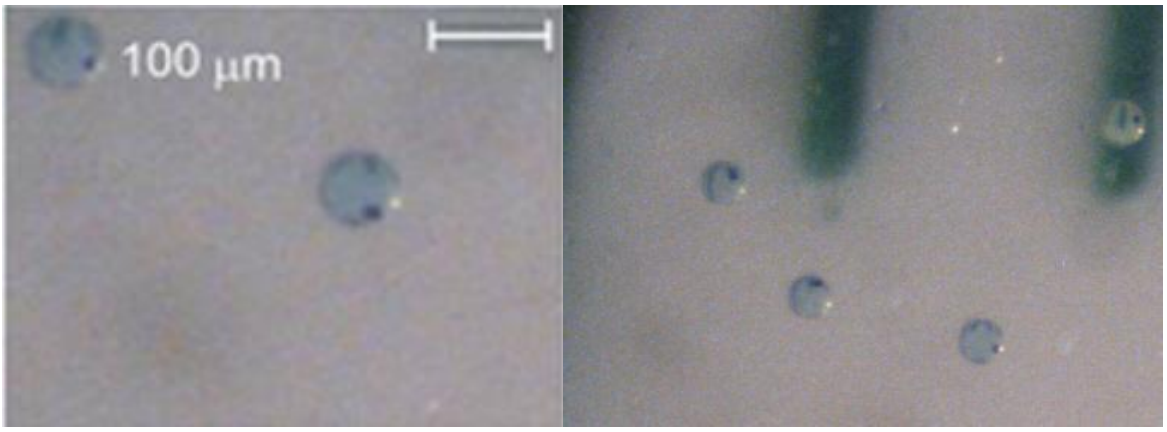

Figure 12. Droplet of water in oil (left) is possible to observe the dispersion of the droplets in the right image

For **Device II** the same principle of the Device I was used, but the main difference is that one fluid is a mix of protein/pectin solution. For this purpose was created a device using glass capillaries as show the figure 13.

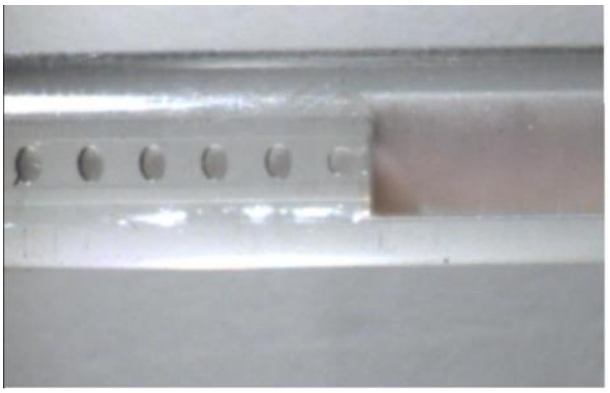

Figure 13. Device II using glass capillary suing sunflower oil and pectin protein mix.

Usually, in microfluidic devices the authors address issues just of well-known fluids, for example synthetic polymers but mostly of them are not food grade. The production of protein/pectin spheres demonstrates the flexibility of the device and the technique versatility to be used with different fluids of distinct nature and especially, the most important characteristic is the possibility to apply this technology in tailored food processes generating microparticles by microfluidic techniques.

Trying to developing innovative technological applications in the food field we have tried to create microparticles totally food grade, in this way using a process with a heat treatment for the **Device III**, where was used a "Y" junction to make flow the solution to form the microparticles and seize the technique. We can observe in the figure 14b the microparticles obtained using the Device III as previously mentioned in materials and methods in the other hand the experimental set up using a glass capillary device represented in figure 14a.

Figure 14. a) Glass capillary device; b) pectin protein microparticles obtained from Device III keeping the monodispersion.

The main importance of this device is to get food grade microparticles. The first problem to resolve was the conservation after their production and mostly, the description of different configuration and devices produced.

For the **Device IV and Device V** as is possible observe the results of the table 1. The sizes of the microparticles are practically the same being a difference between them minimum.

Table 1. Particle size of spheres.

Device	Solution	$Q_{focused}$ (mL/min)	$Q_{focusing}$ (mL/min)	Size (µm)
IV	**Alginate 2% (w/v)**	0.3	0.5	300
IV	**Alginate 2% (w/v)**	0.5	0.7	250
IV	**Alginate 2% (w/v)**	0.8	0.9	200
V	**Alginate 2% (w/v)**	0.3	0.5	280
V	**Alginate 2% (w/v)**	0.5	0.7	240
V	**Alginate 2% (w/v)**	0.8	0.9	180

The objective of adding one more capillary in the **Device VI** (fig. 15) was the production of microparticles with double emulsions have more control and flexibility for encapsulation and, can be achieved through the use of double emulsions, with smaller droplets of a third fluid within the lager drops. The intermediate fluid adds an additional barrier that separates the innermost fluid from the outer fluid, or the continuous phase.

Several authors reported that the use of double emulsions is highly motivated process due their possible applications on food industries to prepared release-controlled substances and to encapsulate food additives, nutrients, drugs. Furthermore, the control of the state of the middle fluid can be exploited for targeted drugs (Utada *et al*., 2005).

Figure 15. Device for microparticles production with two inner capillaries can produce double emulsion

With the **Device VII** was possible to produce mononuclear and multinuclear microparticles as shown in figure 16. The possibility to produce multinuclear or mononuclear microparticles using microfluidic devices is widely studied, the main characteristic of this modality of production allows the possibility to produce combinations of emulsions for example (O/W/O) (W/O/W). Microparticles containing colored sunflower in a fully food grade elements was possible following an emulsification process described by Ren *et al*. (2009), where the control of the outer diameter of emulsion, and the size of their inner oil cores is independent, just adjusting the flow rates and the capillary inner and outer diameter.

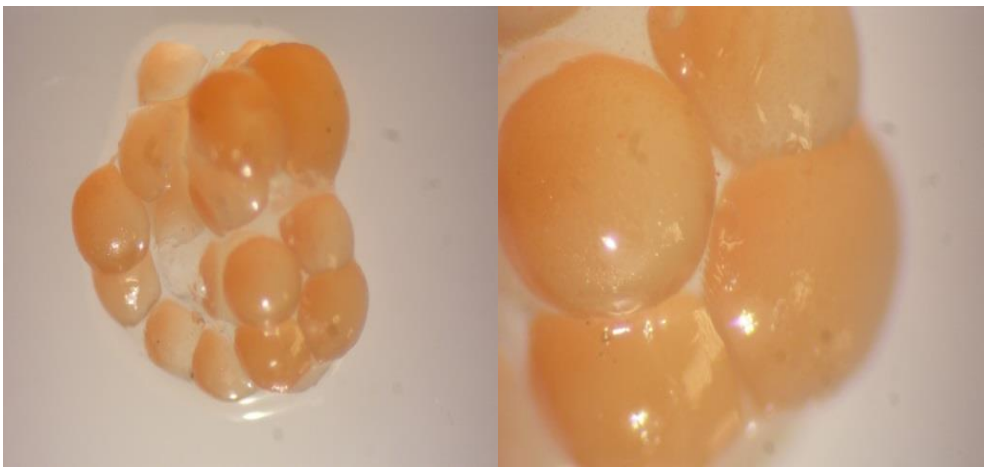

Figure 16. Multinuclear microparticles obtained from Device VI

These results are in agreement with those obtained by Shah *et al*. (2008), in their study, authors defined that the production of single and multiple emulsions as well as the number and size of the internal drops can be control with the flowing rates and suggest the use of a triple emulsion device to favor the monodispersed shells to encapsulate a multiphase core in (W/O/W/O).

For **the Device VII**, the cell encapsulating alginate hydrogel beads of various shapes were successfully produced (figure 17). Tan *et al.* (2007) used this approach to encapsulate biological cell using a non-silanized/silanized devices keeping the gelation conditions without losing cell viability.

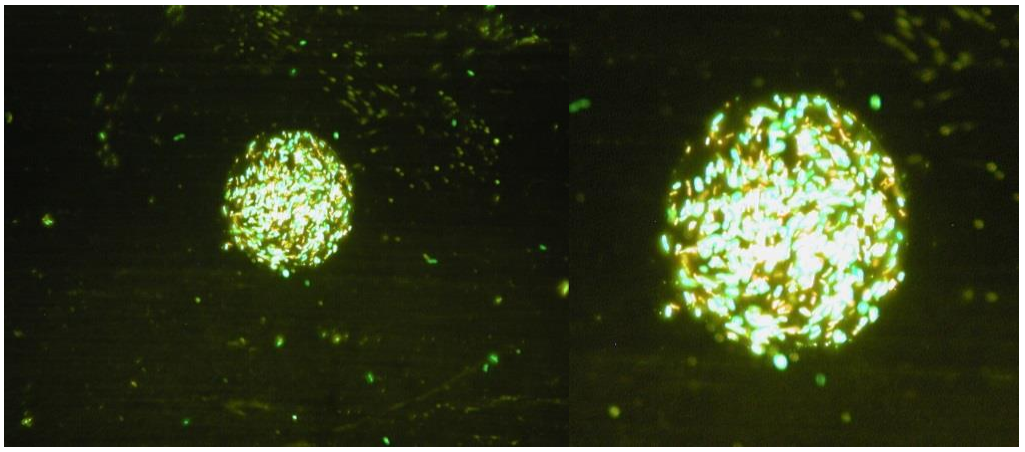

Figure 17. Matrix encapsulation microparticles obtained from Device VI using a special marker and a UV microscope appearing as greenish spots the live cells and brownish the dead cells after the encapsulation process we can see a good viability of cell being microencapsulated in a gentle manner.

In the effort to meet a cleanest process technique, experiments were conducted changing the focusing fluid (sunflower oil) or any other food grade immiscible fluid necessary for the droplets formation choosing gas as focusing fluid.

This apparently little change that differs with the previously techniques described before, becomes in a great challenge because not only was changed the immiscible fluid but also is changed the state of one of the necessary fluid for the microparticles production.

In the axisymmetric smooth system the nozzles are necessary for the production of jets and drops just injecting liquids through them, controlling the flow rate (Montanero *et al*., 2010) the formation of the particles depends to the velocity of the gas stream.

When the gas pressure oscillation is smaller than the surface tension of the fluids in the system, it is possible get a jet breakup allowing virtually a controlled spray, the process happens without strong shear stresses. For this reason this technique is appropriate to handle delicate substances also in the generation moment there are gas oscillations reaching a full turbulent profiles that avoid the coalescence effect (Gañán-Calvo, 1998).

Several prototypes of **Device IX** model, (figure 18) were prepared in different measures in order to compare the results obtained.

Microparticles measures were effectuated with different flow rates in the following figures is evidently the monodispersion and the robustness of the system using two different devices with the dimensions described in the figure 19 both tips "B" class 2b1with an out diameter 1.60 mm inner diameter 338.46 μm, and 2b2 with an out diameter 1.56 mm inner diameter 351.27μm, the same flow rate was used to compare between nozzles the measures averages of each nozzle tip microparticle production obtaining a RSD less than 10% very near to the mentioned by (Morimoto *et al*., 2008) for the monodispersed classification.

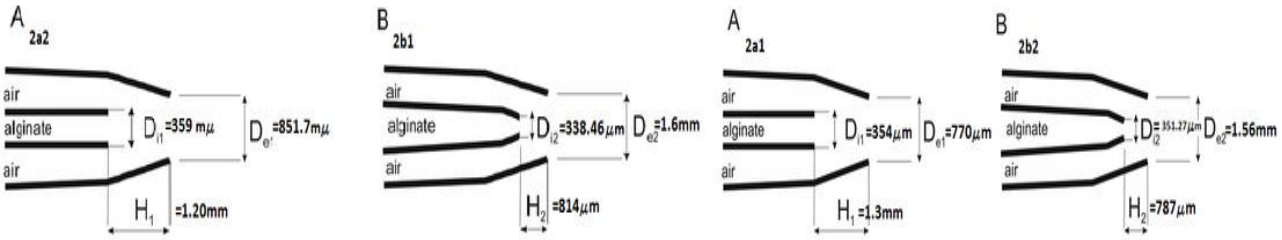

Figure 18. Examples of several devices prepared with their respective measures, the devices were used to compare the dispersion and the repetitively of the devices between batches the results obtained from devices B2b1 and B2b2 are shown on the figure 19.

