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Suspensibilidad y estabilidad de varias microcápsulas que contienen lupulonas

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TÍTULO: **Suspensibility and stability of a variety of lupulone-yielding microcapsules.**

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Resumen

El presente trabajo de fin de grado se basa en la producción mediante el método "spray-congealing" en una planta piloto y estudio de las propiedades de microcápsulas en las que el principio activo es lupulonas con variación del resto de componentes (antioxidantes, emulsionantes y ácidos grasos) para mejorar las propiedades. Las propiedades estudiadas son suspensibilidad en agua, estabilidad en el tiempo tras almacenamiento y medidas de la concentración liberada de las microcápsulas para ser utilizadas como suplemento en la alimentación animal.

Palabras clave

Microcapsulas, spray-congealing, lúpulo, emulsionante

Stability and suspensibility measurements of a variety of lupulone-yielding microcapsules

Bachelor thesis

of

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Erlangen, den 22. Februar 2019

Cecilia Castedo Hernández

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List of abbreviations

Abreviation	Meaning
$A\cdot$	Radical of antioxidant(s)
AH	Antioxidant
Asc	L-(+) Ascorbic acid
CS	capsule suspension
FAU	Friedrich-Alexander-Universität Erlangen-Nürnberg
H_2O	Water
HCl	Hydrochloric acid
HLB	Hydrophilic Lipophilic Balance
HPCE	High Performance Capillary Electrophoresis
MeOH	Methanol
NaOH	Sodium hydroxide
O/W	Oil-in-Water
W/O	Water-in-Oil
$R\cdot$	Alkyl radical
$RO\cdot$	Alkoxy radical
$ROO\cdot$	Peroxy radical
ROH	Hydroxyl compound
ROOH	Hydroperoxide
SoftMG	Softenol 3995 Monoglyceride
SoftTG	Softenol 3196 Triglyceride
Toc	α-Tocopherol
µm	micrometer
cm	centimeter
g	gram
h	hour
kg	kilogram
L	liter
m	meter
mg	milligram
min	minute
mm	millimeter
nm	nanometer
s	second

List of Symbols

Symbol	Meaning	Unit
$\%_v$	Volumetric percentage	-
$\%_{wt}$	Mass percentage	-
A	Area	m^2
c	Concentration	$kg \cdot l^{-1}$
Ø	Diameter	m
m	Mass	kg
p	Pressure	bar; $kg \cdot m^{-1} \cdot s^{-2}$
S	Suspensibility	-
T	Temperature	K; °C
t	time	s
λ	Wavelenght	nm

1 Introduction

There is an increasing tendency to use natural plant compounds that have some antimicrobial activity as a feed for livestock production [1]. One of the reason for this change is the ban on the use of antibiotic growth promoters in the European Union due to concerns about the spread of antibiotic-resistance [2]. For this objective, hops have already been used besides of brewing in many other fields such as medicine, cosmetic and culinary. In fact, the use of hops has more than 200 years of history [3].

Antibiotics can alter rumen fermentation improving rumiant growth and efficiency. The hops plant produces a range of bioactive secondary metabolites, commonly called α- and β-acids. Hop β-acids inhibit rumen bacteria possessing a classical Gram-positive cell envelope. This selective inhibition causes several effects on rumen fermentation that are beneficial to finishing cattle, such as decreased proteolysis, ammonia production and acetate [4].

For this purpose it is necessary to convert the very viscous and sticky hop extract into an applicable form, which is soluble in water. One solution is microencapsulation. At the Institute of Processing Machinery and Systems Engineering (iPAT) of the FAU a process and a production plant for microcapsules by spray-congealing technique was developed. The hop extract is inside of a fat matrix. Some additives such as emulsifiers and antioxidants are added to improve or modify the properties of the capsules. This method provides some interesting features, for example, the hop extract is protected against oxidantion and UV radiation, also the controlled release of active ingredients can be adjusted.

The aim of this thesis is to study the properties of multiple formulations of hop extract microcapsules for their future use as supplement in animal feed. Suspensibility in water and stability of β-acids after storage time were studied. Different combinations of emulsifiers, matrix materials and antioxidants were used to determine the effect they have individually and combined in the earlier mentioned properties. The main objective is to improve the suspensibility of the microcapsules by adding and changing the emulsifiers. Furthermore, an additional test analysing the hop extract release from the microcapsules was carried out with one formulation.

2 Theoretical background

2.1 Microencapsulation of hop extract

2.1.1 Microcapsule structure and encapsulation technique

Microencapsulation is a technology that creates particles enclosing an active substance and the physical state of it can be any. Because of the wide use of this technology, there can be different definitions that can be more precise with the field in which they are used. However, a generic definition can be: the entrapment of a compound or system in a dispersed material to its immobilisation, its protection, its control of transfer, its structure and its functionalization [5]. There is a classification regarding the particle size [6].

Generally microcapsules have two components, core and shell. The core can hold active pharmaceutical material, pigments, proteins, peptides; while the shell can be a wide variety of materials (sodium alginate, gelatine, polyester, etc.) and is referred to as the coating. This material determines the stability of microparticles, the process efficiency and the degree of protection for the core [7]. The core material is gradually diffused through the capsule walls, thereby offering controlled release properties under desired conditions [8]. An important use is the deliver of bioactive components improving their handling properties.

Controlled release is a method by which one or more active ingredients are available at a desired site and time at a specific rate [9]. This technology provides a deliver of compounds such as drugs, fragances and pesticides at a defined rate with efficacy and safety.

Microcapsules may have different morphologies (Figure 2.1); based on this, they can be classified as mononuclear or continuous core/shell, multinuclear or polynuclear, and matrix microcapsule.

The microencapsulation can be made with different techniques, but they are classified in three different methods

- Chemical
- Physico-chemical
- Physico-mechanical

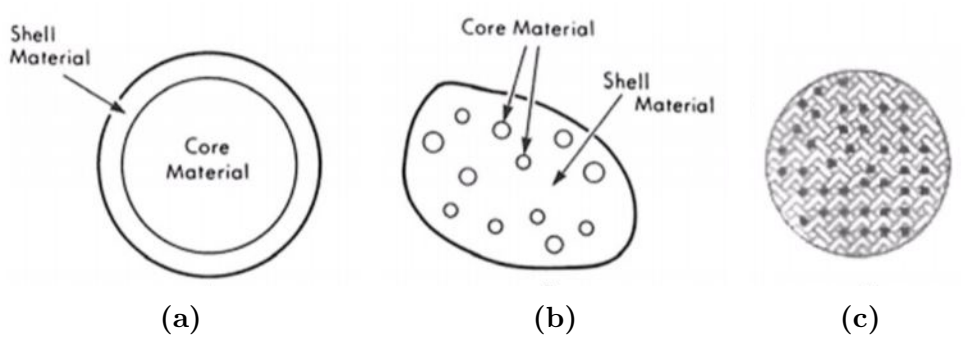


Figure 2.1: Morphology of microcapsules: (a) Mononuclear or Continuous core/shell microcapsule [10]; (b) Multinuclear or polynuclear microcapsule [10]; (b) Matrix microcapsule [11].

Depending on the physical nature of the core material, the technique that is used varies, and with it the size of the microcapsules. The commonly used are fluidized bed or air suspension, coacervation and phase separation, spray drying and spray-congealing, pan coating and solvent evaporation.

The technique used is spray congealing, which is a combination between air spraying and hot melt extrusion techniques. Congealing is called the transition from fluid state to a solid state by cooling. The process time compared to other "particle engineering" technologies like air spraying is shorter due to the absence of solvent and post-processing is typically not required [12].

An schematic representation of the process is shown in Figure 2.2. The matrix material has to be melted and after that the rest of the components such as active ingredients and any other desired are added and finally homogenised. The droplets created by a two-substance nozzle solidify once their temperature goes under the melting point of the mixture. Solid capsules can be collected with a cyclone.

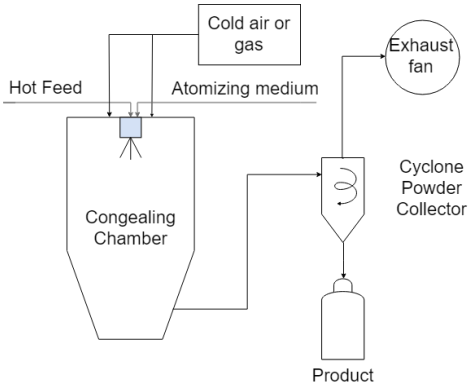
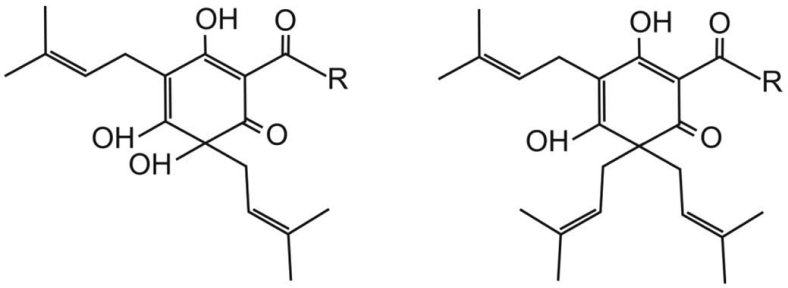


Figure 2.2: Spray congealing method for microcapsule production

2.1.2 Hops

Hops (*Humulus lupulus*) belong to the family Cannabinaceae. The used part of the plant is called cones, which are the inflorescence of the female plant. Inside the cone there are granules, lupulin glands, that contain an essential oil and the hard and soft resins. Among all of this components the most important are the soft resins which consist of α-acids (humulones) and β-acids (lupulones), known as bitter acids (Figure 2.3). They represent 30% of the total lupulin content of hops.



