ENCAPSULATION AND RELEASE OF AMINO ACIDS IN DOUBLE (W/O/W) EMULSION

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Gent, August 2017

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Abstract

Double or multiple emulsions have been under study for several decades, but in the last years there has been an increasing interest in these types of systems, due to the possibility of encapsulation and controlled release of various bioactive compounds. Nowadays, the field where this has major potential for application is in human pharmaceuticals, mainly for the development of drug delivery systems. But other industries, such as food and cosmetics, have also been developing innovative uses for future applications of these systems. Especially in the food industry, this technology is seen as a potential tool for developing functional foods and low fat products.

Despite the progress in the generation of knowledge concerning double emulsions, there is still a lack of understanding on how different parameters contribute to the mechanisms of stability and release. Regarding the latter issue, it becomes very complex considering that different types of compounds with diverse properties can be encapsulated. To our knowledge, few studies have been performed on the encapsulation of amino acids in double emulsions. In our point of view, this topic is of interest because amino acids are the building blocks of peptides, proteins and certain drugs. So, by understanding how these basic units behave, a better understanding can be obtained concerning encapsulation of these macromolecules.

In this study, the encapsulation efficiency of amino acids with hydrophobic (L-leucine and DL-alanine) and hydrophilic (L-glutamine and L-serine) side chains in water-in-oil-in-water (W/O/W) emulsions was investigated, considering the role of the droplet size and entrapped water volume fraction of the double emulsion.

To quantify the amount of release, two spectrophotometric methods were evaluated, in which both are based on the specific reaction of trinitrobenzenesulfonate with the amino groups of amino acids. The first method consists of quantifying the amino acid concentration by means of a standard curve, and the second by the least-squares adjustment of a mathematical model based on the absorbance spectrum. Both methodologies showed similar results, hence both are suitable for measuring the amount of amino acids released in double emulsions.

The double emulsions showed no variation in the entrapped water volume fraction during storage for 16 days at 4°C. However, the droplet size increased during storage for all double emulsions. Moreover, double emulsions were observed by microscopy, where it was showed that they maintained their form as double emulsions during the evaluation period. The results showed that L-leucine is released to the external phase at a faster rate than the other amino acids. This might be explained by the distinctive hydrophobic properties of this amino acid, which can facilitate the diffusion across the oil layer. Also, the effect of the hydrophilic emulsifier polysorbate 80 at different concentrations (2, 1 & 0.5 wt%) in the release of L-leucine was evaluated. The release profile of the amino acid showed to be dependent on the amount of emulsifier. However, there is still a need to better understand how the mechanism of release changes as function of the concentration of this emulsifier.
Chapter 1
Literature review

1.1 Colloidal systems

Colloidal systems have been under study for a long time. In 1845, Francesco Selmi described these systems as “pseudo solutions”. Because of their lack of transparency, they couldn’t be considered homogeneous solutions. Then in the 1850s, Michael Faraday did studies on dispersions of gold in water, where he concluded that lyophobic dispersions can only be kinetically, but not thermodynamically, stabilized. Until 1861, Thomas Graham defined colloidal systems as “systems in which the dispersed particles are sufficiently large enough (above $10^{-9}$ m) that they do not display a significant diffusion coefficient in comparison with small molecules” (Ritzoulis & Rhoades, 2013).

Nowadays, it is generally accepted that colloids are any particle with dimensions between $10^{-9}$-$10^{-6}$ m. However, it is important to consider that colloid science involves many disciplines, such as physics, biology and material science. Therefore, the size range of the particles is a subjective parameter according to the field of application. So, the particle size might not be the only requirement to define a colloid (Hiemenz & Rajagopalan, 1997).

Despite of the arbitrariness of colloidal particle sizes, all colloidal systems are composed of a dispersed and continuous phase. The former refers to the particles and the latter to the phase in which the particles are dispersed. Depending on the physical state of the phases, the colloidal systems can be characterized into specific systems, some examples are shown in table 1.1 (Ritzoulis & Rhoades, 2013).