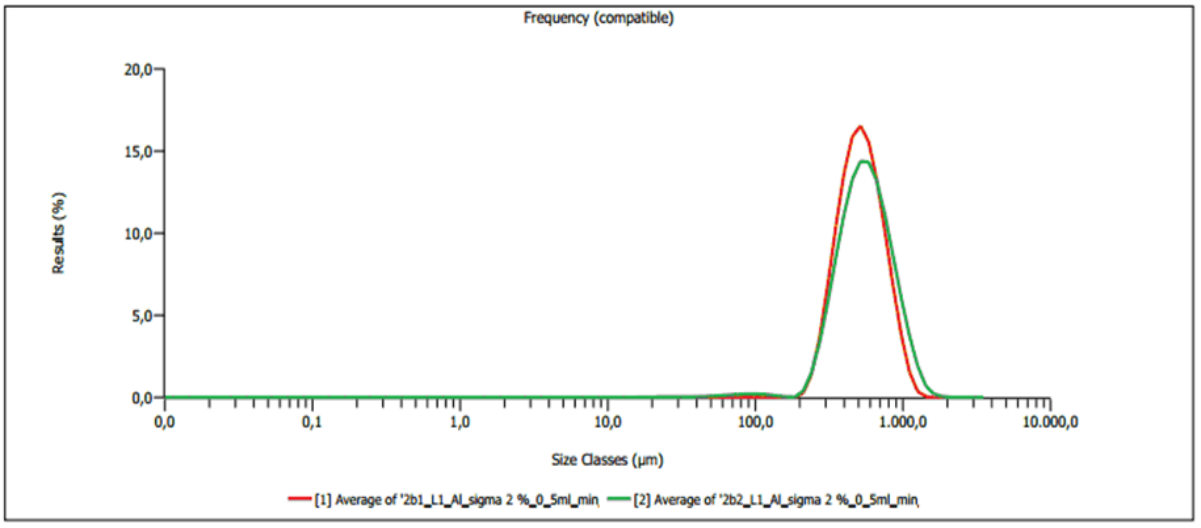

Figure 19. Comparison of two different devices prepared with very similar sizes in their geometry measures, the experiment was conducted keeping constant the flow ratio, disperse phase flow rate Q_1=0.5 mL7min and continuous phase flow rate Q_2= 1 mL/min. The mean sizes of the classes in this experiment were for Dv 10= 327µm, Dv 50=531 µm, Dv 90=869 µm, also using the averages of minimum 20 runs of the light scattering and being the averages of 3 different batches. The respective RSD expressed in % for this experiment is Dv 10= 0.90, Dv 50=4.60, Dv 90=9.2 confirming the good monodispersed microparticles obtained with both devices.

The reproducibility of the system was confirmed producing with the same nozzle tip different batches afterwards analysing the microparticles generated experimental condition were kept the same by the production of each batch, the results of this experiment are shown on the table 2.

An example of the controlled and desired size microparticle production is shown in the figure 21, using a nozzle of the **Device IX**. Microparticle production measures were performed fixing the flow rate of the disperse phase in 0.5 mL/min, in this case sodium alginate from Sigma 2% (w/v), using for the microparticle production a gas value from 0.5 L/min, 1.0 L/min 3.0 L/min and it is possible to observe the increasing size varying the focusing fluid (gas).

Device **IX**	Dv 10 (µm)	Dv 50 (µm)	Dv 90 (µm)
	222	335	497
	221	336	504
	220	336	509
	220	338	511
	220	335	503
Mean	220	336	505
Std Dev	0.919	1.2	5.15
RSD (%)	0.417	0.357	1.02

Table 2. Measures obtained from device prepared using the same geometry of the **Device IX** microparticles sizes are reported as Dv10, Dv 50, and Dv 90 that represents in (%) respectively 10, 50, 90 of the population the values obtained of RSD are less than mentioned by Morimoto *et al*. (2008), as particles monodispersed classification.

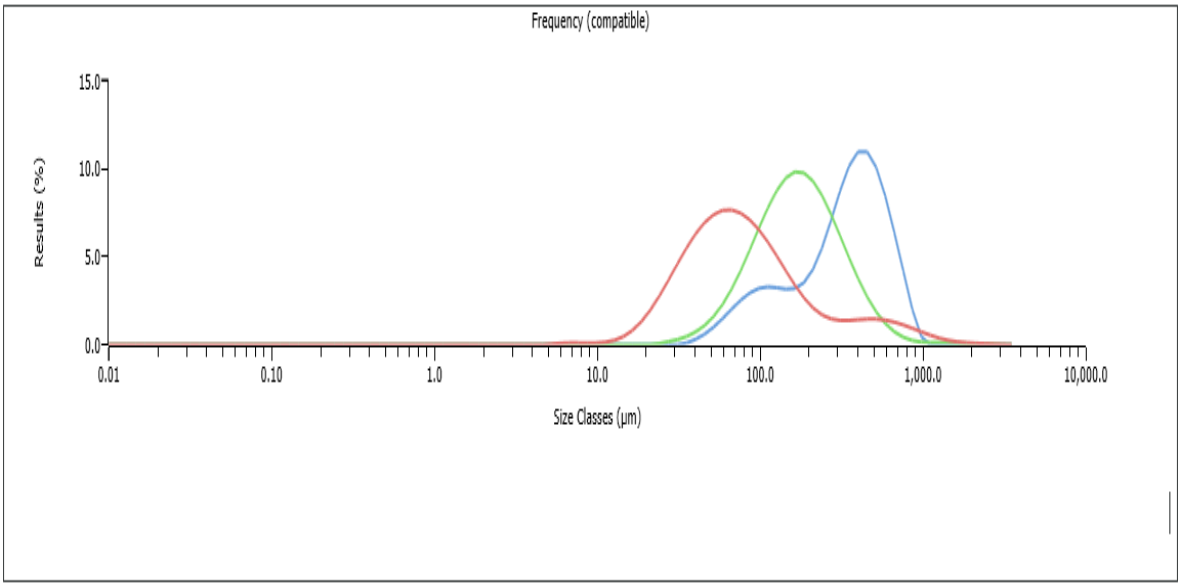

Figure 21. Example of the microparticles produced using a Device IX geometry, changing the continuous flow rate (gas). It is possible to observe the size changes of the particles produced. The flow rate for the disperse phase in all curves is 0.5 mL/min and for continuous phase 0.5 mL/min (blue), 1 mL/min (green), 3 mL/min (red).

The morphology of microparticles of different sizes produced are shown in figure 22, where is possible to observe, using different approaches in a stereoscopic microscope from left side the smaller to larger (right side). For c) it is shown microparticles without suspension fluid.

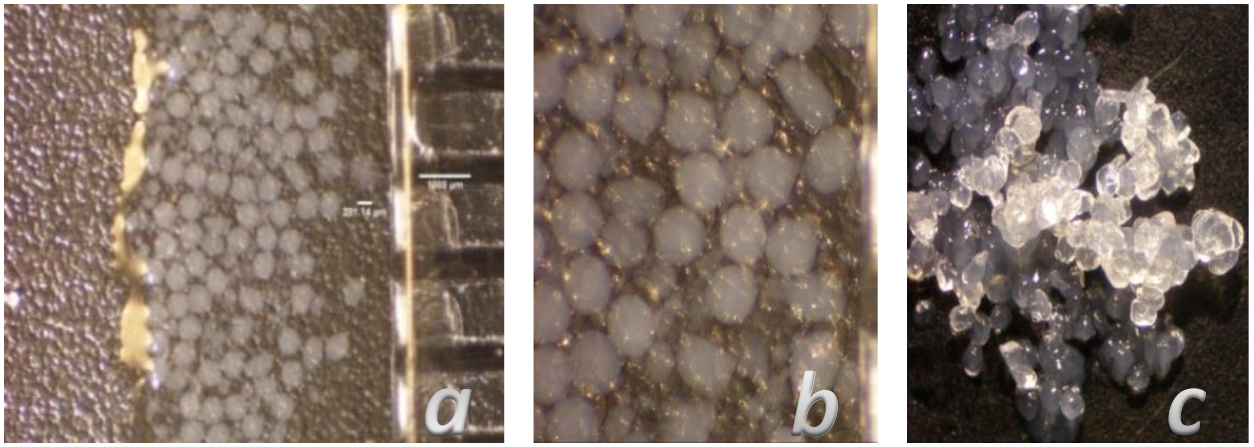

Figure 22. a) microparticles of mean size 230μm. b) different approach.
c) microparticles prepared without suspension (drained).

Furthermore, a SEM image analysis was made to the obtained microparticles figure 23 using the **Device IX.**

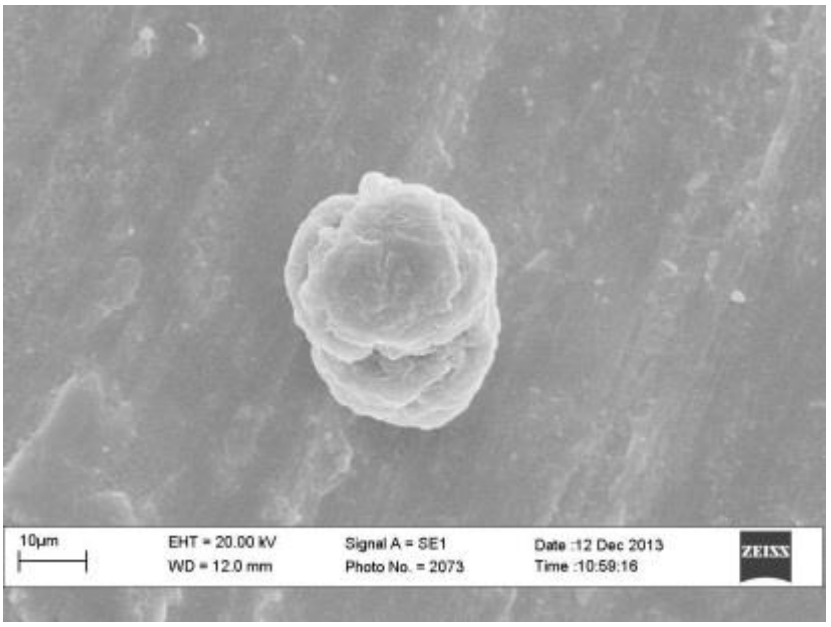

Figure 23. SEM image analysis of microparticles produced using the Device IX (gas-liquid) is possible to observe a microsphere with 9X zoom and 20kv.

3.2.3 Conclusion

The main goal of the devices designed was successfully achieved the production of microparticles very near to the characteristics that we have previewed, also where tested using diverse food grade materials opening a wide range of possibilities of the applications using food

grade reagents such as alginate, pectin and protein mainly. Furthermore, there are more reagents that can be suitable to be used with our devices may be more available to produce the microparticles due to the nature of them because is necessary to remember the differences between materials derivate from biological sources and from chemical synthesis. The irregularity on the biological components will be always an important parameter to consider when the microencapsulation materials are selected, however thanks to the versatility in microencapsulation, ingredients for food. Was possible to obtain good results when the tests of microparticles were conducted where was used mainly sodium alginate, protein, pectin, etc.

On the other hand still remaining some technical problems for the improvement of the device that were presented when the tests were conducted that can be solved using several possibilities for the device construction changing materials, the concentricity, kind of fluid that will be used setting up better, and the device geometry used to produce the microparticles there is the possibility to add a precise regulation on the separation between the inner capillary and the outlet orifice there are many more options to improve the microcapillary device all those must be studied with accuracy.

3.2.4 References

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3.3 Topic III Improvement of the microparticles production process

3.3.1 Materials and Methods 3

Reagents: Calcium oleate, calcium linoleate, Sodium alginate medium viscosity W201502 and oleic acid from Sigma-Aldrich from Sigma-Aldrich (England, UK), sodium alginate S550, 6021, 3001 were a courtesy from Cargill (France). Sunflower oi1was purchased at a local market, Sodium oleate Sigma-Aldrich.