(a) **(b)**

Figure 2.3: Structures of Hop α-acids (a) and β-acids (b) [13, edited].

Within this work, an extract with lupulones should be encapsulated. These β-acids present a very poor solubility in water due to their hydrophobic chains in their structure [14]. Bitter acids can be oxidized very easily by air [15] and have a deterioration in time accompanied by a strong odor. Pure β-acids at 20 °C under open air conditions decompose approximately 50% after one month [16]. If the products tested are pressed hope cones instead of pure acids, after 12 months of storage at the same conditions, the decrease in β-acids composition is 51–83%. The oxidation rate depends on the storage temperature [16]. The bitter acids have intrinsic anti-oxidant activities. To avoid the oxidation of the lupulones another antioxidants are added so they are oxidized instead of the bitter acids [17].

The main products of natural aging hops, under open air conditions are hulupones and humulinones [18].

2.1.3 Antioxidants

Oxidation is a reaction which cause free radicals, highly reactive species which have one or more electrons in their outermost shell. Free radicals can react indiscriminately with any molecules, radicals or non-radicals [19]. If the reaction is with a stable molecule a chain reaction starts.

Antioxidants react with the free radicals (favor entrapment of radicals $R\cdot$, $RO\cdot$, $ROO\cdot$, $\cdot OH$, etc.) ending the chain reaction. The activity of antioxidants depends of many factors, lipids nature, the presence of other antioxidants, oxygen pressure, temperature and antioxidant concentration [20]. If this is not optimal, the opposite effect could happen, and become prooxidants instead of antioxidants. For this reason it is important to determine the optimal concentration.

The mechanism of free radical chain reaction can be divided in three stages: initiation, propagation and termination. On the first stage, RH turns into an alkyl radical $R\cdot$ [20].

$$RH \rightarrow R\cdot + H\cdot$$

The chain free radical propagation occurs. Oxygen reacts with the radical and forms peroxides and a peroxyl radical $ROO\cdot$.

$$R\cdot + O_2 \rightarrow ROO\cdot$$
$$ROO\cdot + RH \rightarrow ROOH + R\cdot$$
$$ROOH \rightarrow RO\cdot + \cdot OH$$

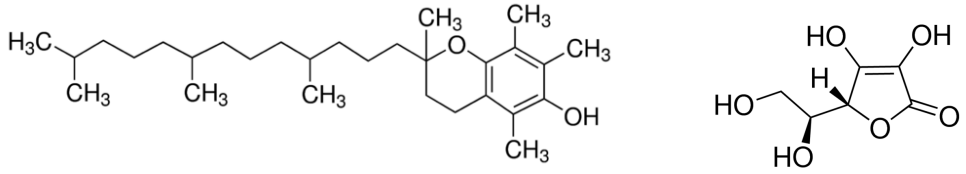
On the last stage, two radicals could lead to a nonradical adduct and end the free radical chain.

$$R\cdot + R\cdot \rightarrow R - R$$

When one antioxidant (AH) is in the same phase as the substance the chain reaction can be stopped by reaction with the radicals [21].

$$R\cdot + AH \rightarrow ROH + A\cdot$$
$$ROO\cdot + AH \rightarrow ROOH + A\cdot$$
$$R\cdot + A\cdot \rightarrow RA$$
$$ROO\cdot + A\cdot \rightarrow ROOA$$

Antioxidants can be classified in two big groups, enzymatic are produced in the human body and non-enzymatic are needed for the metabolism but not produced in the body [22]. The antioxidants used belong to the non-enzymatic category and inside of this wide group, they belong to vitamins. DL-α-tocopherol and L(+)-ascorbic acid are both commonly known as Vitamin E and C, respectively. DL-α-tocopherol is lipid-soluble which makes



(a) **(b)**

Figure 2.4: Structures of (a) DL-α-tocopherol [23] and (b) L(+)-ascorbic acid [24]

it very useful in the microcapsules. The structures of each antioxidant can be seen in Figure 2.4

Vitamin E. There are 4 different tocopherols but their structure is characterized by a saturated side chain consisting of three isoprenoid units. The activity of each one varies with the number and position of the methyl groups on the ring and the configuration of the asymmetric carbons in the chain [25]. **α- tocopherol** is the most abundant and biologically active form of tocopherols.

It is an important synthetic antioxidant. It is mainly in vegetable oils where it carries out an antioxidation function protecting the tissue. The hydrogen in the hydroxyl group on the aromatic ring is donated to the free radical what gives the molecule the antioxidant property [26].

L(+)-Ascorbic Acid is considered one of the most important substances in the metabolism of organisms. Humans cannot synthesize this vitamin because they do not have the enzyme required. However, some animals and plants have the ability and synthesize it from D-glucose. The intermediate ascorbyl radical has delocalized electrons therefore, it reacts with free radicals making the propagation of the reactions finish [27].

There is also evidence to suggest that α-tocopherol and ascorbic acid can function together in a cyclic-type of process [28], in which the α-tocopherol (Toc) is converted to an α-tocopherol radical ($Toc\cdot$) by the donation of the hydrogen, and the radical can be reduced to the original form by ascorbic acid (AsH), changing into semihydroascorbic acid radical ($As\cdot$), which can be further oxidized to give dehydroascorbic acid (DHAs) [29]:

$$Toc + R\cdot \rightarrow Toc\cdot + RH$$
$$Toc + ROO\cdot \rightarrow Toc\cdot + ROOH$$
$$Toc\cdot + AsH \rightarrow Toc + As\cdot$$
$$As\cdot \rightarrow DHAs + H\cdot$$

2.1.4 Emulsifiers

Because the active ingredients used are not easily soluble in water emulsifiers must be used to get the functionality that it is expected together with good wettability and suspensibility. These surfactants create connections with both hydrophilic and lipophilic molecules. This mechanism allows the capsules to be dispersed into water. This happens because emulsifiers are amphiphilic, (Figure 2.5) and get adsorbed in the interface between the oil and the water phase [30] lowering the surface tension. An scheme of how the molecules are arranged in a W/O emulsion is shown in Figure 2.5. Emulsions can be oil-in-water (O/W) or water-in-oil (W/O). They have the ability to alter surface and interfacial properties and to self-associate and solubilize themselves in micelles. Having the tendency of lowering interfacial tension [31].

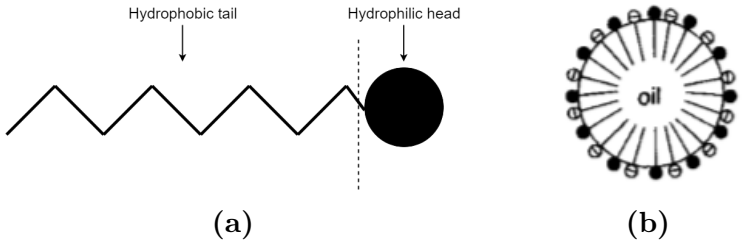


Figure 2.5: (a) Structure of an amphiphilic molecule and (b) adsorption of the emulsifier at an oil droplet [32, p.83]

When the concentration of emulsifiers is high, organized aggregates of larger molecules (about 20-100 molecules/micelle) are formed. Lipophilic parts of the emulsifier associate in the interior of the aggregate, and the hydrophilic parts face the aqueous medium [33].

An important concept in emulsifiers is the hydrophilic-lipophilic balance, HLB, which relates the lipophilic parts and the hydrophilic parts in the emulsifier's molecule. Even with their amphiphilic nature, they can be easier soluble in water or oil; this tendency is expressed in the HLB. For O/W emulsions, the HLB should be between 8 and 18. The ones used in the production have and HLB higher than 10. The value of HLB varies with temperature, specially the non-ionic emulsifiers [31, p.91]. The nature of these emulsifiers can have a influence on droplet size distribution.

2.1.5 Principles of suspensibility

A suspension is a solid-liquid dispersion with particle size greater than 1 μm [34]. The dispersed solid phase is usually referred to as the internal phase and the continuous phase as the external phase.

In suspensions there is a thin region, known as the interface, whose properties are very important due to a high interface area. Each interface has a certain free energy per unit area which has a great influence in the stability and structure of the dispersion [31, p.53]. Since the dispersion is thermodynamically unstable, the system has a tendency to revert spontaneously to a lower free energy system through flocculation, this could be prevented with an energy barrier [32, p.154].

Dispersed substances can come together in different ways: sedimentation, aggregation and coalescence. In **sedimentation** two separate layers of dispersion with different concentration are created as a result of a density difference between the dispersed and continuous phase. **Aggregation** occurs when two or more particles clump together but keeping the total surface area. **Coalescence**: two or more particles fuse together creating a single unit, hence, the total surface area is reduced.