<table>
<thead>
<tr>
<th>Phase</th>
<th>Continuous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid</td>
<td>Minerals</td>
</tr>
<tr>
<td></td>
<td>Salami</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquid</td>
<td>Margarine</td>
</tr>
<tr>
<td></td>
<td>Butter</td>
</tr>
<tr>
<td></td>
<td>Expanded polystyrene</td>
</tr>
<tr>
<td></td>
<td>Sponges</td>
</tr>
<tr>
<td></td>
<td>Bread</td>
</tr>
<tr>
<td></td>
<td>Whipped Cream</td>
</tr>
<tr>
<td>Gas</td>
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</tbody>
</table>
1.2 Emulsions

Colloidal systems, which are made up of a liquid continuous and dispersed phase, are known as emulsions. Yet, sometimes, emulsion also refers to dispersed fat or oil in gel or solids, like the cases of butter and sausage, respectively (Ritzoulis & Rhoades, 2013). In order to have an emulsion, the following conditions must be met: both phases need to be immiscible with each other, work is needed to disperse one liquid into the other, and an appropriate surfactant needs to be used (Chen & Tao, 2005).

Because the dispersion consists of two immiscible liquids, the dispersed phase tends to arrange in spherical shape, in order to minimize the contact surface between the two liquids (Ritzoulis & Rhoades, 2013). The size of the droplets that are formed has an important effect on stability, because as the droplets are smaller they are more stable against processes like creaming, coalescence and flocculation (Walstra, 1993).

The type of surfactant that is needed depends of the type of emulsion. As there are two immiscible liquids involved, an oil and an aqueous phase, there are two possible types of emulsions, an oil dispersed in water or water dispersed in oil. The former type needs a hydrophilic surfactant, and the latter a hydrophobic surfactant (Ritzoulis & Rhoades, 2013). The surfactant play an important role because it decreases the interfacial tension, promoting smaller droplets and preventing recoalescence (Walstra, 1993).

1.3 Double emulsions

One emulsion can be used as the dispersed phase of another emulsion, meaning that an oil in water emulsion can be homogenized in oil leading to an oil in water in oil (O/W/O) emulsion, or a water in oil emulsion can be homogenized in water leading to a water in oil in water (W/O/W) emulsion (Ritzoulis & Rhoades, 2013). These complex multiple emulsion systems are known as double emulsions (Garti & Bisperink, 1998).

In 1925, multiple emulsions were described by Seifriz, but it has not been until the last 30 years that the research on these systems has become more relevant (Garti & Bisperink, 1998). The most common forms are W/O/W, but O/W/O emulsions can also be used in specific applications. W/O/W emulsions consist of small water droplets dispersed inside fat globules, which are dispersed, in turn, in a continuous aqueous phase (Garti & Bisperink, 1998; Jiménez-Colmenero, 2013; Pays, Giermanska-Kahn, Pouligny, Bibette, & Leal-Calderon, 2002). This compartment structure, a unique characteristic, has been of interest because of the potential use as reservoir of encapsulated substances to be released under different conditions (Pays et al., 2002). Therefore, application of double emulsions applies to different scientific fields, especially food, cosmetics, pharmacology and separation science (Garti & Bisperink, 1998).

The W1/O/W2 system consists of three phases as shown in figure 1.1, an internal (W1), lipid (O) external (W2) phase, the lipid or oil phase is located between the aqueous phases, which are separated
by two types of interfaces that are stabilized by means of hydrophilic and lipophilic surfactants (Jiménez-Colmenero, 2013).

![Diagram](image1)

**Figure 1.1** Schematic representation of W<sub>1</sub>/O/W<sub>2</sub> emulsions (Jiménez-Colmenero, 2013).

The size of the dispersed globules in the double emulsion tends to be very heterogeneous. On one hand, there are globules that can measure around 15-50 µm, which usually have a high number of entrapped water droplets, around 50-100 water droplets. On the contrary, there are also small globules around 2-5 µm, which contain either a single or few droplets of water. So, there are different types of these emulsions according to the internal water distribution as shown in figure 1.2 (Garti & Bisperink, 1998).