Materials: Microsyringe pumps Aladdin (USA), 60 mL plastic syringe Plastipak (UK), Olympus stereoscopic microscope (Japan), Polarized microscope Zuzi 501 (Spain), camera coolpix 900 Nikon (Japan), Media cybernetics image pro plus (USA), Image Pro Plus (Media Cybernetics, Inc. (USA), tubing and fitting from exacta (Italy), microfluidic devices (I),(II), Hitachi CCD camera KP-FD202GV (Japan).

Calcium oleate and calcium linoleate oleate was prepared as described by Harrison (1924) using oleic acid instead sunflower oil on the corresponding case.

Image analysis. It was effectuated using the Image Pro Plus software. To reach a good consistency in our measurements, images were taken using a polarized microscope and a digital camera attached on the microscope, using at least 3 microparticles batches produced and processed in the same way. There were collected more than 1500 microparticles by experiment, classified and counted from at least 3 randomly selected images. Afterwards, the software was programed to classify automatically in 4 population classes and results are expressed as relative frequency. Instead, for roundness determination the first steps of classification were followed and images were selected. The roundness classification follows an interval of software values obtained from 0.80 to 1.25 being 1 a circle, the calculation used by the software is for the diameter mean average length of diameters measured at 2 degree intervals and passing through object's centroid.

Sodium alginate solutions. Solutions at 1% (w-v) were prepared dissolving in distilled water additives such as calcium oleate, calcium linoleate, and sodium oleate, then were mixed using a magnetic stirrer and added in 75 mL sunflower oil at 35°C and let stirrung 5 hours or until the solution becomes homogeneous. Afterwards, the solution was loaded in a 60 mL syringe, and placed in the mycrosyringe pump, both pumps were set up with a previously selected flow rate completing the microparticles production with the method previously described and using calcium chloride as solidification bath.

3.3.2 Results and Discussion 3

Once the devices were improved and solved the major technical problems, like leaks, decentered position between capillaries, etc., it still remained the microparticle production efficiency problems after the droplet production. The methodology remains the same described on the topic II for particle production, but there is a difference, solutions were adjusted using other type of sodium alginate, using oleic acid, or using additives with a calcium component. This step was made before the calcium chloride bath operation to begin the gellification when the droplet formation starts inside of the capillary device. Making this modification using additives, and changing the technique previously used trying to solve the coalescence effect,

consistent in the aggregation of the generated droplets each other producing and undesired effect affecting the microparticles monodispersity and possibly affecting the shape.

Considering important points in the coalescence dynamics in the microparticles production, the main causes of the coalescence are the contact between droplets, a gradual thinning of the film (wall of the droplet) to a few amstrong, breaking then allowing the coalescence, and the increment on the microparticles size or even without formation, the timing of coalescence depends on the rate of draining. The breaking of that film often the breaking happens before the other two conditions are satisfied (Chhabra, *et al.*, 2007).

A part of our experimentation consisted on the use of calcium oleate, calcium linoleate, sodium oleate as an inonic source added to the oily phase (figure 5a), and using just oleic acid instead of sunflower oil. In some tests as a continuous phase, the (cation) sources were mixed. Once the prepared solutions were ready with the additives, were used to produce the microparticles of sodium alginate.

Also is possible to observe the coalescence effect on alginate microparticles production without use additives the images on the figure 1 can illustrate the problem.

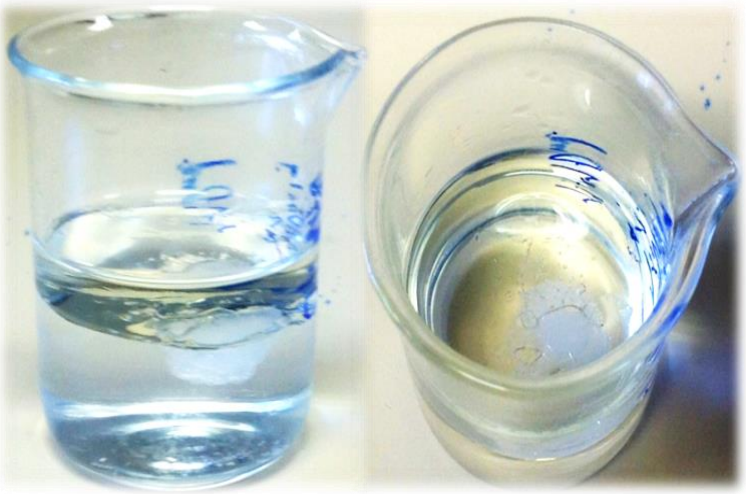

Figure 1. Example of test for microparticles production with coalescence results using sunflower oil for the continuous phase and Cargill S550 as discontinuous phase, using the Device II the most important problem to solve for the process of microparticle production.

The process of hardening microparticles or “gelation” is important, there are two ways to explain how gelation happens, and are called internal and external gelation. Actually, it is difficult to use a polysaccharide for microparticles production because is necessary to use ions for example $CaCl_2$ for the gelation process, but is not soluble in the oil phase, necessary in the microparticles production process. Furthermore, it is possible to use another calcium sources like calcium citrate, and through a pH reduction to facilitate the gelation process (Poncelet *et al.* 1995). Due to insolubility of the calcium we have prepared an oily phase with calcium content using additives.

Calcium linoleate was added to the oil phase, using the same procedure describe above to dissolve the calcium source. Best result was obtained using 0.1 g in 75 mL of sunflower oil.

Also, oleic acid was used to replace sunflower oil as a continuous phase while the discontinuous phase was tested using different sodium alginates: Sigma-Aldrich, Cargill type 6021, 3001, S550. Once the particles were produced, were conducted the analysis changing different type of alginate an image process software image pro plus (fig.2). Each single image was processed using the processing image tools: Erode, despeckle, watershed, brightness, and finally a threshold treatment the pictures were made keeping the same zoom conditions of any photograph tests were evaluated the most important attributes of the microparticles, size (fig. 3) and roundness (fig. 4). Relative frequency distribution was calculated in both cases.

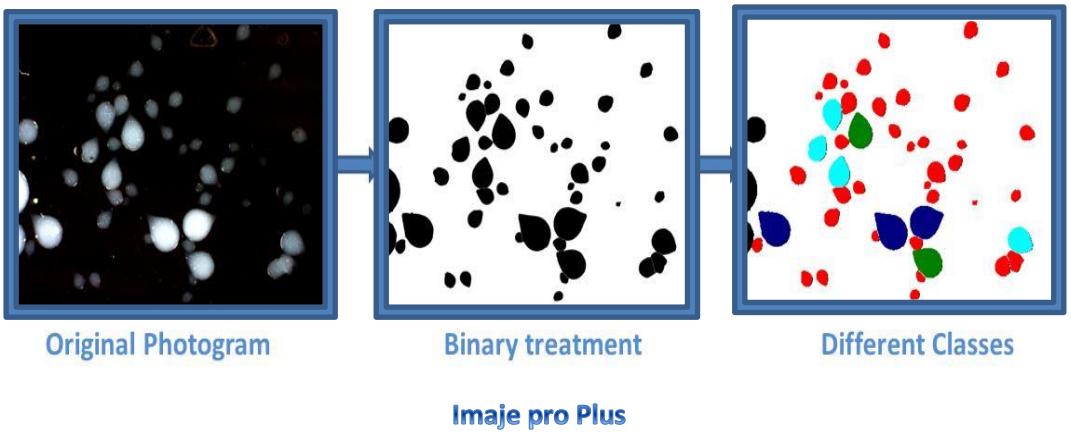

Figure 2. Microparticle production image analysis was used to evaluate the changes in the process of microparticle production comparing using or not additives to avoid the coalescence. The process consists in to obtain an image from the optical microscopy and adjusting the image properly to begin the image treatment finally the image is analyzed using the software Image Pro Plus as previously mentioned.

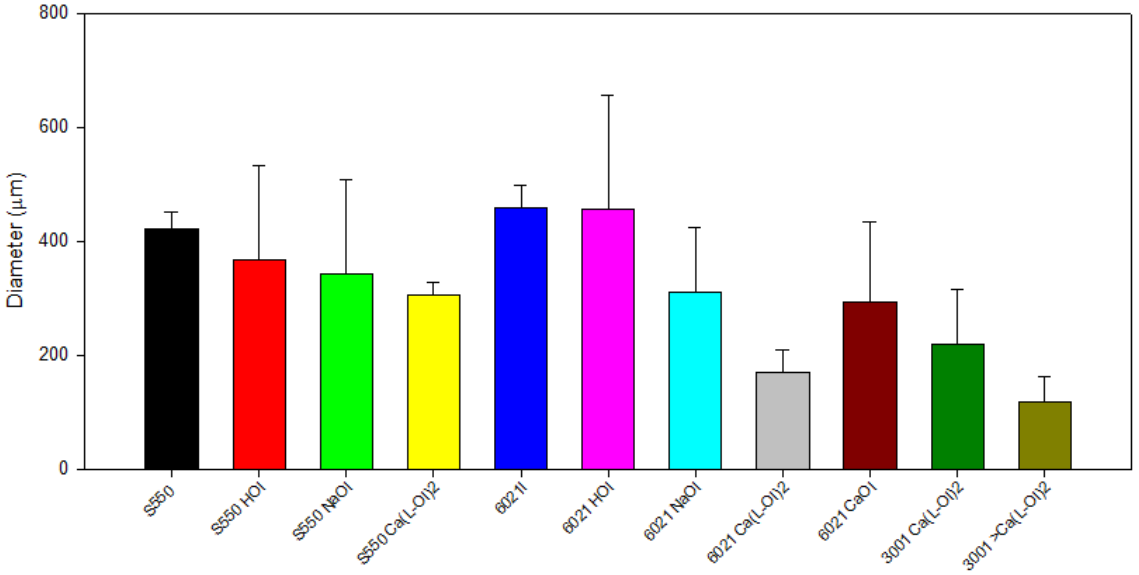

Figure 3. Comparison of each type of sodium alginate used to conduct the tests comparing the microparticle size using the same flow rate, and experimental conditions just adding the respective additive (cation source). For each sodium alginate test: Without additive, using oleic acid (HOl) as continuum phase instead sunflower oil, sodium oleate (NaOl), calcium linoleate oleate $Ca(L\text{-}Ol)_2$, there are reported as relative distribution of the microparticle. With the sodium alginate 3001 from Cargill were used just the representative results using the calcium linoleate oleate and a test using double quantity of additive.

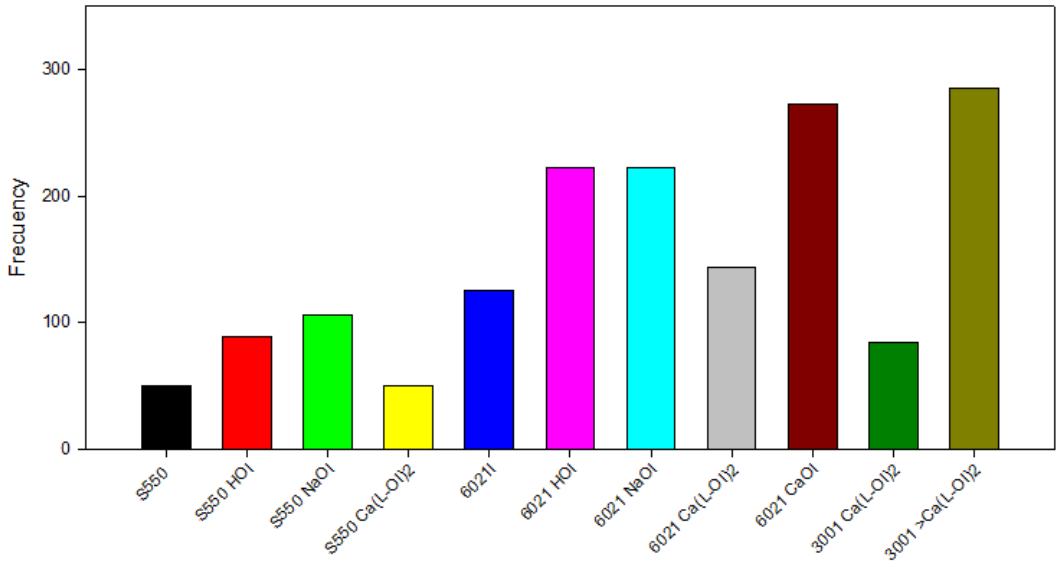

Figure 4. Comparison of each type of sodium alginate used to conduct the tests comparing the microparticle Roundness using the same flow rate, and experimental conditions just adding the respective additive (cation source). For each sodium alginate test: Without additive, using oleic acid (HOl) as continuum phase instead sunflower oil, sodium oleate (NaOl), calcium linoleate oleate $Ca(L\text{-}Ol)_2$, there is reported the frequency considering the number of occurrences within a interval from 0.80 to 1.25 of the microparticle obtained being the circle the value 1.