It is possible that aggregation of particles leads to coalescence and the formation of larger droplet units separate the phases. While in aggregation the species keep their identity, in coalescence they become part of new species [31, p.117].

To consider a dispersion stable it should not vary the total number of particles, nor the particle size distribution in time.

There are four types of stability: kinetic which is associated with the prevention of coagulation by an energy barrier; thermodynamic in which the disperse phase has lower free energy than the coagulated; electrostatic where electrostatic repulsion forces produce a stabilization, if particles in the liquid medium possess the same charge in the surface the attraction between them is worse, consequently the stability is better. And the last one steric stability which can be achieved by adding macromolecules to the system [35].

Stability of the colloidal systems can be described by the Deryagin, Landau, Verwey and Overbeek theory (DLVO). The interactions between the particles can be divided into the van der Waals attraction forces and repulsive electric double layer forces. If the sum of the repulsion forces is higher than that of the attraction forces, the described system can be considered stable [36].

2.2 High Performance Capillarity Electrophoresis

The principle behind this separation technology is the different migration time of substances in an electrical field. One advantage is that this technique is fast and allows convenient quantitative analysis of components in the mixture, the analyte interacts only with the buffer and the electric field [37]. Also another advantage is the diverse application range, while in the introduction of this technique was conceived for the analysis of biological macromolecules it has proven good separation in different compounds such as amino acids, chiral drugs, vitamins, organic and inorganic acids, oligonucleotides and DNA restriction fragments as well [38].

The instrumentation used is very simple and a schematic diagram is shown in Figure 2.6. The buffer is placed in two small glass vessels each one has the end of the silica capillary inside. The composition of the buffer is the same as the one within the capillary. Electrodes are in the reservoirs connected with a power supply, so the electric field is created.

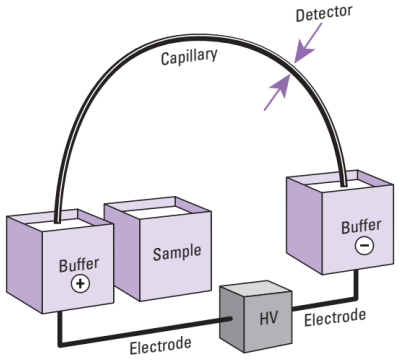


Figure 2.6: Scheme of a generic HPCE [38, p.12]

Each substance has a different migration speed, due to different factors such as charges, size, interaction with the capillary or buffer and concentration, the solution can be separated. After a period of time, the separated sample zones reach the region of the optical window where spectrophotometric detection takes place. There are multiple detection methods, but UV-Visible is one of the most used. The detection range is from 200 nm up to the visible spectrum [37].

3 Material and Methods

This chapter explains the production of microcapsules along with materials, equipment and methods.

3.1 Materials and equipment

3.1.1 Materials

The laboratory equipment used is listed in Table 3.1

Table 3.1: Summary of the material

Material	Code	Supplier/Manufacturer
Beaded rim bottle ND11 1,5 ml	LC 23.1	Carl Roth
Beaker (150/400/1000 mL)	several	DURAN Group, Simax
Büchner funnel, ⌀70 mm	TS439	Carl Roth
Celulose Filter, ⌀ 70 mm, Pores⌀ 2-3µm	AP 51.1	Carl Roth
Disposable pipette, 5 ml	LW 4728	Alpha Laboratories
Disposable syringe, 10 ml/20 ml	0058.1/00.59.1(Carl Roth)	Braun GmbH
Erlenmeyer flask	several	Simax
Filter mat	ET 3, EV 3,15 mm	Telle
Filtring flask	several	DURAN Group
Hose, Tygon Noprene	AH 64.1 (via Carl Roth)	Saint Gobain
Magnetic stir bar	several	several
Parafilm	H666.1 (via Carl Roth)	Bemis Company
Pipette, glass 1 ml		
Pipette tip, 1000µl	HL 70.1	Carl Roth
Pressure vessel		FAU
Snap cap, PE	LC 87.1	Carl Roth
Snap-cap vial, 25 ml	LC 84.1	Carl Roth
Spatula	several	several
Syringe filter, RC-Membrane, 0,45 µm	PP 42.1	Carl Roth
Volumetric flask, 25 m	several	DURAN Group
Weigh boat	HS120224	Sigma-Aldrich

The chemical materials used as matrix are listed in Table 3.2.

Table 3.2: List of chemical products used for microcapsules matrix

Designation	Order number	Supplier/Manufacturer
Sunflower oil		Hangzhou Dayangchem
Canola oil	20161005	Hangzhou Dayangchem
Softenol 3995 (MG)	507065	Cremer Oleo
Softenol 3169 (TG)	507056	Cremer Oleo

The antioxidants used in the microcapsules are listed in Table 3.3.

Table 3.3: List of antioxidants used for microcapsules production

Designation	Lot number	Manufacturer
DL-α-Tocopherol $\geq 96,0\%$	446251368	Carl Roth
L-(+)-Ascorbic Acid $\geq 99,0\%$	457264333	Carl Roth

The emulsifiers used in the microcapsules are listed in Table 3.4.

Table 3.4: List of emulsifiers used for microcapsules production

Designation	Lot number	Manufacturer
Glycasol	1014	GlaconChemie
C-1816, HLB 16	6X082211	Mitsubishi-Chemical Foods
SE-11, HLB 11	-	Guangxi Gaotong Food Technology
SE-15, HLB 15	GB8272-2009	Guangxi Gaotong Food Technology
Tween 20, HLB 16,7	9127.2	Carl Roth
Tween 24, HLB 16,5	P-4089	Croda International

Emulsifiers SE-11 and SE-15 are sucrose-fatty acid esters being the only difference the HLB. Tween 20 and 24 are polysorbates nonionic surfactants. Glycasol is a monovalent primary alcohol, formally described as methanol substituted by a cyclic ether group [39].

The hop extract used in the microcapsules is listed in the Table 3.5.

Table 3.5: Hop extract composition

Designation	Lupulone	Humulone
β-Extract	$7.7\,\%_{\mathrm{wt}}$	$23.1\,\%_{\mathrm{wt}}$

3.1.2 Equipment

Table 3.6: Summary of the used equipment

Device Name	Code	Supplier/Manufacturer
^{3D}HPCE	G1600AX3DCE	Hewlett-Packard
Heating cabinet	U-10	Memmert
Incubator	MIR-154	Sanyo
Magnetic stirrer	RCT B	IKA Labortechnik
Peristaltic pump	PA-SF	IKA Labortechnik
- Head	Masterflex P/S Easy Load II	Thermo Scientific
- Motor	7AA63M04	Elektra Hangelsgesellschaft
Precision scale	A200S	Sartorius Analytic
Pipette, 1000 µl	Pipetman DH57562	Gilson
Production plant		
- Pressure reduction valve	E/W 2000-02	E· MC
- Hollow cone Nozzle white	Albuz ATR	Agrotop
- Flat Nozzle orange	AT AVI-80-01	Agrotop
- E-Motor	WF10DR56L4	SEW
- Conveyor belt		Engineering Kitz
Scale	1518 B MP8-1	Sartorius
Spraying System	self-constructed	FAU
- Pressure reduction valve	LT 2000	Spectron Gas Control Systems
- Heating two substances nozzle		Red Lion
- Industrial vacuum cleaner	Professional NT 57/1	Kärcher
- Oil bath	C-MAG HS 7	IKA Labortechnik
- Peristaltic pump	PA-SF	IKA Labortechnik
- Spraying tower	Self-made	FAU
- Thermometer, digital	ETS-D5	IKA Labortechnik
- Utra Turrax	T18 digital	IKA Labortechnik
- Water bath	RCT basic	IKA Labortechnik
- Cyclone	Self-made	FAU
Ultrasound bath	RK 106	Bandelin Sonorex
Vacuum Pump	Rotavac valve tec	Heidolph Instruments
Vortex mixer	L46	GLW

3.2 Methods

3.2.1 Microcapsules production

The method used for the production of the microcapsules is spray-congealing. The process flow diagram of the spraying process is represented in Figure 3.1

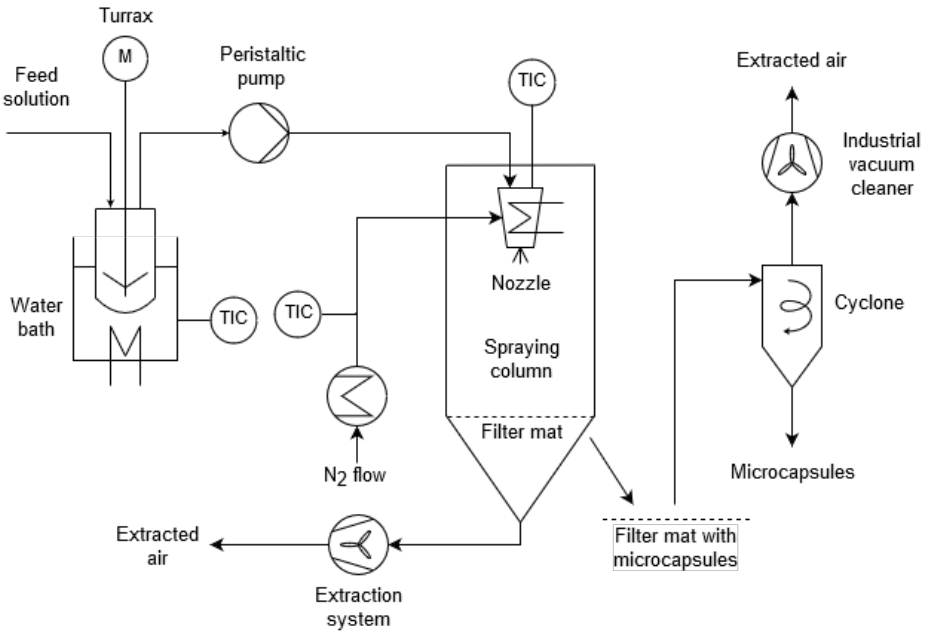


Figure 3.1: Process flow diagram of the spraying plant

The production of microcapsules is for every powder very similar. The matrix material has to be preheated to be in liquid phase so it can be added to the β-acids. The whole equipment is heated, such as the nozzle (80 °C), the water bath (80 °C) for the solution and the nitrogen (100 °C). In this moment a filter mat can be placed between the bottom of the spraying tower, its height is 5.3 m and diameter 0.45 m, and the air extractor. It takes approximately 30 minutes to reach the temperatures.