![Diagram](image2)

**Figure 1.2** Different types of W/O/W emulsions according to the amount of internal water distribution (Garti & Bisperink, 1998).

### 1.3.1 Preparation

The preparation process of double emulsions generally consists of two steps of emulsification using two types of emulsifiers (figure 1.3). Considering a W/O/W emulsion, first the water in oil (W<sub>1</sub>/O) emulsion is prepared under high shear conditions, using a hydrophobic emulsifier to stabilize the system. Then the second emulsification step is performed at lower shear conditions in order to avoid the rupture of the first emulsion. In this step, a hydrophilic emulsifier is used to stabilize the external interface between the oil and the external water (Garti & Bisperink, 1998). If homogenization is too
mild, the resulting system is highly polydispersed, but if it is too intense, the encapsulation is less efficient (Jiménez-Colmenero, 2013).

For the preparation of these systems, the surfactant with a low hydrophilic to lipophilic balance (HLB) is first dissolved in oil. In the next step, water is added and a W/O emulsion is formed. Then the system is emulsified again in an aqueous solution of surfactant with a high HLB number to produce a W/O/W emulsion. These two types of surfactants mix at the water-oil interface, having an effect on the lifetime of the films and their permeation properties. Therefore, the type of surfactants that are used, have an influence on the stability (Pays et al., 2002). The hydrophobic emulsifier, located in the oil globule should be at a higher concentration than the hydrophilic, in order to have a stable system (Garti & Bisperink, 1998).

![Figure 1.3 Schematic illustration of the two-step production of W/O/W emulsions (Garti & Lutz, 2004).](image)

Regarding the lipophilic emulsifiers, polyglycerol polyricinoleate (PGPR) (E-476) shows enhanced stability with different oils and hydrophilic surfactants in double emulsions. PGPR is permitted for use in foods as an additive and is used in the production of chocolate to reduce viscosity (Schantz & Rohm, 2005). But its use in food applications is highly regulated and it has been shown that in concentrations above 5%, an unpleasant off-taste is detected (Dickinson, 2011). Nevertheless, PGPR is by far the most common and effectively used low HLB emulsifier and it is considered as a reference when developing multiple emulsions with other lipophilic emulsifiers or partially substituted with other compounds, such as protein and/or polysaccharides (Jiménez-Colmenero, 2013).

On the other hand, for hydrophilic emulsifiers, different types of emulsifying food proteins have been used, such as whey proteins, sodium caseinate, etc. Also, hydrocolloids such as gum arabic, xanthan gum, modified starch, and some soluble polysaccharides like pectin, alginate or gellan may be used. These types of emulsifiers also act as stabilizing agents due to their thickening/gelling properties, helping to enhance stability and reducing release problems (Jiménez-Colmenero, 2013). From the synthetic emulsifiers, polysorbates are hydrophilic nonionic surfactants that have an excellent
capacity as emulsifiers and lubricants in food products, especially in ice cream, whipped cream, and nondairy cream alternatives (Lu et al., 2014). In addition, polysorbate 80 has been used in the preparation of nanocapsules containing food-grade ingredients (Esmaeili & Gholami, 2015).

1.3.2 Instability

Similar to simple emulsions, double emulsions are thermodynamically unstable due to the excess free energy related to the surface of the emulsion droplets. This excess surface free energy results from the cohesive forces of the molecules of one liquid being stronger than the cohesive forces between two liquids. In emulsions, the interfacial area increases considerably, which leads to an unstable system that tends to return to the initial two phase system with a minimum interfacial area (Jiao & Burgess, 2007). This phenomenon leads to the disruption of emulsions during storage, especially when exposed to environmental stress like temperature changes and mechanical forces (Jiménez-Colmenero, 2013; Mezzenga, Folmer, & Hughes, 2004).

The kinetic stability depends on three mechanisms: electrostatic, steric and mechanical stabilization (Garti & Lutz, 2004). The electrostatic mechanism is based on the repulsion that occurs between charged particles, as a result of an interaction of the electrical double layers surrounding these particles (Tadros, 2013). The mechanical stabilization mechanism involves the incorporation of small solid particles into the emulsion, which will act as mechanical barriers to coalescence if interfaces are absorbed. The steric mechanism implies the use of macromolecular surfactants, that are able to completely cover the external interface improving the stability of double emulsions (Garti & Lutz, 2004).