To avoid the coalescence effect of the not solidified droplets, there were conducted different tests adding calcium oleate, calcium linoleate oleate, sodium oleate, oleic acid, and changing the type of alginate. Improvements were observed achieving a sphere hardening without coalescence being this great problem for the microparticle formation.

There are to solve the problem of coalescence reported in literature several alternatives for example the charging droplets with a high voltage electrode (400-1400 V). Studies conducted charging droplets due to an electrostatic repulsion, create a radial surface stress which neutralizes the stresses generated by the surface tension as reported by Brandenberger *et al*., 1999. Another example where is used the coalescence effect as a vantage is generating the microparticle production without the use of any kind of surfactant because the alginate bead formation happens, thanks to the coalescence of the alginate with the $CaCl_2$. The microparticles are produced with a narrow distribution reported by (Sugiura *et al*., 2005).

It is possible to observe how the technique works with the use of our technique modification there are two examples showing how works the variation shown on the figure 5a. A sample of microparticles was obtained using additives instead another example is shown in the figure test conducted without using any kind of additive, and without technique modification (figure 5b). The coalescence using additives in our system, improves the microparticles formation avoiding the coalescence, and clearly agreeing with our experiments the figure 5a.

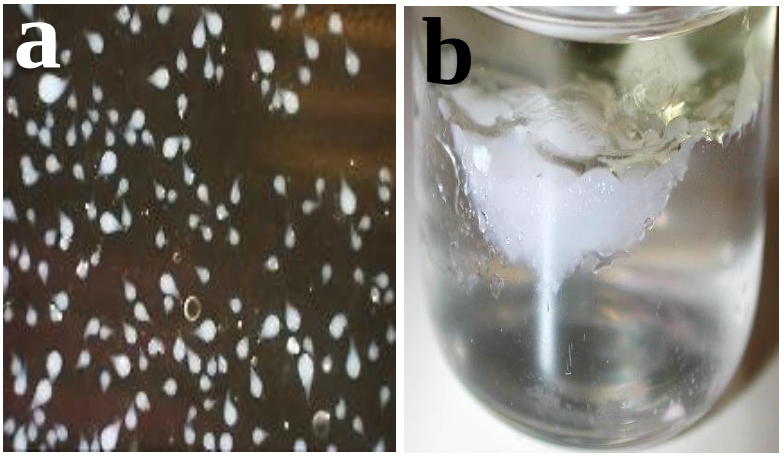

Figure 5. The images show the two effects obtained in the microparticle production using the Device II. a) Example of microparticles that were produced using calcium additive: Ca(L-Ol)2 calcium linoleate oleate from sunflower oil and using a different type of sodium alginate Cargill 6021 tangible improvement on the technique reducing the coalescence effect.
b) Using sodium alginate without using calcium additive on the process of microparticle production was used the Device II the same used in the image a) in image b) Is possible to detect the effect of coalescence too marked there was no particle formation. Image a) was obtained from optical microscopy using an objective 10X and dark field.

Size and distribution of microparticles produced are determined by different factors such as concentration, alginate molecular structure, especially in presence of surfactants, calcium source (fig. 6), initial and final pH, and finally the technique used. The interfacial tension that governs the rates of dispersion and coalescence processes reflects in the droplet size and finally the bead size (Poncelet *et al*., 1999).

Figure 6. Microparticles with good monodispersion using the Device II, also the shape is improved is possible to observe even a progress comparing with the Figure 5. a). This sample was produced using a calcium additive $Ca(L\text{-}Ol)_2$ calcium linoleate oleate from sunflower oil adjusting the quantity being the optimal founded 0.1g in 75 mL of sunflower oil the images were obtained from optical microscopy using an objective 10X.

Additional particularities were founded using an optical microscopy with a crossed nicols. It was observed the same organized and repeated figure in all the microparticles in a cross shape (fig. 7).

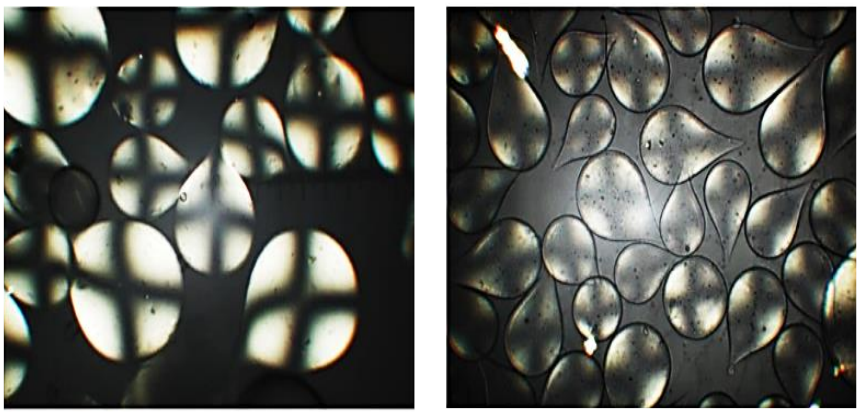

Figure 7. Microparticle produced using the Device II for this case sodium alginate used from Cargil 6021 1% (w/v) for the sample preparation using CaCl2 2% (w/v) optical anisotropy detected by observation under crossed nicols. This effect on the alginate beads generating a birefringence pattern of crossed dark lines conferred to the alignment of the alginate molecules perpendicular to the direction of Ca+2 flow the images were obtained from a crossed nicols microscopy 20X.

This effect discovered more than 50 years ago in anisotropic structure of the alginate gel seems alginate that have an anisotropic structure has not been studied very well, the birefringence grade depends on the polymer concentration and the polymer orientation (Maki *et al*., 2011), this open a wide field of research trying to seek any others practical or industrial applications of this properties.

Our results are in agreement with other authors, that used cations such as Cu, Co, Zn, and Ca allowing anisotropic comportment gelation thanks to the association of the chains segments and cations evidenced nanofibrillar morphology. This effect depends of the type of cation and mainly mannuronic/guluronic ratio, so in this way is possible to control the structure that could be of useful for industrial applications (Agulhon *et al*., 2011).

Results are also in agreement with Maki *et al*. (2009) who observed the pattern using a crossed nicols in the presence of Ca^{+2} due to the alginate molecules radially oriented in the spherical gel very similar as in our experiments.

Another interesting characteristic is that the anisotropic hydrogels registered self-assembled bundles organized in *perpendicular* direction to the Ca^{+2} diffusion showing improved characteristics as higher elastic modulus and tensile fracture strain/stress being observed *in situ* (Wu *et al*., 2011).

Different experiments were conducted to understand the structural change, and the results correlated to the birefringence changes the test was prepared adding to 5 g of sodium alginate Cargill 6021 microparticles solidified with a solution of $CaCl_2$ 2% (v/w) then the microparticles immerged in a solution of NaCl 10% (v/w) for 2 hours (fig. 8) the image show possible structural change.

The birefringence changes considerably and get vanished after addition NaCl, evidently there is a structural change in the sodium alginate matrix showing instability in contact with the NaCl solution because the cross-linking effect is destroyed by the ionic strength also there is an exchange of Na+ due to the high concentration of NaCl (Wu *et al*., 2011).

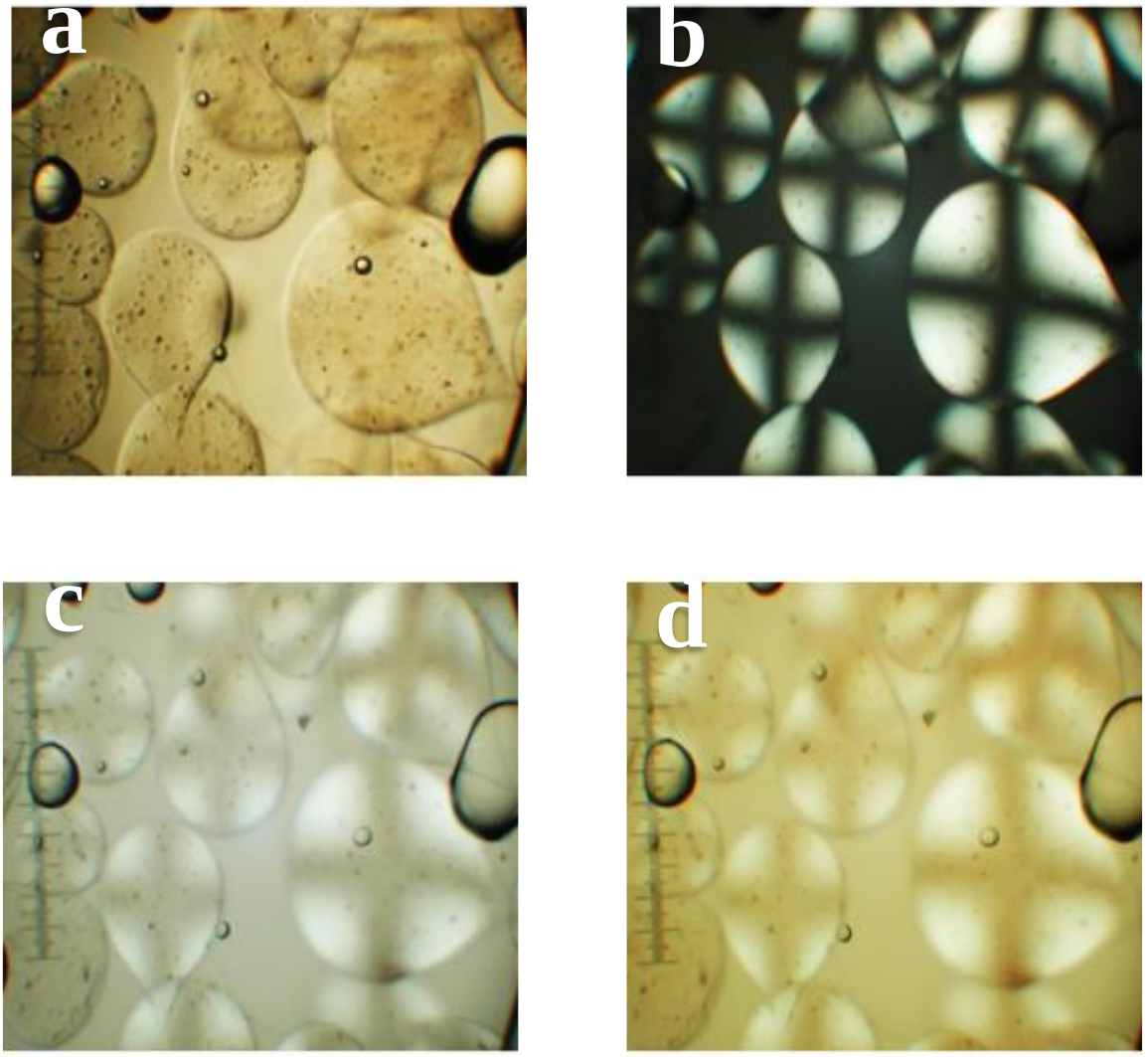

Figure 8. Observation of the same microparticles in different stages of the anisotropy images obtained from microscopy crossed nicols a) Image without use polarized filter, b) Applied polarized filter, c) There is possible to see the vanished cross, effect of the anisotropic material, d) Adjusting the polarized lens repeating the same effect than the image. Images were obtained from crossed nicols optical microscopy using an objective 10X, scale is 1.0mm.