Once the matrix material had melted, it was added to the active component; this is an important step so it has to be done carefully, to achieve the precision needed the last grams will be added with a plastic pipette. In addition, any other components that are in the microcapsules composition such as emulsifiers and antioxidants are added after the matrix. After this, it went into the water bath with the mixer, that allows getting an homogeneous mix. The rotational speed of the Ultra-Turrax is 11000 rpm.

The flow of nitrogen starts at this moment 2 min has to pass before the spraying at a pressure of 5 bar, turning the valve to adjust it if needed.

The tube used to get the mixture to the nozzle was previously cutted making one of the ending angular and inserted carefully in the nozzle, and through the pump. Making sure it does not move while the process was going.

At the beginning, the speed of the peristaltic pump should be the maximum until the mixture gets to the nozzle, this is the way to avoid that the mixture starts to solidify before getting to the nozzle. Once the mixture is in the nozzle the spraying begins and a sound can be heard, in that moment the velocity must be changed to 10 rpm. The speed of the Turrax is lowered during the process therefore no foam is created. The spraying finishes when the mixture is over.

A bottle for residues is places right below the nozzle, and the feed is changed to hot water with soap so the nozzle and the tube does not get clogged with the residues of the solid mixture. This operation goes for a few seconds, the rest of the water with soaps is used to clean the *Turrax* and then the beaker which contained the mixture. Boiling water from the 1000 ml beaker is used again as the feed for the pump in order to clean it.

The filter is taken out, and the particles are collected in a plastic container with the use of a cyclone.

The parts that have been in touch with the microcapsules are carefully cleaned with compressed air. The tube used to suck up the microcapsules, the cyclone and the filter, which will be used for multiple microcapsules production. The powder is stored in a incubator at 20 °C. All the batches produced are listed in Chapter 7.1.

3.2.2 Stability of active ingredients

In this work the effects of different composition in microcapsules are studied. For every powder produced, the concentration of lupulones is externally analysed. Of every fresh powder two amber glass bottles with 1,2 g are filled. One of them is stored at 54 °C for one week and the other one is sent on the production day. Every powder produced contains 10 $\%_{wt}$ of lupulones. The results are expressed relative to the initial concentration.

3.2.3 Suspensibility test of microcapsules

The aim of this test is to know how the microcapsules are going to behave when they are mixed with water. No floating or sedimentation is desired. It

is going to be compared the microcapsules that exist in the top and bottom $10\%_{\text{v}}$ of a 250 mL graduated cylinder, with a 30 minutes sedimentation.

In a 150 mL beaker, 0.83 g of the powder and 50 g of tap water are weighted. This solution is taken to a magnetic mixer where it is mixed for 1 min at level 7. Once the time is over, the solution is poured into the 250 mL graduated cylinder, taking care that all the powder gets into it and nothing is left in the beaker. A rubber stopper is introduced at the top, then inverting the cylinder 30 times, the suspension is mixed. After this, the cylinder is placed at the table and must wait 30 minutes for the precipitation.

(a) **(b)** **(c)**

Figure 3.2: Equipment and steps for the suspensibility test. (a) Graduated cylinders (b) Peristaltic pump (c) Vacuum and Büchner funnel

The top $10\%_v$ are removed with a disposable pipette and filtered, the $80\%_v$ left are removed with a pump, taking away always the surface top, avoiding to introduce the tip further because the removal would not be the desired. The $10\%_v$ left are filtered with a Büchner funnel, with a filter paper (pore size 2-3 μm). 24 hours have to pass before weighting the powder in order to be completely dry (Figure 3.3).

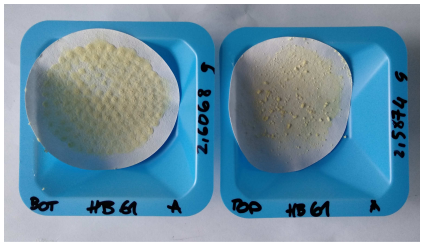


Figure 3.3: Paper filter of both top and bottom $10\%_V$

To determine the suspensibility is used the Equation 3.1.

$$S = \frac{10}{9}\frac{100 \cdot (m_1 - m_2)}{m_1} \tag{3.1}$$

Where m_1 is the mass of the microcapsules, and m_2 the mass of the top or bottom 10%.

Each sample is duplicated, and averaged the result. There will be two different suspensibility results, one for the top 10% and the other one for the bottom 10%. The value used for comparison is the suspensibility at the bottom, a perfect suspensibility is 100%, but it has to be taken into account the value of the top and also the visual perception of the powder once it is in the graduated cylinder.

3.2.4 Solubility test of L(+)-Ascorbic Acid

A solubility test with L(+)-ascorbic acid and different emulsifiers is carried out, because it needs to be dissolved in the mixture before spraying, otherwise it would block the nozzle.The test consists in adding ascorbic acid to emulsifier in the same ratio that exits in the microcapsules at room temperature and the same solutions are taken to the incubator at 54 °C for 4 hours. At the end the vortex mixer is used to help the dissolution.

The solubility of L-(+)-ascorbic acid in water follows the equation 3.2, [40].

$$\ln x = A + B(T/K) \tag{3.2}$$

Where x is the mole fraction of ascorbic acid and the parameters A and B for water as solvent and mean root square are listed in the Table 3.7

Table 3.7: Solubility parameters Ascorbic Acid

Solvent	**A**	**B**	$\mathbf{10^3\sigma}$
Water	-11,698	0,0278	0,37

3.2.5 Determination of active ingredients release

The release of the lupulones inside of the microcapsules once they are mixed in water is the key for to obtain the results wanted. To know the concentration High Performance Capillary Electrophoresis (HPCE) technique is used.

The HPCE has three steps, a preconditioning followed by measuring and postconditioning. The whole process takes around 70 – 80 min depending on the selected options. The injection of the sample is done by pressure at 50 mbar for 4 seconds. The wavelength selected in which the detector works is

Figure 3.4: HPCE device

$\lambda = 330$ nm. All the steps that the instrument does and the configuration of the device are listed in Chapter 7.2.

The preparation of the sample is as it follows: 2 g of the powder and 75 g of water are weighted in a 150 ml beaker, followed by stirring at 800 rpm for 10 minutes. The stirred solution is filtered with a Büchner funnel to remove the microcapsules. With a disposable syringe and a filter, the solution is filtered and also the buffer. 3 ml of the filtered solution are mixed with 3 ml of the buffer which is the sample. To begin with the analysis 1 ml of the sample is poured in a 1.5 ml glass snap cap and placed in the ^{3D}HPCE. The preparation of the buffer is explained in chapter 7.2.

The determination of the release of the β-extract is carried out differently due to the solubility of the active ingredient in methanol, which has a high volatility. Instead of a 150 ml beaker, an Erlenmeyer flask is used with a rubber stopper.

The results obtained are plotted as an electropherogram that represents the absorbance (mAU) vs the time (min). A scheme of a generic graph is shown in Figure 3.5. The data extracted from the graph is the area below the line of absorbance for each peak. To get a value of concentration the quotient be-

tween the area and the time is used because it is more precise than using only the area due to the differences that can happen in different measurements.

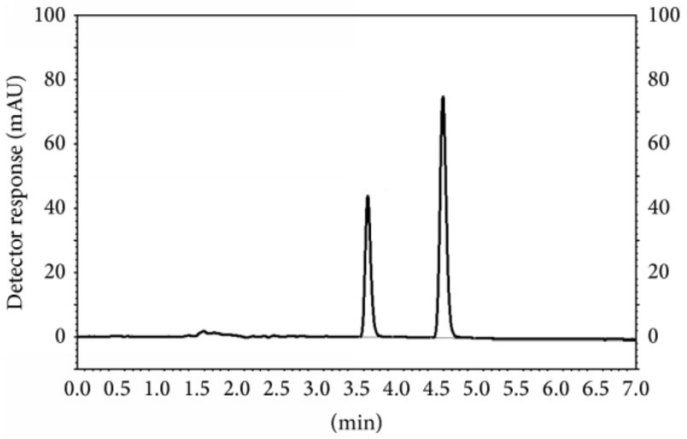


Figure 3.5: Generic electropherogram [41]

4 Results and discussion

This chapter first reviews the effect of antioxidants in stability and suspensibility. Followed by the effect of emulsifiers and matrix material. The active ingredient release was studied in one of the powders.