In W/O/W emulsions, the hydrophobic and hydrophilic emulsifiers may interact at the external water-oil interface and interfere with each other’s stabilizing performance (Jiao & Burgess, 2007). Typically, the instability of these multiple emulsions can be classified in different levels: between small internal droplets, between large globules, and between the globule and the small droplets dispersed within it (Ficheux, Bonakdar, & Bibette, 1998).

The main physiochemical mechanisms of these instabilities are illustrated in figure 1.4, where two main types can be distinguished, coalescence and water diffusion. For coalescence, there are two possibilities. First the coalescing of oil droplets, forming bigger oil droplets that will lead to faster creaming. Second, coalescing between the droplets of the internal phase, which causes a coarsening of the system. Regarding the water diffusion, a net water transport happens when there is a difference in osmotic pressure between the internal and external phase, so the oil droplets may either loose or gain water depending on which aqueous phase has the higher osmotic concentration (Mezzenga et al., 2004).
Figure 1.4 Representation of possible instabilities occurring in W/O/W emulsions (Mezzenga, et al., 2004).

To prevent compositional ripening, it is recommended to add oil insoluble electrolytes. These molecules help to counteract the Laplace pressure difference between the internal water droplets and balance the osmotic pressure of the two aqueous phases (Garti & Bisperink, 1998).

Polymeric amphiphiles are used as surfactants to improve the stability of the double emulsions, by the formation of polymeric thick films in the oil-external phase interface, which enhance the steric and mechanical stability and slow down the rate of coalescence (Garti & Bisperink, 1998). Because they do not form micelles, they do prevent reverse micelle transportation in the oil phase (Garti & Lutz, 2004). Some examples of polymers are proteins such as bovine serum albumin, gelatin and casein (Garti & Bisperink, 1998).

Other ways of improving the stability of double emulsions is by reducing mobility within the double emulsion by using high viscous oil or gelation of the oil or the aqueous phase. These methods are based on depletion stabilization, which consists of using gelling agents in one of the phases to reduce the mobility. Moreover, food grade surfactants, like proteins and polysaccharides, are used because they create complexes that improve stability more than protein alone by making a type of a microsphere because the interfacial film turns so thick and multilayered (Garti & Lutz, 2004).

1.3.3 Release of entrapped compounds

As mentioned before, W/O/W emulsions have a potential use for the encapsulation, protection and release of hydrophilic components (Jiménez-Colmenero, 2013; Tammak et al., 2016). This is possible because the oil fraction acts as a membrane, separating the two aqueous phases. This membrane can be considered semipermeable or fully permeable, depending on the circumstances of the release of the solute (Magdassi & Garti, 1984). Currently, several types of mechanisms on how the entrapped chemical substance in a double emulsion is released have been proposed. But, there is still not a full consensus on these mechanisms and how to control them (Garti & Lutz, 2004).
Pays et al. (2002) described two ways of how the release occurs. The first type is due to the coalescence between the internal droplets and the globule surfaces, where the film rupturing is initiated by spontaneous formation of a small hole. The second type is known as compositional ripening and occurs without film rupturing. Instead, the entrapped chemical crosses the oil phase by diffusion or permeation. This mechanism is similar to the chemical exchange across phospholipid bilayers in biological membranes. In the latter case, the molecular flow of the entrapped compound can be described by Fick’s law.

Several studies have described other three major possible mechanisms explaining the diffusion of water and water soluble compounds through the oil phase (figure 1.5). These are reverse micellar transport, diffusion across a very thin lamella and hydrated surfactant transport (Garti & Lutz, 2004).