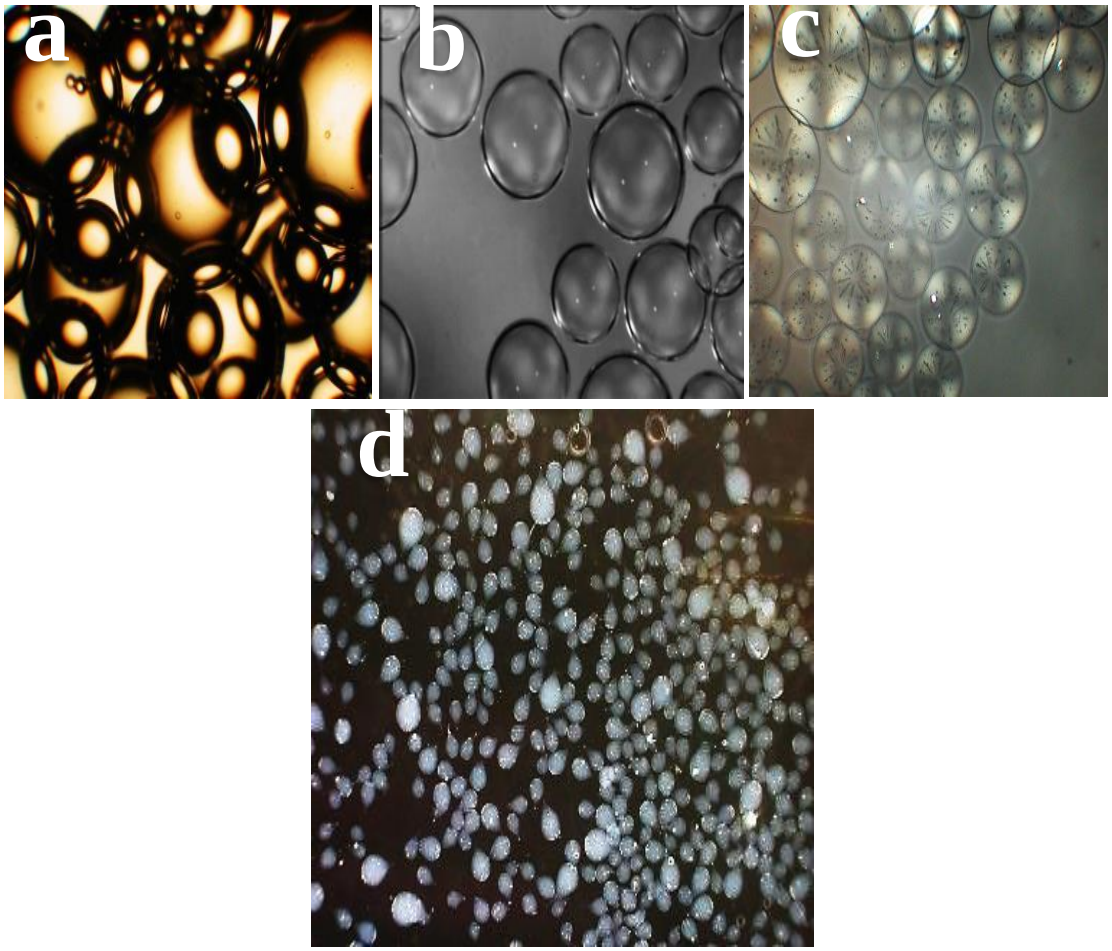

Figure 9. Example of gellification process stages of the sodium alginate droplets to produce sodium alginate beads. Images a) and b) obtained using a CCD camera used to understand the coalescence dynamics, instead for image c) polarized microscopy was used and finally used for c) stereoscope microscopy with a zoom of 10 X.
a) First stage of the droplets just poured on a $CaCl_2$ 2% (w/v) produced from the Device II,
b) After 30 seconds after poured the droplets remaining on the solidification bath of $CaCl_2$,
c) 2.5 minutes after poured almost gelled, d) Microparticles obtained and hardened in CaCl2 2% (w/v) solution after 10 minutes of produced.

3.3.3 Conclusion

We can conclude that the use of various additives such as calcium oleate, calcium linoleate, and sodium oleate helps to produce microparticles of sodium alginate resolving in a great part the problem of coalescence, one of the main problems for the formation of microparticles. Due to aggregation of the droplet before the solidification, methodology operation has being improved and the microparticles are produced reaching a good grade of dispersion in consequence a low grade of coalescence.

Also we found that birefringence effect in the microparticles is another important aspect of the experiments conducted, because it can open another important aspect of the sodium alginate microparticles that can be used in different industrial applications, helping to develop various applications to industrial scale for example using this microparticles as an biological indicator of the release of NaCl in the packaged foods and several examples like this can be reached in a short time making the necessary studies.

3.3.4 References

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3.4 Topic IV Industrial Application of Microfluidic Technique in a frozen baked-bread.

3.4.1 Materials and Methods 4

Reagents. Sodium alginate medium viscosity W201502, essential oil rosemary oil Spanish W299200, Span80, Tween20 Sigma-Aldrich (Italy), Sodium alginate Cargill S550, Cargill 6021, Cargill 3001 (France), sunflower oil Carrefour (Italy), distilled water, soft wheat flour Barilla "00" (Italy), compressed yeast (Italy).

Materials. Device IX (reference at topic II), microsyringe pump Aladdin 220 (USA), plastic connectors, fittings, and tubing were purchased from Exacta Optech (Italy). Plastic syringes were purchased from Plastipak (UK), EVO 40 SEM, Zeiss, (Germany), Ika Ultra Turrax T18 Basic homogenizer (Germany), Compressed Gas source (Italy), PET Film (Di Mauro Italy), Electric Oven from Moretti Forni (Italy), planetary kneader model KSMC50 KitchenAid (USA), Dough laminator Zmatic FS (Italy), Blast chiller Sirman (UK), Agilent 6890N gas-chromatograph with an Agilent 5973N mass spectrometer (USA), Dynamic head space Tekmar Instruments, (UK), 3000 Malvern (UK).

Emulsion preparation. Emulsion was prepared using flow focusing technique. The sodium alginate was dissolved in distilled water obtaining a concentration of 2% (w/v) avoiding bubbles. Diverse quantities were tested to determine the best formulation evaluating the particle size and separation of phase as stability indicator. Reagents were weighted and then poured in a flask and were homogenized for 60 seconds at 15,500 rpm using a homogenizer for about 1 minute.

Emulsion particle size. The emulsion was analysed using light scattering, adding 500ml of distilled water as a dispersant medium and a refraction index of 1.33, then samples were added until obtain a minimum laser obscuration of 15%.

Experimental set up. The syringe was filled with the prepared emulsion, the Device IX (gas-liquid system) connected to the syringe that eventually could be placed over the micro-syringe pump and was configured to a flow rate of 0.50 mL/min, and also the gas source was connected and the flow rate was regulated to 1 L/min.

Bread preparation. Was prepared using soft wheat flour, deionized water, salt, and compressed yeast (table 1). Dough was prepared using a planetary kneader, equipped with a 4.800 L bowl, on medium speed for 1 min, followed by 2 min at high speed. 200 g of dough were taken just after mixing and placed on a flat surface where it could expand in every directions without constrains during leavening and baking processes.

The dough was incubated at 36 ± 1°C and 70% R H for a time corresponding to the maximum volume of the dough. After the leavening phase, the dough was laminated (thickness of 2.0 cm and diameter of 30 cm) by means of a dough laminator. Baking took place inside a conventional electric oven at 300°C for 2 minutes, and then dough was cutted using a metallic mold with 10 cm of diameter.

Table 1. Ingredients use for the bread preparation.

Ingredients	%
Soft wheat flour	60.9
Yeast	1.7
Vegetal Oil	1.6
Salt	1.8
Deionized Water	34

Microcapsules addition. Once the bread was prepared and baked the samples were completely frozen, using a blast chiller at -23°C for about 25 minutes. Then microcapsules were applied using the device IX positioned 60cm above the sample, depositing the microcapsules with the aroma encapsulated for 10 seconds with the flow rates fixed at 0.5ml/min for microsyringe pump and 1 L/min for the gas source. Samples were packaged in PET packets and stored at -23 °C until its use.

Aroma measurement The Volatile organic compound VOCs were isolated using the purge and trap concentrator Teledyne dynamic head space equipped with a Tenax trap. A purge vessel containing the sample was connected to the purge and trap unit. The ad hoc experimental purge and trap settings are indicated in Table 2.

VOC analysis was performed using a gas-chromatograph equipped with a mass spectrometer and a HP-5 MS capillary column 5%-phenylmethylpolysiloxane, 30 µm 0.25 mm inner diameters 0.25 mm. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The oven temperature program was as follows: 45°C, 7°C/min ramp to 100°C for 15 min; and 5°C/min ramp to 150°C for 20 min. The mass spectra were generated at 70 eV. The VOC identification was achieved by comparing the mass spectra with those of the data system library of the gas chromatograph–mass spectrometer (GC–MS) device (NIST 02 and WILEY 275). Concentrations of each analyzed compound were expressed as % of total compounds.

Table 2. Purge and trap conditions

PURGE	
Valve oven temp.	150°C
Transfer line Temp.	150°C
Sample Mount temp.	90°C
Purge ready temp.	60°C
Dryflow standby temp.	175°C
Standby flow	10 ml/min
Pre-purge time	0.50 min
Pre-purge flow	40 ml/min
Sample heater	On
Sample preheat time	3.00 min
Preheat temp.	30°C
Purge time	15.00 min

Purge temp.	60°C
Purge flow	40 ml/min
Dry purge time	2.00 min
Dry purge temp	150°C
Dry purge flow	100 ml/min
DESORB	
GC start	Start of desorb
Desorb preheart temp.	250°C
Desorb drain	off
Desorb time	5.00 min
Desorb temp.	250°C
Desorb flow	100 ml//min
BAKE	
Bake time.	10.00 min
Bake temp	270°C
Dry flow temp.	250°C
Bake flow	300 ml/min

3.4.2 Results and Discussion 4

For a consumer, flavor and aroma are one of the most important parameters correlated to food quality related manly with the freshness of the food. Also are considered a mix of odor, tastes and stimuli that can produce different sensations on consumer (Firestein 2001).

Moreover, aroma is compound by diverse odorous molecules, usually have low molecular weight and can be generated during the production process or can be added to the food product. All these parameters determine the volatility of compunds and their resistance to mass transfer (Druaux and Voilley 1997; Van Ruth and Roozen 2002; De Roos 2003).

Therefore, encapsulation is a technological tool that helps to protect the aromas and flavors when a new product is designed and improves their functionality in the product (Porzio 2007a; Angelich 2005).

We have designed an emulsion to be used for aroma microencapsulation starting with 4 combinations on the emulsion ingredients. The results obtained as best formulation to be used in the encapsulation was the emulsion 1 because have the best ratio in quantity of essential oil and droplet size principally there was tested using a medium concentration of rosemary oil, having the smallest particle size and the best stability over time as shown on table 3 and figure 1. For the emulsion 4 it was observed a fast phase separation for this reason it was not consider for the next experiments.

Table 3. The variation on the essential oil quantity.