4.1 Effects of adding antioxidants in the formulation

Results of stability and suspensibility with the addition of antioxidants and solubility of one of the antioxidants can be found in this section.

4.1.1 Solubility test of L(+)-Ascorbic Acid

Ascorbic acid is only soluble in water at room temperature, in the rest of emulsifiers crystals are visible. Therefore, ascorbic acid is not dissolved and got deposited at the bottom. Due to this result, using 'normal size' ascorbic acid but with previous solution in the emulsifiers is not feasible because it is not dissolved and it could block the nozzle.

Dissolving the ascorbic acid in water before spraying, 2.04 g ascorbic acid were solved in 8.18 g of water. The maximum amount of solution in the microcapsules is $1\,\%_{\mathrm{wt}}$.

A comparison of the suspensibilities of the microcapsules produced with finely ground L(+)-ascorbic acid (HB49) and the one produced with L(+)-ascorbic $\mathrm{acid}_{(\mathrm{aq})}$ (HB51) is shown in Figure 4.1.

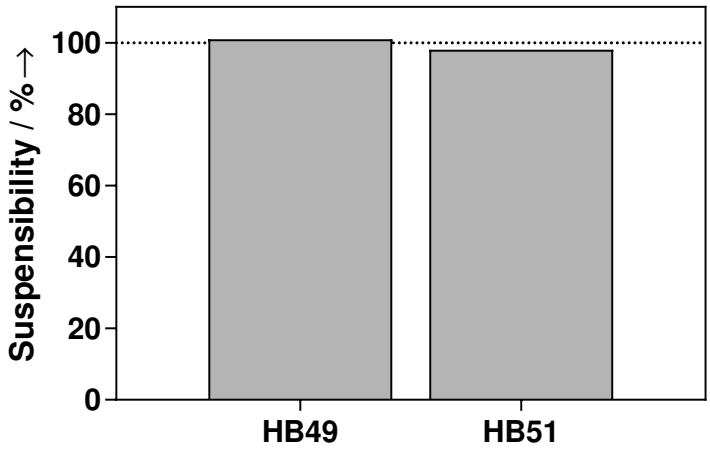


Figure 4.1: Suspensibility of HB45 and HB51, 1 d after production.

Although the suspensibility of HB49 powder is 3 % higher than HB51, the difference is not substantial and the use of ascorbic acid in solution is cheaper than the finely ground antioxidant.

4.1.2 Stability of lupulones in the microcapsules

The active ingredients contained in the microcapsules, lupulones, are susceptible to oxidation. Because of that, antioxidants are added to avoid the degradation while the microcapsules are stored, or at least improve the stability. The methodical procedure to determine the content of active ingredients after a defined time is described in Chapter 3.2.2.

Figure 4.2 shows the stability results of the batches produces with variations of the antioxidant used. All the data is given in $\%_{wt}$.

The reference batch composition is listed in Table 4.1

Table 4.1: Composition of HB37

Batch	**Matrix** /$\%_{wt}$	**Active Ingredient** /$\%_{wt}$	**Emulsifier** /$\%_{wt}$
HB 37	Sunflower oil, 70.71	β-Extract, 14.2	Tween 24, 15

The lupulones show a degradation over time in every batch relative to the start value (100%). When there are no antioxidants, the lupulone reach a value of 83.9 % of the original quantity. The best result achieved is when 0.2 $\%_{\mathrm{wt}}$ of ascorbic acid and 0.5 $\%_{\mathrm{wt}}$ of tocopherol are used together, 89.6 %.

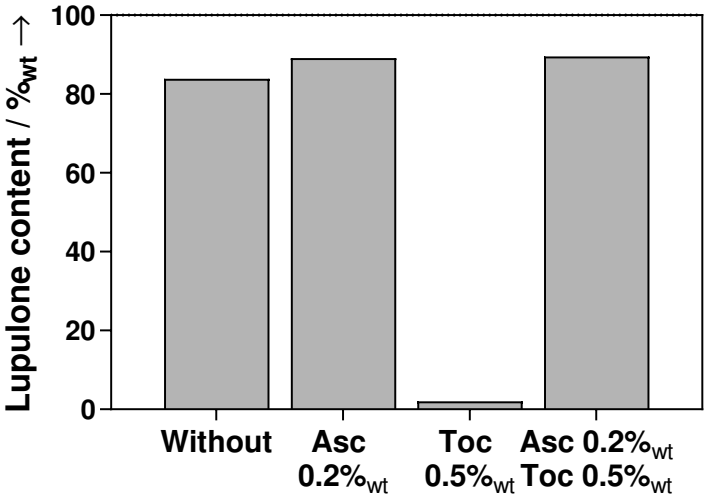


Figure 4.2: Results of stability tests of lupulone for individually added antioxidants compared to HB37 (Whithout).

When ascorbic acid is used alone the result is 89.13 %. The worst result, and therefore highest degradation is when only tocopherol is used. Only 2.0 % of the original lupulone remain.

The difference in the efficiency of both antioxidants is that while the molecular structure of ascorbic acid makes it hydrophilic, tocopherol is lipophilic. Ascorbic acid reacts with the amphiphilic emulsifiers on the microcapsule surface protecting it against free radicals. Tocopherol instead, does not accumulate on the surface and is located closer to the centre of the capsules. It is possible that tocopherol works under these conditions as a prooxidant instead of antioxidant, passing their negative charge to the active ingredient.

However, the combination of the two of them achieve the best results. This is because they have a synergistic operation. With the presence of ascorbic acid the inhibit capacity and induction period of tocopherol are increased [42].The mechanism is that tocopherol is the main antioxidant in the system and tocopheryl, tocopherol radical, can be regenerated by ascorbic acid due to the low redox potential of ascorbic acid [43].

4.1.3 Suspensibility of microcapsules

Some preliminary results can be determined in the first steps when the suspension is in the measuring cylinders. Pictures were taken to see the differences between each powder that show a clear difference in suspensibility. The results of the suspensibility test are shown in Figure 4.2.

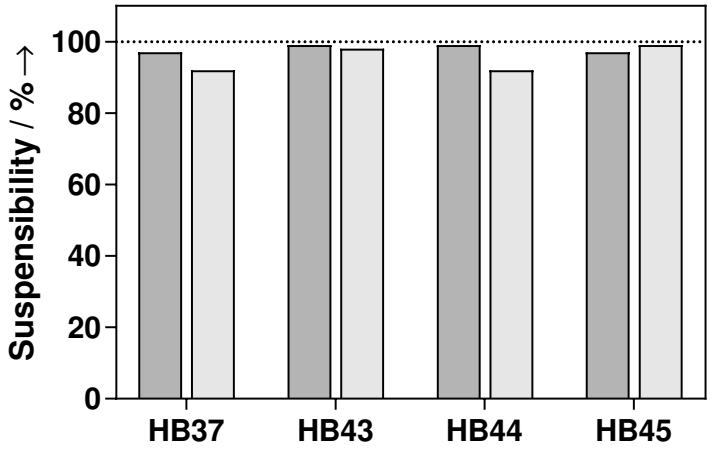
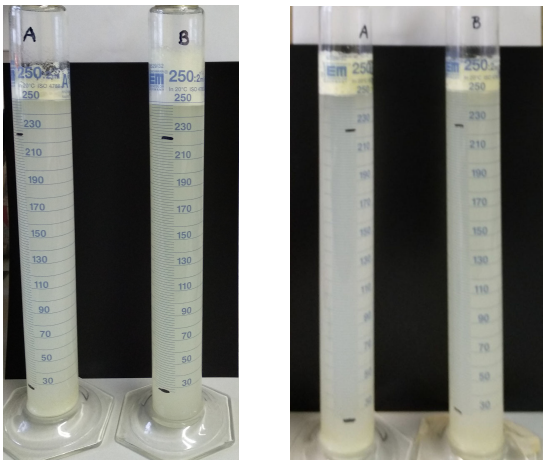


Figure 4.3: Suspensibilities of different microcapsules after 1 d (■) and after 7 d (□).

All the batches have suspensibilities higher than 90 %. The highest value of 99 % corresponds to HB43 and HB44 one day after production. On the other hand, the lowest of 92 % is for HB37 and HB44 after 7 d. Over time the suspensibility is worse for every powder except for HB45 that is slightly better with 2 %

Despite the good results of suspensibilities, the microcapsules are not well distributed inside the measuring cylinder, shown in Figure 4.4. There is a foam layer on top composed mostly by the microcapsules hence, the rest of the cylinder has a low quantity of microcapsules.

The addition of antioxidants does not affect significantly the suspensibility.



(a) **(b)**

Figure 4.4: Suspensibility test of (a) HB37 and (b) HB45.

4.2 Using different emulsifiers in the formulation

The batches HB48, HB49, HB50 and HB55 are created to check which emulsifier is better when it is used alone. To check whether using double amount of emulsifier, and which combination of sucrose ester and Tween 20 reaches a better stability, HB53 and HB54 are produced. The addition of softenol monoglygeride, sucrose esters and Glycasol, using softenol triglyceride as the matrix matrix material, was tested with HB59 to HB61.

4.2.1 Suspensibility of microcapsules

Figure 4.5 shows the results of suspensibilities of the batches in relation to HB45. Top 10%$_v$ is represented as well. The value that is referred to is the bottom one unless otherwise stated.

If Tween 20 (HB49), SE-15 (HB50) and SE-11 (HB55) are used as emulsifiers, the suspensibility is over 100 %, being the highest for the batch produced with SE-15, 110 %. If the suspensibility value is over 100 % indicates that the capsules are floating, hence the mass at the bottom is lower than an ideal suspension. The lowest value is for the powder with C1816 (HB48) after being stored, 91%. The high results of the batches with SE-15 and SE-11 have a correlation with the low results, suspensibility below 45 %, of the top 10%$_v$.

Due to a lower hydrophilic nature than Tween 20, sucrose esters (SE-11,

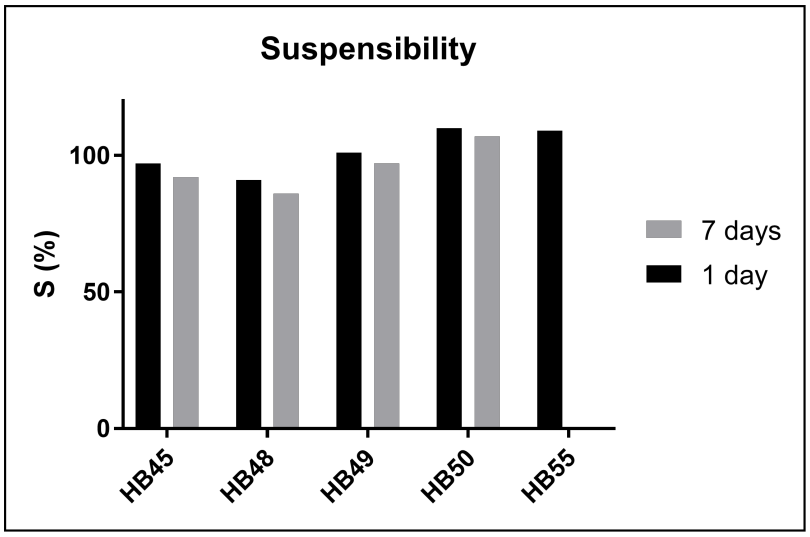


Figure 4.5: Suspensibilities of different microcapsules, bottom 10%$_v$ (■), top 10%$_v$ (□).

SE-15), if used as the only emulsifier do not perform properly and the microcapsules float on the surface instead of being suspended in the cylinder.

The results of adding more than Tween 20 as emulsifier, for both bottom and top 10%$_v$ can be seen in Figure 4.6.

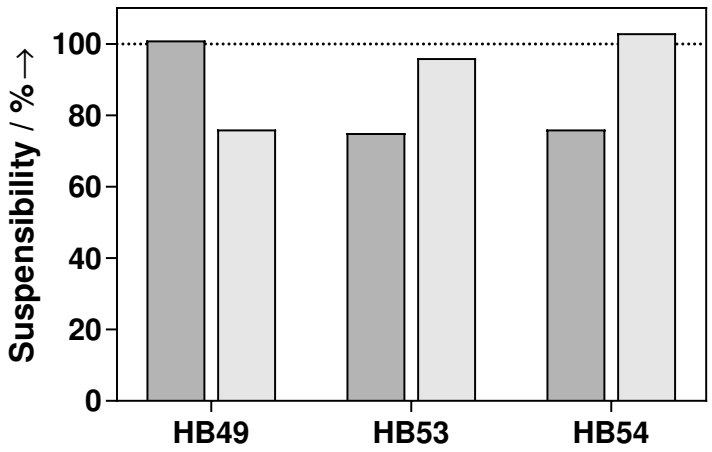


Figure 4.6: Suspensibilities of different microcapsules with more than one emulsifier, bottom 10%$_v$ (■), top 10%$_v$ (□).

The batch HB49 that only contains Tween 20 as emulsifier reaches a value of 101 % (bottom 10%$_v$) while if more emulsifiers are added C-1816 (HB53)

and SE-15 (HB54) the suspensibility is significantly lower, 75 % and 76 %, respectively. Both additional emulsifiers are sucrose esters, which are less hydrophilic than Tween 20. This causes an accumulation of polar molecules on the surface, which leads to the creation of lumps of microcapsules that sediment faster lowering the suspensibility. These results are supported with the values of top suspensibility, being in batch HB54 higher than 100 %.

Figure 4.7 shows the results of different emulsifiers with softenol triglyceride as the matrix material.

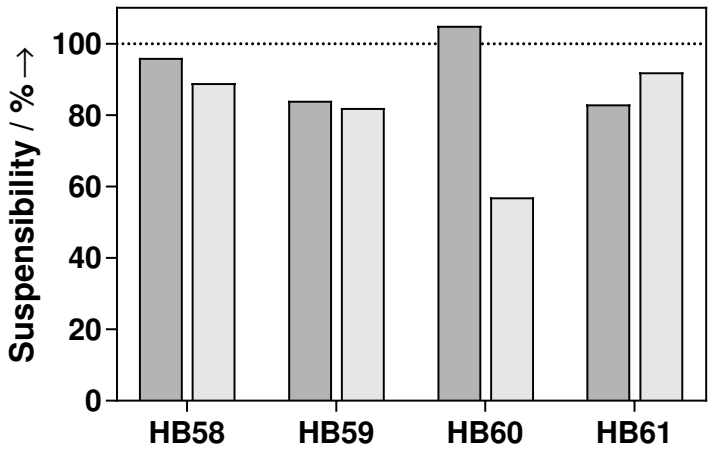


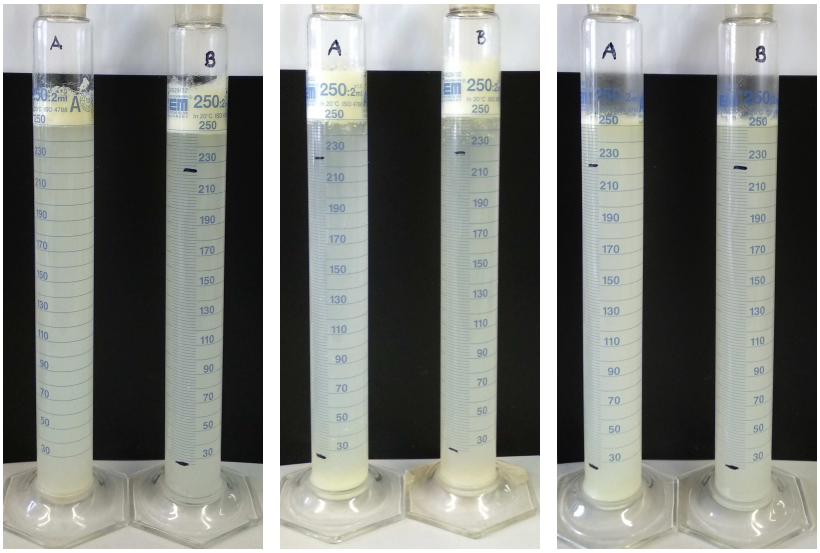
Figure 4.7: Suspensibilities of different batches (HB58 - HB61), bottom $10\%_v$ (■), top $10\%_v$ (□).

In this last comparison, when softenol is the matrix material, the highest realistic suspensibility is reached by the batch HB58, 96%. The suspensibility reached by HB60 is not reliable because is higher than 100 % and floating microcapsules appear it can be seen in Figure 4.8. The addition of monoglyceride in batches HB59, HB60 and HB61, contrary to what was expected due to the polarity of the molecule, lowers the stability of suspensions. Comparing HB59 to HB58, suspensibility is lower in both, top and bottom $10\%_v$. The addition of Glycasol to the rest of sucrose ester emulsifier increases the overall suspension stability.

Despite the numeric results, HB61 is the one with better results. In Figure 4.8 the measuring cylinders can be seen, and while the batches HB58 and HB60 have a thick layer of foam, the one in HB61 is very thin, and the water is less translucent than the in the other two.

The Figure 4.9 shows the evolution in time of suspension stability.

After storage time the suspensibility decreases. This behaviour is similar in



(a) **(b)** **(c)**

Figure 4.8: Suspensibility tests of (a) HB58, (b) HB60, (c) HB61.