Test	Emulsion 1 Weigth (g)	Emulsion 2 Weigth (g)	Emulsion 3 Weigth (g)	Emulsion 4 Weigth (g)
	Medium	More Oil	Less Oil	(<) Emusifier
Alginato	44.00	39.00	46.50	44.50
Rosemary Oil	5.00	10.00	2.50	5.0
Span 80	0.50	0.50	0.50	0.25
Tween 20	0.50	0.50	0.50	0.25
Size averages(µm)	1.63	5.42	2.23	3.32

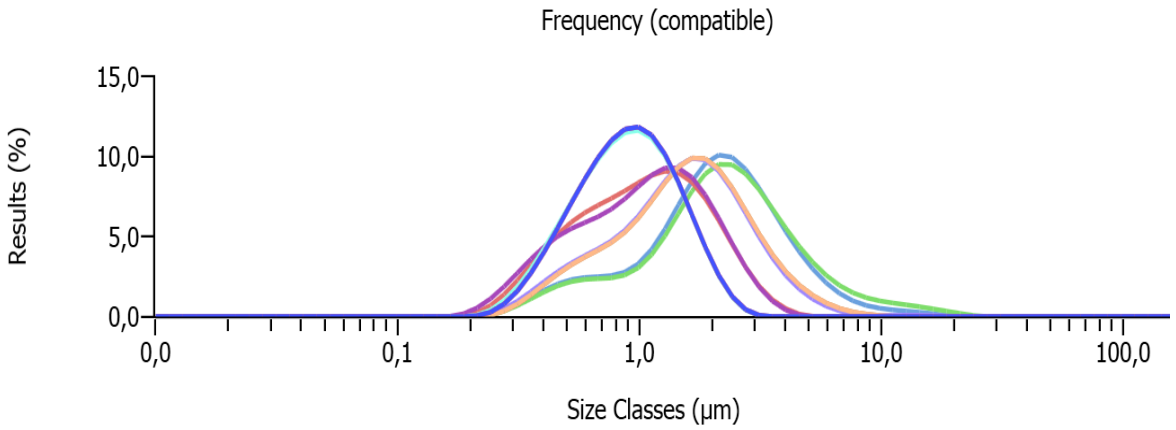

Figure 1. Size of classes of 4 different preparation of the emulsion in base to the essential oil quantity and measures of the emulsion being in blue a medium oil quantity, more oil quantity with green line, less quantity of oil on violet line, less quantity of emulsifier on orange line.

The different emulsions were observed and a phase separation was evidenced at diverse times, to understand visually better the emulsion stability is possible to observe it on figure 2. Also there were conducted tests to know the differences of the emulsion size without gelification and with $CaCl_2$ gelification. The microparticles solidified will be applied over a baked-frozen product, there is a clear example about the differences on the measures of both microparticles in figure 3.

Moreover it was observed that incrementing the rosemary oil concentration increases the particle size and decreases the emulsion stability over time.

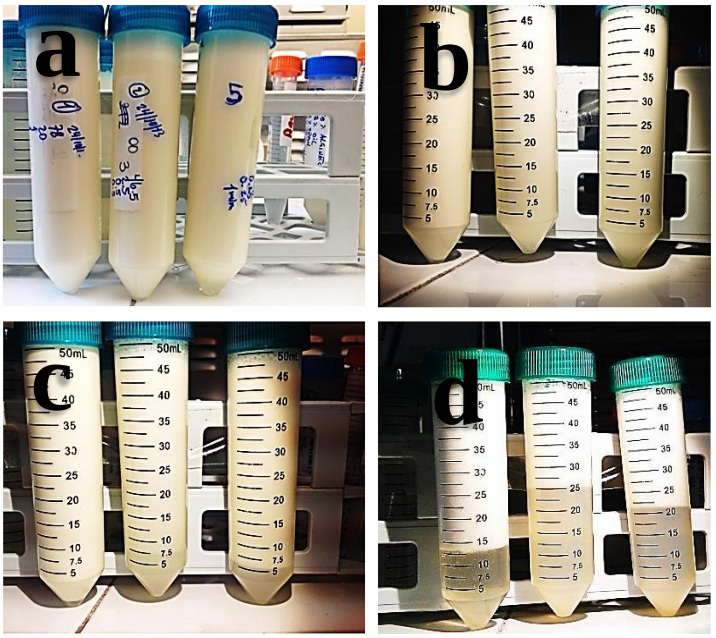

Figure 2. From left to right emulsion 1 a) Emulsion stability after 3 hours b) Emulsion stability after 6 hours c) Emulsion stability after 3 hours 8 d) Emulsion stability after > 24 hours in this photogram is evident the phase separation.

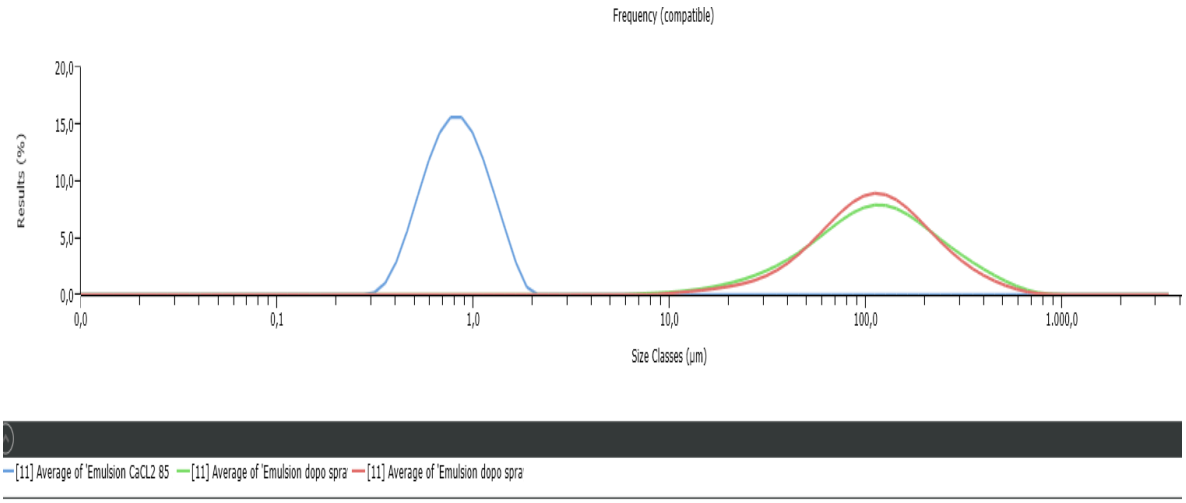

Figure 3. The blue curve shown the comportment of droplet size of the emulsion before the $CaCl_2$ solidification process and after the solidification process (right) there are two curves from different batch of production of microparticles to confirm the variability there was not necessary to confirm the left curve because agrees totally with the results reported on figure 1. It is possible to observe the tailored bread with a palatable rosemary flavor using microparticles obtained from a microfluidic Device IX

The Device IX used in a real baked-frozen bread industrialized process have diverse advantages because, using the microfluidic techniques it is possible to improving the traditional process being the aroma on the frozen and baked products, one of the most important characteristics that the final consumer can evaluate being mainly the aroma.

Also easily applicable any other frozen product after baked, improving the aroma emitting "aromatic" vapors or eliminating any other unpleasant or disliking aroma produced by food additives etc.

For this reasons we have conducted tests to obtain this desired effect adding the previous prepared emulsion rosemary oil added on the last operation to the end of the production process before the packaging operation.

The rosemary essential oil composition is fundamentally formed by monoterpenes and monoterpene derivatives (95–98%), the rest (2–5%) are sesquiterpenes (Angioni *et al*., 2004; Diaz-Maroto *et al*., 2007). The principal volatile compounds in rosemary are camphor and 1,8-cineole, followed by borneol, verbenone, a-pinene and camphene (Pino *et al*., 1998; Rao *et al*., 1998; Diaz-Maroto *et al*., 2007, Szumny *et al*., 2010). On table 3 we can found an example of rosemary chemical composition of the crude oil and all the fractions characterization effectuated using the techniques C-NMR spectroscopy and GC, which means that all components reported (Pintore, *et al.*, 2002).

Experiments to understand the level of aroma permanence and conservation using the microparticles test were conducted using a gas-chromatograph procedure there is on table 4 an example of the aromatic componets of the *Rosmarinus officinalis L*. this precise variety from Corsica and Sardinia to illustrate the kind of components founded in this type of aromatic plant.

In our tests conducted on the GC, results not shown a significant difference between samples prepared with microcapsules made with encapsulated rosemary oil and control samples prepared in the absence of microparticles.

This may be due to the low concentration of particles and another hypothesis could be the low concentration of oil used in the emulsion.

Table 4. Chemical composition of samples of *Rosmarinus officinalis L*. oil from Corsicaand Sardinia (% on BP-1).

No	Compound	Retention Indices		Corsica		Sardinia	
		BP-1	BP-20	C1 (%)	C2 (%)	S1 (%)	S2 (%)
1	Tricyclene	921	1021	0.3	0.1	0.2	0.2
2	α-Thujene	923	1021		0.1		
3	α-Pinene	931	1021	24.6	13.7	20.2	14.7
4	α-Fenchene	942	1056				0.1
5	Camphene	944	1065	6.6	2.4	3.5	3.0
6	Verbenene	947	1123	0.8	1.2	0.4	0.4
7	1:3-Octenone	957	1307			0.1	0.1
8	1:3-Octenolo	960	1440	0.1		0.2	0.3
9	β-Pinene	971	1109	1.9	1.1	0.5	0.4
10	Myrcene	980	1157	3.0	0.7	0.9	0.9
11	α-Phellandrene	997	1160	0.3	0.1	0.3	0.1
12	δ-3-Carene	1005	1144	tr	0.1		
13	α-Terpinene	1009	1176	0.2	0.1		
14	*p*-Cymene	1012	1265	3.0	1.5	1.2	1.1
15	Limonene	1021	1199	4.6	2.0	3.2	1.4
16	1,8-Cineole	1021	1208	3.5	3.4	11.3	4.9
17	β-Phellandrene	1021	1208	1.2	0.3	0.2	0.1
18	(Z)-β-Ocimene	1025	1227	0.2	0.1	0.3	0.1
19	γ-Terpinene	1048	1241	0.4	0.4	0.3	0.2
20	Fenchone	1071	1401				tr
21	*p*-Cymenene	1073	1431		0.1	0.1	0.1
22	*trans*-Linanol oxide	1073	1469			tr	0.1
23	Terpinolene	1079	1277	0.1	0.4	—	0.3
24	*cis*-Sabinene hydrate	1083	1538	tr			
25	Linalool	1083	1538	4.6	1.8	1.0	1.0
26	Chrysantenone	1100	1500	0.8			
27	Campholenal	1102	1481			0.1	0.2
28	*allo*-Cymene	1118	1370			tr	
29	Camphor	1122	1509	8.7	2.9	11.5	14.1
30	*cis*-Verbenol	1124	1645	0.1	0.6		
31	*trans*-Verbenol	1128	1669	0.3	0.6		
32	*trans*-Pinocarveol	1130	1654	0.1	0.1	0.3	0.3
33	Pinocarvone	1140	1561	0.3			
34	Lavandulol	1150	1675		0.3		
35	*iso*-Pinocamphone	1150	1538	0.5	0.5	0.5	0.6
36	Borneol	1150	1691	6.2	6.7	7.1	7.3
37	*iso*-Pinocampheol	1162	1716		0.3		
38	Terpinen-4-ol	1162	1593	0.9	1.3	1.0	1.1
39	α-Terpineol	1174	1687	0.8	2.4	2.3	2.9
40	Estragole	1176	1662		0.3		
41	Myrtenol	1181	1782	0.4	1.7		
42	Verbenone	1183	1701	4.4	20.3	15.7	24.9
43	α-Campholenol	1185	1782	0.6	0.9		
44	*trans*-Carveol	1198	1825	tr			
45	Citronellol	1208	1755		0.4		
46	Neral	1214	1677		0.2		
47	Geraniol	1234	1836	0.3	6.2		
48	Geranial	1240	1742		0.2		
49	Bornyl acetate	1272	1573	13.6	17.0	11.3	12.0
50	Pinocarvyl acetate	1281	1648	0.4	0.1		0.1
51	Neryl acetate	1341	1725		0.2		
52	Geranyl acetate	1359	1748		0.6		
53	Jasmone	1367	1933	0.1		0.1	0.1
54	Methyl-eugenol	1369	1639		0.4		
55	β-Caryophyllene	1421	1587	2.0	0.6		
56	α-Humulene	1453	1658	0.3	0.2		
57	α-Curcumene	1472	1763		tr		
58	Spathulenol	1567	2113		0.2		

tr, trace.

Is possible to observe diverse images obtained from SEM of alginate microcapsules with rosemary essential oil loaded, on the figure 4. The samples were taken from a suspension in water with low concentration of microspheres.

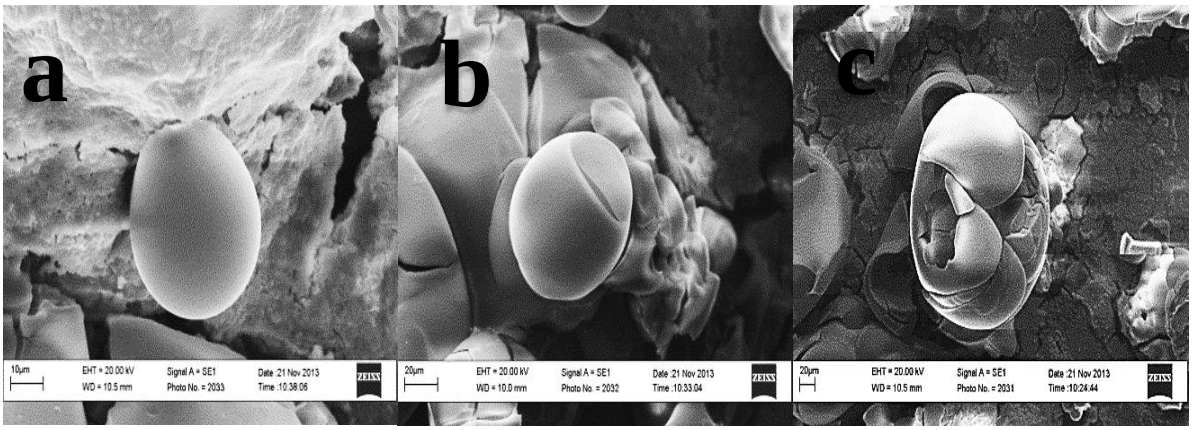

Figure 5. Analysis of SEM images was effectuated observing different stages of the microparticles of alginate solidified with $CaCl_2$ entrapping essential oil a) microparticle undamaged after the $CaCl_2$ bath 9X, 20kv b) semi damaged microparticle 9X, 20kv c) damaged microcapsule example after the $CaCl_2$ bath 9X, 20kv.

3.4.3 Conclusion

The application of the microcapsules in form of matrix sodium alginate demonstrates being a available method to be used in an industrial process, specifically in this case for a baked frozen food product, achieving the main objective that is to capture a controlled quantity of essential oil, to protect it from the low temperatures until the frozen product is delivered to the final consumer.

Minimum quantities of microencapsulated essential oil are applied to the frozen product due to the cost of the natural essential oils. However, this small quantities are often enough to be perceived by the consumer. The accuracy injection of the aromatic compound allow a real optimization about the costs that others techniques do not have this is a very valuable vantage in an industrial process we can assume that encapsulation is a valued tool for the design of food attributes on products for instance the encapsulated aroma improve aroma functionality and stability in the food product.

Aroma encapsulation has many advantages in industrial processes like ease of handling, low evaporation, degradation, interaction with other components in the food product, safety reducing flammability risk, no concentrated aroma oil handling, creation of visible and textural effects, adjustable aroma properties particle size, structure, oil or water dispersible, controlled aroma release, aroma differentiation, aroma retention, also claims that it is growing faster than the aroma market.

The demonstration of the microencapsulation of essential oil as a good tool to avoid high volatility improving the stability of released volatiles and other negative effects inherent to the nature of essential oil, improving the possibility of storage can be used in many food applications.

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APPENDIX 1

***EFFoST* Annual Meeting 2011. Berlin**

Microfluidic focusing flow technology for micro droplets generation of protein/pectin complexes

Cristian Davalos Saucedo, Prospero Di Pierro, Paolo Masi, Giovanna Rossi Marquez, Fabrizio Sarghini

Highly structured protein/polysaccharide complexes may display better functionality than proteins and polysaccharides alone (Ye, 2008). The manipulation of protein/polysaccharide interactions (Dickinson, 2008) can represent an important tool to modify the microstructure of the composite systems to produce edible films. The use of edible films to produce microcapsules (encapsulation of aroma compounds, vitamins, and additives) confers to them the status of active films. There is a growing demand in the food, personal care, agricultural, and pharmaceutical industries for new encapsulation techniques with defined mechanisms for the release of active ingredients. Microencapsulation defined as "the technology of packaging solid, liquid and gaseous materials in small capsules that release their contents at controlled rates over prolonged periods of time". We have recently obtained and characterized a whey protein and pectin (P/P) soluble complex with dimensions of 5-7 m diameter which shown good properties to be used as a material for active films production. The characteristics of adsorbed complexes, the structures of mixed biopolymer interfaces, are still poorly understood. A proper understanding and control of the P/P interactions should help in designing emulsified films by microdroplet. Microfluidic represents a promising tool for generation of micro droplets. Monodispersed microdroplets generation of protein/pectins aggregates introduces additional challenges, due to the molecular structure of the complex subjected to aggregation in presence of an adverse pressure gradient or unbalanced electrical charges. As a consequence, direct extrusion in coaxial borosilicate capillaries resulted to be inappropriate, due to protein structuring. In this work a monodispersed droplets generation of P/P in oil was obtained by using an opposing focusing flows in a coaxial capillaries device (see Fig. 1a) and varying the ratio between P/P inner flow rate and oil and consequently controlling the droplets radius. A typical experimental dripping regime is shown in Fig. 1b. Effect of relative ratio between P/P-OIL was investigated, and results are presented for different dripping regimes.

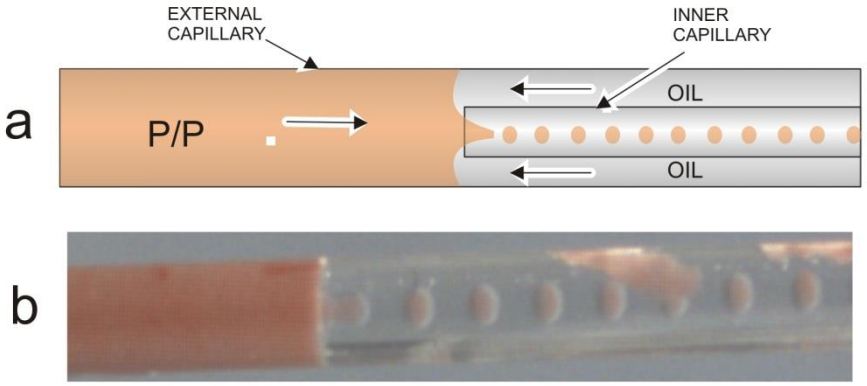

Fig.1. Focusing flow device configuration (a) and experimental dripping regime (b)

APPENDIX 2

Inside Food Symposium 9-12 April 2013, Leuven, Belgium

Alginate beads production using a liquid sheet pre-gelification technique

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ABSTRACT

Alginate micro-bead production represents an interesting technological application in many fields such as pharmaceutical, food, and cosmetics. Usually the studies of micro droplet or micro-bead production in micro channels formed in different geometries and using different techniques (mostly T channel or flow-focusing devices) have been the subject of many research studies using pure, well characterized solutions and do not take into account the behaviour and interaction of food grade and natural products. The possibility of using air as focusing flow (Bong et al., 2010) in microfluidic devices to produce sodium alginate micro-bead introduce some advantages; for example, the use of different focusing fluids like oil frequently requires complicate production processes, introducing a barrier to the interaction of alginate drops with the calcium ions solution during gelification phase and requiring a posteriori filtering and washing procedure. Moreover, direct immersion of liquid alginate drops in a calcium chloride bath to induce gelification usually happens at relatively high speed, inducing a bead shape deformation due to inertial effects. Such deformation effects can represents a problem, especially when drop encapsulation is required and can affect for example controlled release properties. Aim of this work paper is to introduce a mild pre-gelification technique interposing a liquid sheet of calcium chloride vertically flowing in the path of liquid alginate drops, where the micro-bead shape fixing process starts "on the fly", and eventually finish the process in a liquid bath. Effect of calcium concentration in the liquid sheet and effects on shape preservation shows that this procedure represents an effective method for the sodium alginate micro-bead production (shape fixation), with minimal shape loss.

1 Introduction

Industrial scale production of possibly monodispersed alginate beads with desirable characteristics such as controlled size and shape is a desirable target in food industry. Several mechanical and chemical methods had been introduced to produce particles with narrow size distribution in a single step approach, (e.g. milling, oil-water and water-oil emulsions, coacervation, spinning disk, atomization, vibrating nozzle) (Schneider *et al.*, 2011).

Microfluidic devices plays an interesting role in microbeads production due to their relatively cheap technology, and several devices based on different approaches had been proposed; they seem to work quite well on a laboratory scale with fluid mixtures of quite simple rheology and well known properties. Nonetheless the scale-up of such technologies to the industrial scale often represents a powerful challenge, as several additional characteristics are required: devices should be easy to build, possibly arranged in parallel arrays for mass production with minimal interference between the single devices, the separation between fluids and microbeads should be simple, and possibly cheap.

Among many possibilities, flow focusing is a microfluidic technique where two or more phases of liquid or gases are co-axially focused and then forced through a small orifice. The flow rate of the outer phase, called the continuous phase (CP), usually exceeds that of the inner dispersed phase (DP), typically by ten to thousand times depending on the fluid characteristics. The DP is thus forced into a narrow jet and obliged by DP confinement to flow at the orifice. Due to the rapid change in pressure chamber to the outlet and the prevailing effects of shear stress, fluid dynamics instabilities appear, and the jet breaks up into droplets after passing the orifice (Schneider *et al.*, 2011).

Micro-beads can be prepared using polysaccharides such as alginate, a water soluble linear polysaccharide extracted from brown seaweed. It is composed of alternating blocks of 1–4 linked α-L-guluronic and β–D-mannuronic acid residues. Depending on the source of the alginate, the molecules can be composed of three types of blocks: polymannuronic acid blocks (MM), polyguluronic acid blocks (GG) and mixed blocks (MG). The amount of the elements (M and G) varies with the source of alginate (Herrero, *et al*., 2006).

Alginate is often used as nutraceutical and active compounds in both matrix and drop in drop encapsulation. A possible flow focusing configuration requires oil as CP, allowing a very good control of geometrical properties of beads in the extruding phase, although it involves complicate production processes, introducing a barrier to the interaction of alginate solution with the calcium ions solution during gelification phase and requiring a posteriori filtering and washing procedure to recover the beads.

The possibility of using a gas as continuous phase introduces several advantages if compared with the previously mentioned liquid-liquid configuration: easier separations of the different phases, cheaper production costs and the possibility of using three dimensional arrays for mass production with minimal interference distance. On the other hand, the reduced shear stress at the phase interface and different inertial properties of the gas-liquid mixture requires relative high gas velocities involving flow instabilities (Si *et al*, 2009) and turbulence, with a more difficult operational control of drop properties.

This technique was used for active compounds microencapsulation (Gundabala, *et al*., 2013) using a configuration in co-focusing flow (gas-liquid) in which viscous (Cohen, *et al*., 2001; Blanchette and Zhang, 2009) and/or pressure (Gañan-Calvo, *et al*., 1998) forces stretch an interface until small DP jet is emitted.

Gas-liquid flow focusing approach uses the pressure gradient induced by an outer gas stream to confine and 'focus' a steady liquid jet of the dispersed phase. When the liquid microjet and the co-flowing gas stream cross a discharge outlet whose diameter is much larger than that of the microjet, a jet instability is generated, inducing a jet break-up and allowing mass production of ultrafine and almost monodispersed spray, microcapsule or microspheres (beads) (Acero, *et al*., 2012). This technology is still in maturation and there are not enough theoretical information about the shape of the alginate beads, as instabilities dominating jet breakup of complex rheological liquid such those used in food industry still represent a theoretical challenge.