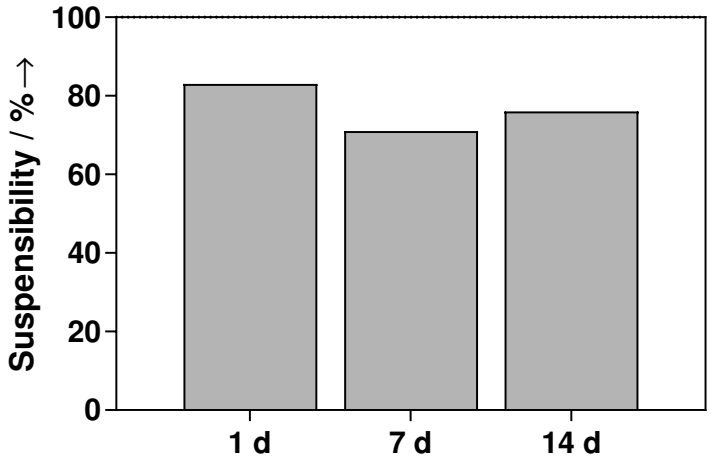


Figure 4.9: Suspensibilities of HB61 over time.

most of the batches, however, the drop of HB61 is bigger than in previous batches reaching a suspensibility of 71 % in 7 d and 76 % after 14 d. After 7 d, the microcapsules stay at the top and sediment during the 30 min, but they can easily move and be suspended again along the cylinder.

4.2.2 Stability of lupulones in the microcapsules

The Figure 4.10 shows a comparison of the lupulones' amount in the microcapsules after 14 d and 28 d, respectively.

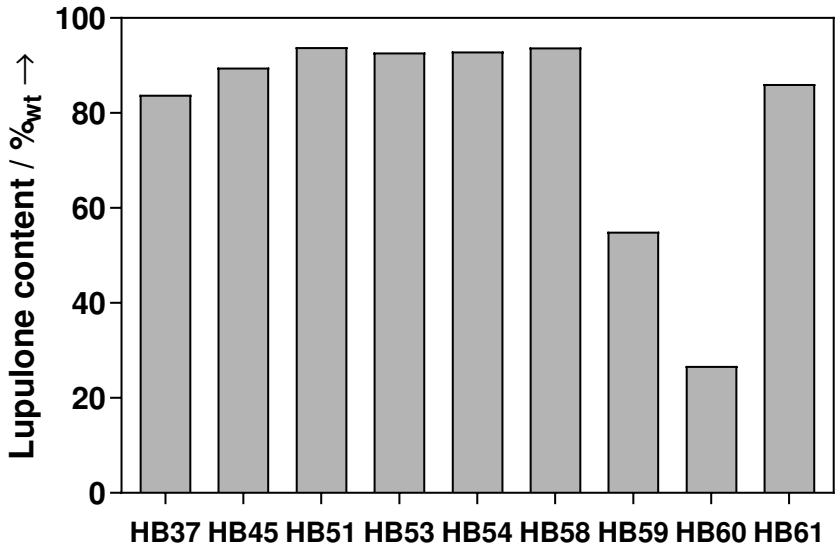


Figure 4.10: Lupulone contents after 14 d (HB37, HB45, HB58, HB59, HB60 and HB61) and 28 d (HB51, HB53 and HB54).

Batches from HB51 to HB54, and HB58 have lupulone contents higher than 90 % of the initial value after one week, batches from HB59 to HB61 have the lowest stabilities, 55 %, 27 % and 87 % respectively. It must be noted that batches HB51-HB54 were stored at 50 °C for four weeks instead of 54 °C for 14 d. Batches with softenol monoglyceride in their composition have worse stabilities. An additional emulsifier together with Tween 20 has no effect on the stability. Higher stabilities are achieved in batches HB54 and 58.

After the results from the previous chapter, the stability of HB61 is important, even though is not the best one.

4.3 Effects of different matrix materials

The batches HB38, HB58 and HB52 are created to check which matrix material has a better performance in both lupulones' stability and suspensibility.

4.3.1 Stability of lupulones in the microcapsules

The Figure 4.11 shows the lupulone stability of the batches, compared each one to the one with same composition but with sunflower oil as the matrix material (HB52 and HB51).

The use of canola oil have no effect on the stability neither the use of softenol triglyceride, but if softenol monoglyceride is used the stability lowers drastically 38.81 %.

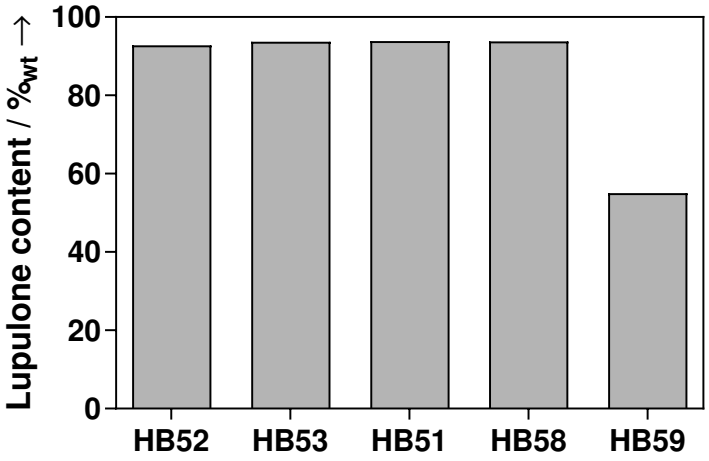


Figure 4.11: Stability of lupulone with variation in matrix material.

4.3.2 Suspensibility of microcapsules

The Figure 4.12 shows compared each one to the one with same composition but with sunflower oil as the matrix material.

When canola oil is used only with emulsifier (HB38), the suspensibility is slightly lower; but when the microcapsules have antioxidants too (HB53), the suspensibility is better. Softenol triglyceride as the matrix material (HB58) reduces the suspension stability.

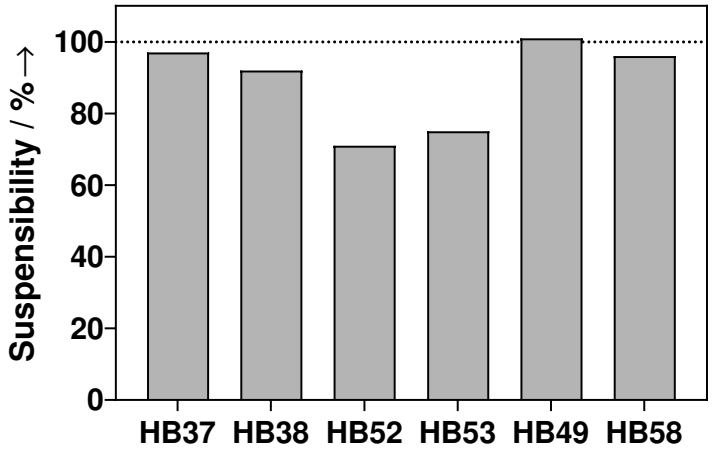


Figure 4.12: Suspensibilities of different microcapsules.

4.4 Lupulone release and stability

The aim of this section is to evaluate the lupulone release of the batch HB61. During the chemical analysis three peaks appear in the electropherogram. To decide if they correspond to the extract or to other substances of the microcapsules, a test with only the extract is carried out.

This test revealed that only the two last peaks correspond to the hops β-acids, so only this two are taken into account to determine the total area.

The concentration (C) is calculated with the calibration curve shown in Equation 4.1 [44], where A is the area of one peak, and t is the time of that peak maximum value of concentration that can be achieved is 2,597 g/L.

$$C[g/L] = \left(\sum A/t - 0.28\right) \cdot \frac{2}{50.0901} \tag{4.1}$$

Figure 4.13 shows the released amount of active ingredient of the microcapsules the next day after production and 7 d later.

The amount released is extremely low in both cases, 6.08 % after 1 d and 5.43 % after 7 d. After time, the release is lower. Structural changes and oxidative degradation influence the released amount of active ingredients. In this case, in Chapter 4.2.2 the active ingredient stability was high, so oxidative degradation is not primarily responsible for the low release. One possible explanation is that emulsifiers are mostly on the surface of the capsules which makes the solvent difficult to diffuse into the capsule hence, the active in-

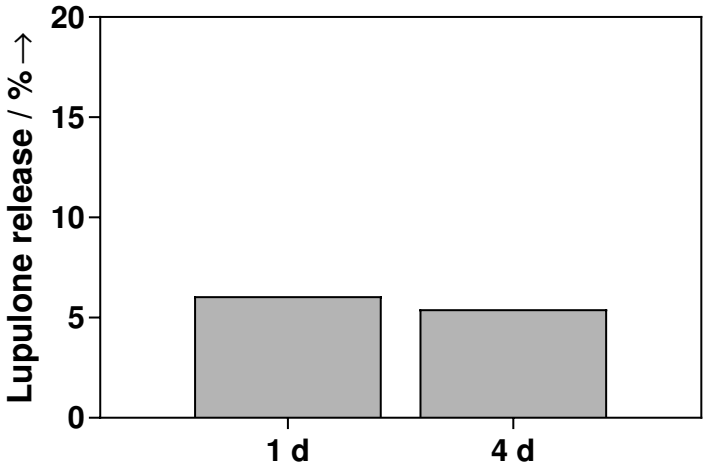


Figure 4.13: Released amount of active ingredients in HB61 1 d after production and 4 d later.

gredients are not dissolved. These emulsifiers on the surface can go into the continuous phase and create micelles, making the transportation of active ingredient not possible.

5 Conclusion

The aim of the present work was to investigate the properties of microcapsules containing hops' β-acids (lupulones) as active ingredients, to use them as animal feed additives for chicken and cows. Lupulones benefit digestion, and behaviour. To study the properties different batches from HB37 to HB61 were produced by spray-congealing technique. All batches have the same amount of β-acids. Changing the rest of the components the influence of them in the properties studied could be seen, individually and altogether.