In this work both a numerical and an experimental analysis was adopted, aimed to understand the jet break-up mechanisms of the liquid DP in gas in a simple microfluidic configuration, and then an experimental setup was used introducing a mild pre-gelification technique, obtained interposing a vertical liquid sheet of calcium chloride solution in the path of liquid alginate drops where the micro-bead gelation process of shape fixation starts "on the fly".

2 Materials and methods

Characterization of alginate microbeads sizes was quantitatively obtained using a light scattering particle size measuring instrument (Mastersizer 3000, Malvern Instruments), and optically with an optical microscope with 40X magnifying power stereomicroscope equipped with a digital camera. Illumination was provide by using a UV lamp at 390 nm wavelength.

The quite simple microfluidic geometry shown in Fig. 1 represents exactly the experimental setup, including the presence of the vertical liquid screen obtained using a slot in a polycarbonate horizontal tube, while the microfluidic device consist in two coaxial capillaries, one with the inner radius equal to 200 ㎜ for the DP and an extrusion hole radius equal to 400 mm.

Calcium chloride solution was prepared using AppliChem GmbH Ottoweg Calcium chloride dried powder pure at 97% in a baker stirred on a warm hot plate 20 °c until the powder was dispersed in distilled water at 1% in weight.

Alginate solution was obtained by using Sodium Alginate from Sigma-Aldrich Algin, Alginic acid sodium salt from brown algae, for R&D use. The solution was mixed at 30 °C for 12 hours and then coloured using UV activated red florescent Createx water based colour.

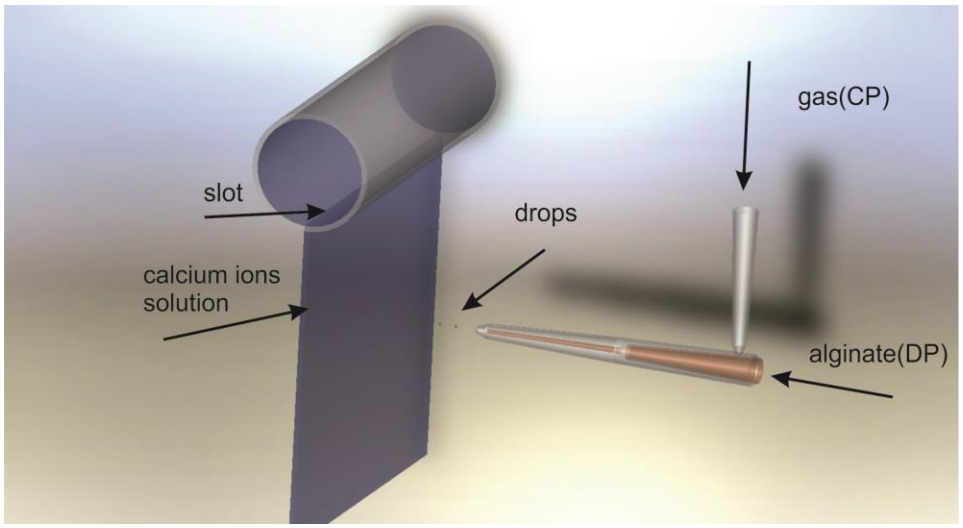

Fig. 1 Schematic representation of the main experimental setup.

3 Results

3.1 Numerical Results

In order to obtain a better understanding the dynamics of drop formation and a model prevision of their possible shape and dimension at the exit of the microfluidic device, a numerical simulation was performed using computational fluid dynamics. The numerical solver adopted in this work is OpenFoam (Openfoam v2.2.0) , a free, open source CFD software package. Several mesh configurations were tested, ranging from $150x10^3$ up to $2x10^6$ control volumes. A Large Eddy Simulation approach was used for turbulence modelling of the gas phase using a scale similar model (Sarghini *et al*, 1999), while CP-DP interaction was analysed by using a Volume of Fluid (VOF) approach (Hirt & Nichols, 1981). A value of 65 mN/m of relative surface tension between alginate solution and air was set (Babak *et al*, 2000), while rheological properties was obtained experimentally by using a Rheometrics RFS II rheometer.

Several ratio between DP and CP were tested, but results are shown only for CP mass flow rate of 1 L/min and DP mass flow rate of 0.3 mL/min.

In Fig. 2a the jet instability and in Fig. 2b the associated isosurface of DP (cut-off at 0.92 due to numerical smearing effects) are shown. A very good agreement is obtained between the predicted size of the alginate drops (about 500mm) and shapes: two main geometries can be recognized, one more spherical and a secondary more ogival, and a comparison with a picture of alginate beads obtained using an optical microscope is showed in Fig. 2c shows a strong similarity.

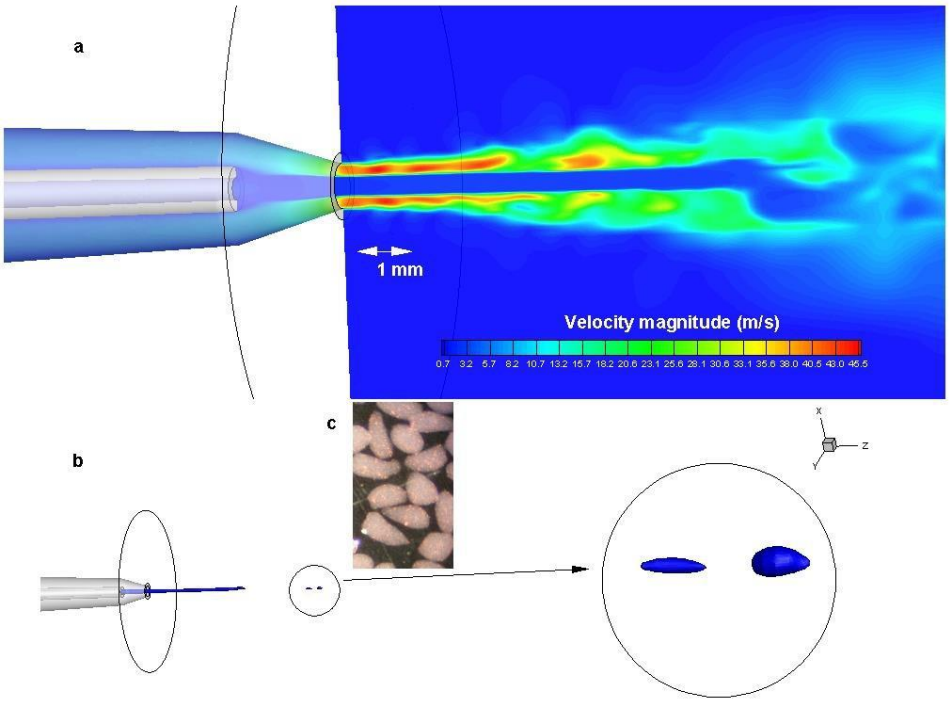

Fig. 2 Instantaneous snapshot of velocity magnitude flow field and isosurface of liquid alginate jet and drops before shape fixing compared with experimental results.

3.2 Experimental results

The main target of this work was to test the efficiency of a vertical liquid sheet containing calcium ions to induce a fast gelification while the drops are still "flying". Experimental results were performed using several CP/DP mass flow rate ratios: in Fig.3 a comparison between beads obtained with the use of the vertical liquid sheet (Fig.3a) are compared with those obtained without (Fig. 3b): the former are collected by directly filtering the falling film, the latter using an open collector tank positioned at 1.2 m of distance from the microfluidic device. The screen was positioned at 0.15m from the drop formation zone, as the high gas speed velocity would result in a liquid sheet break-up is positioned at a nearer distance. As highlighted in the previous section referred to numerical results, the "original" shape of the drops is not exactly spherical, due to the jet break-up dynamics, and numerical predicted shape is very similar to those obtained using the liquid screen. On the contrary, drops collected after the splash into the collector tank present a more spherical shape, due to the aerodynamics pressure regularization effects during the flight, but a wider size dispersion is present, caused by the impact and fragmentation of the drops on the liquid tank surface.

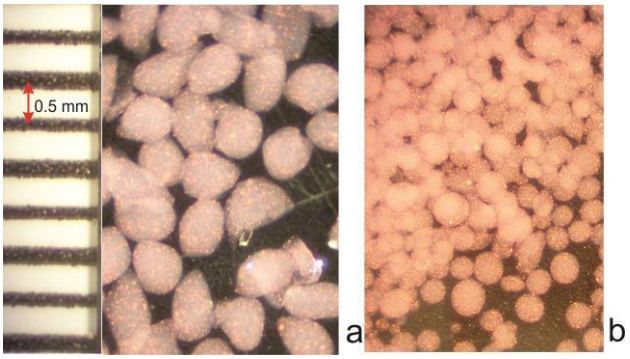

Fig. 3 Image analysis of alginate beds obtained with (a) and without liquid sheet (b).

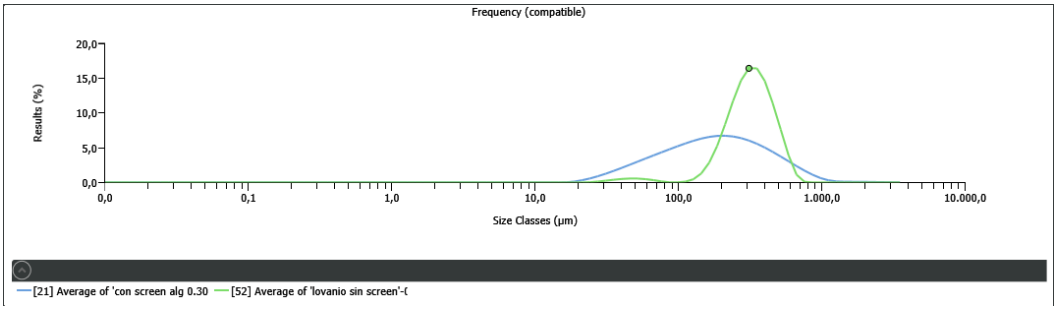

Fig. 4 Particle size distribution of alginate beads with (blue) and without (green) screen obtained with Mastersizer 3000

Results obtained by using optical image analysis were confirmed by using a quantitative test with the light scattering particle size analyser, and results are shown in Fig. 4. Both effects (reduction of medium size and a wider size distribution) are evident. Quantitative results need to be refined as in this case the obscuration parameter was at the optimal limit for this type of test.

4 Discussion

A first result is the quite good agreement between the shape and dimension theoretically predicted by the numerical simulation and the experimental ones, showing that the fixation process on the liquid sheet can really improve shape fixation of alginate droplets before external uncontrolled mechanical and pressure driven parameters can vary size distribution and shapes.

Shape fixation can be performed just at the exit of the microfluidic device and this technique can be adjusted to a wide range of configurations.

The adopted microfluidic geometry, very simple, requires very high gas velocities to confine the liquid jet, introducing turbulent flows ($Re=3x10^4$) for the gas phase, and a more careful parametric study is required to optimize diameters, internal configurations and mass flow rates. Production of smaller beads using the geometrical configuration adopted in this work would trigger the spray regime, introducing a wide distribution of beads sizes.

A second consideration is referred to the physical characteristics of the thin liquid sheet, not fully investigated; as a matter of fact the difficulty to obtain a very thin uniform sheet obliged us to produce a relatively thick sheet (1-2 mm), in which the alginate drops were entrapped and dragged down together with the calcium ions solution. Probably a surfactant is required to control the liquid sheet surface tension. Effect of liquid drag on drops shape deformation before complete gelification was performed still need to be investigated.

5 Conclusion

The results obtained showed that the proposed approach (a gas dispersed phase plus a liquid sheet to induce an immediate gelification) represents a viable solution for alginate beads production, at least for matrix encapsulation of nutraceutical. The microfluidic device proposed in this work is very simple and cheap, and probably something better could be used in the future. Geometric parameters are fundamental in order to control jet instabilities dominating the drops break-up dynamics (Vega *et al*, 2010), and optimal shape is required and need to be further investigated. More extensive tests are now in progress to analyse interference effects in multiple array configurations and to study the drag effect on the drops flowing with the liquid sheet respect to gelification time.

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