Antioxidants were added to the formulation HB37 in order to improve the stability of the active ingredients in time. A combination of L(+)-ascorbic acid and DL-α-tocopherol ($0.2\,\%_{\mathrm{wt}}$ and $0.5\,\%_{\mathrm{wt}}$) achieve the highest stability, remaining in the capsules after two weeks approximately 90% of the initial amount of lupulones. The addition of these antioxidants does not influence the suspensibility.

Besides the active ingredients stability, the suspensibility was studied. Because of the matrix material and active ingredients nature, emulsifiers had to be added. Different possibilities were evaluated to replace Tween 24. This emulsifier cannot be used in animal feed due to legal restrictions. The use of sucrose ester emulsifiers (SE) reduced drastically the suspension stability, microcapsules floated on the surface and the suspensibility was higher than 100%. On the other hand, when Tween 20 was used the numerical results were worse. They were slightly higher than 100%, but an improvement could be seen in the thinner layer of foam that was on the surface, but still most of the capsules were in that layer. Doubling the emulsifier amount up to 30% by adding sucrose esters did not improve the suspensibility, but changed the behaviour and capsules sedimentated instead of floating. Therefore, the results were worse when Tween 20 was used together with other emulsifiers. Over time all the batches had a lower suspensibility.

Matrix material also contributes to all the previously mentioned properties. Sunflower oil was used for most of the batches. To improve the results canola oil was tested and although the stability was similar to sunflower batches, the suspensibility was worse. Due to this result, canola oil was discarded. With Softenol 3169 triglyceride the stability of lupulones was similar but the suspensibility is slightly reduced. The addition of monoglyceride to the formulation decreases drastically its stability and slightly the suspensibility creating a thicker foam layer.

An additional batch was created combining sucrose ester emulsifiers C-1816 and SE-11 and Glycasol together with softenol triglyceride and monoglyc-

eride as the matrix material. This batch turned out to be the best one in suspensibility, it did not have an evident foam layer on the surface and the capsules were suspended in the cylinder. However, after 7 d of storage the suspensibility has deteriorated and many of the capsules went to the bottom of the cylinder and others floated. With a slight movement of the cylinder the capsules can be dispersed again.

In terms of active ingredients stability the batches HB54 and HB58 were the best. Due to the good suspensibility of batch HB61 and to combine both properties as good as possible, the stability of HB61 is considered to be quite good. Hence, a release of active ingredients test was run and only 6.08% were released 1 d after production, decreasing to 5.43% after 5 d. It is important to check next how all the properties studied change with longer storage time.

Ultimately with HB61 a formulation with good suspensibility and stability is provided, which can be the starting point for further improvements.

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7 Appendix

7.1 Produced batches

Table 7.1: Composition of all produced batches

Batch	Date	Composition	Amount($\%_{wt}$)
HB37	14/11/2078	Sunflower oil	70.71
		Tween 24	15.00
		Beta extract	14.29
HB38	14/11/2018	Canola oil	70.71
		Tween 24	15.00
		Beta extract	14.29
HB39	27/11/2018	Sunflower oil	85.71
		Beta extract	14.29
HB40	27/11/2018	Sunflower oil	85.21
		Tocopherol	0.50
		Beta extract	14.29
HB41	27/11/2018	Sunflower oil	85.51
		Ascorbic Acid	0.20
		Beta extract	14.29
HB42	27/11/2018	Sunflower oil	85.01
		Tocopherol	0.50
		Ascorbic Acid	0.20
		Beta extract	14.29
HB43	04/12/2018	Sunflower oil	70.51
		Tween 24	15.00
		Ascorbic Acid	0.20
		Beta extract	14.29
HB44	04/12/2018	Sunflower oil	70.21
		Tocopherol	0.50
		Tween 24	15.00
		Beta extract	14.29
HB45	04/12/2018	Sunflower oil	70.01
		Tween 24	15.00
		Tocopherol	0.50
		Ascorbic Acid	0.20
		Beta extract	14.29

Table 7.1: Composition of all produced batches

Batch	**Date**	**Composition**	**Amount**($\%_{wt}$)
HB48	12/12/2018	Sunflower oil	70.01
		C-1816	15.00
		Tocopherol	0.50
		Ascorbic Acid	0.20
		Beta extract	14.29
HB49	12/12/2018	Sunflower oil	70.01
		Tween 20	15.00
		Tocopherol	0.50
		Ascorbic Acid	0.20
		Beta extract	14.29
HB50	12/12/2018	Sunflower oil	70.01
		SE15	15.00
		Tocopherol	0.50
		Ascorbic Acid	0.20
		Beta extract	14.29
HB51	18/12/2018	Sunflower oil	69.16
		Tween 20	15.00
		Water	0.85
		Tocopherol	0.50
		Ascorbic Acid	0.20
		Beta extract	14.29
HB52	18/12/2018	Canola oil	55.01
		Tween 20	15.00
		C-1816	15.00
		Tocopherol	0.50
		Ascorbic Acid	0.20
		Beta extract	14.29
HB53	18/12/2018	Sunflower oil	55.01
		Tween 20	15.00
		C-1816	15.00
		Tocopherol	0.50
		Ascorbic Acid	0.20
		Beta extract	14.29
HB54	18/12/2018	Sunflower oil	55.01
		Tween 20	15.00
		SE-15	15.00
		Tocopherol	0.50
		Ascorbic Acid	0.20
		Beta extract	14.29

Table 7.1: Composition of all produced batches

Batch	**Date**	**Composition**	**Amount**($\%_{wt}$)
HB55	18/12/2018	Sunflower oil	70.01
		SE11	15.00
		Tocopherol	0.50
		Ascorbic Acid	0.20
		Beta extract	14.29
HB56	08/01/2019	Softenol 3169 (TG)	85.71
		Beta extract	14.29
HB57	08/01/2019	Softenol 3169 (TG)	70.71
		Tween 20	15.00
		Beta extract	14.29
HB58	08/01/2019	Softenol 3169 (TG)	69.21
		Tween 20	15.00
		Tocopherol	0.50
		Ascorbic Acid (aq)	1.00
		Beta extract	14.29
HB59	08/01/2019	Softenol 3169 (TG)	54.21
		Tween 20	15.00
		Softenol 3995 (MG)	15.00
		Tocopherol	0.50
		Ascorbic Acid (aq)	1.00
		Beta extract	14.29
HB60	08/01/2019	Softenol 3169 (TG)	54.21
		C-1816	15.00
		Softenol 3995 (MG)	15.00
		Tocopherol	0.50
		Ascorbic Acid (aq)	1.00
		Beta extract	14.29
HB61	10/01/2019	Softenol 3169 (TG)	22.81
		Softenol 3995 (MG)	11.40
		C-1816	15.00
		SE-11	20.00
		Glycasol	15.00
		Tocopherol	0.50
		Ascorbic Acid (aq)	1.00
		Beta extract	14.29

7.2 Buffer and device settings of HPCE

The chemicals used for the buffer (500 ml) are listed in Table 7.2. These are mixed in a beaker with 400 ml of distilled water. The pH is 9 adjusted with a solution of 1M $NaOH_{(aq)}$. After the desired pH is reached, it is poured in a 500 ml flask, and filled up with distillate water and shake.

Table 7.2: Chemicals used for the buffer of the ^{3D}HPCE

Chemical	Quantity
Sodium dihydrogen phosphate dihydrate $NaH_2PO_4 \cdot 2H_2O$	0,78 g
$C_4H_{11}NO_3$	1,514 g
$C_{12}H_{25}NaO_4S$	3,605 g
Methanol, MeOH	50 ml
Water, H_2O	Around 400 or 500 ml

Table 7.3: Device settings of $^{3D}HPCE$

Parameter	Configuration
Lift Offset	4 mm
Temperature in the capillar tube	30 °C
Injection	pressure: 50 mbar, period: 4 s
Polarity	positive
Alarm lower limit	5 µA
Replenishment-System	Buffer-Inlet and Buffer-Outler; 1,4 mm
Stop time	25 min or 30 min
Measurement A	321 nm ± 20 nm; Ref 400; ± 40 nm
Measurement B	390 nm ± 400 off
Measurement C	370 nm ± 20nm; Ref 400 ± 40 nm
Measurement D	330 nm ± 20nm; Ref 400 ± 40 nm
Measurement E	345 nm ± 20nm; Ref 400 ± 40 nm

Table 7.4: Programs for operation and cleaning of the $^{3D}HPCE$

Program	**Process**
Preconditioning	5 min buffer
Measuring	4 s Injection, 30 min measuring time in buffer
Postconditioning	5 or 10 min MeOH 5 min distilled water 5 min 0.1 molar $NaOH_{(aq)}$ 5 min distilled water 5 min 0.1 molar $HCl_{(aq)}$ 5 min distilled water 5 min buffer
Cleaning and conditioning	15 min 1 molar $NaOH_{(aq)}$ 10 min Distilled water 15 min 1 molar $HCl_{(aq)}$ 10 min distilled water 15 min buffer
Rinsing with distilled water	25 min distilled water