![Figure 1.5](image)

In the case of entrapped electrolytes, monomeric emulsifiers have shown transportation of these electrolytes from the internal to external phase, by a reverse micellar mechanism. When the osmotic pressure is not equalized, external water can flow into the internal phase or vice versa. The migration direction is determined by the osmotic pressure gradient. It has been demonstrated that water molecules and water soluble compounds are transported via reverse micelles at significant rates even if there is no osmotic pressure gradient, but if a sufficient number of reverse micelles exist in the oil phase (Garti & Lutz, 2004).
In most cases, hydrophobic surfactants are added in great excess, so aggregation of reverse micelles can occur and the water and water soluble compounds can be solubilized in the globules. It has been demonstrated that reducing the amount of hydrophobic surfactant decreased release rates, by limiting the formation of reverse micelles. Also, the release can be slowed down by using polymeric amphiphiles that cannot form reverse micelles (Garti & Lutz, 2004).

Nevertheless, Bahtz et al. (2016) demonstrated that the presence of PGPR in an oil phase and in contact with water, leads to spontaneous emulsification of water droplets in the oil phase, and the number of droplets increased as the concentration of the surfactant increased. This water droplet formation is primarily due to the entrapment of water at the interface from the bulk phase into PGPR micelles. Also, they described that at concentrations above the critical micellar concentration (1.8% wt), the viscosity of the oil phase increases and because of this, droplet formation and the number of entrapped water droplets are restricted.

In the case of hydrated surfactants transport mechanism, it takes place when the osmotic pressure between the internal and external phases are different. The hydrophilic part of the surfactant links to the water or water soluble molecules at the interface of the oil and low concentrated aqueous phase, the surfactant-water linkage diffuses through the oil phase, and at the high concentration aqueous phase it dehydrates. If the solute concentration is equal in the internal and external phase, there is no osmotic pressure difference, the solutes do not diffuse but water molecules are still able to diffuse in any direction by this mechanism (Benichou, Aserin, & Garti, 2004).

Wen & Papadopoulos (2000) observed different water transport rates between visually contacting and non-contacting W/O and O/W interfaces, and concluded that each case has a different mechanism. In the case where there is contact between internal and external phases, the water transport is mainly realized by the diffusion of hydrated surfactants. Where these phases are not in contact, the transport of water is due to spontaneously emulsified droplets and reverse micelles. Also, they concluded that water transport by hydrated surfactants is faster than spontaneously emulsified droplets and reverse micelles. So, the first may be the primary method for water migration in double emulsions.

Thin lamellar diffusion occurs when internal droplets move towards the external interface inducing a fluctuation in the thickness of the oil layer (Garti & Lutz, 2004). In this scenario, the water or water soluble molecules pass through a thin lamellae of surfactants in the outer interface (Cheng et al., 2007). This mechanism can release the entire internal water droplets to the external phase without causing coalescence (Garti & Lutz, 2004).

1.3.4 Potential uses

Double emulsions have been used for controlled release of the target compound at a controlled rate in function of environmental stress, protection of susceptible compounds from deteriorative reactions with other hydrophilic ingredients, and the inhibited release of some undesirable components that provide unpleasant properties (Tannak et al., 2016).
Different industries are using double emulsions for micro-encapsulation, for example in pharmaceuticals as carriers of hormones and steroids, and in cosmetics for creams with encapsulated compounds. In the food industry, due to the enclosing of nutritional and bioactive compounds, double emulsions offer a significant potential in the development of functional foods (Jiménez-Colmenero, 2013).

There are three main reasons for considering W/O/W for healthier food applications: to reduce the fat content and to provide healthier fatty acid profiles, to reduce the sodium content and encapsulation of bioactive compounds. From the latter application, the following advantages have been reported. First, the functional ingredients can be trapped in the internal phase and released at a controlled rate or in response to specific environmental triggers, like in the mouth, stomach or small intestine. Second, functional ingredients can be protected from chemical deterioration by isolating them from other water soluble ingredients, which they might normally react with. Third, water soluble functional ingredients that have undesirable sensory properties can be trapped inside the internal phase in a way that these properties are not perceived in the mouth during mastication (Jiménez-Colmenero, 2013).

The following functional components have been reported to be encapsulated in multiple emulsions: minerals, vitamins, amino acids, polyphenolic compounds and microorganisms. Regarding amino acids, L-tryptophan has been evaluated in double emulsions because it is an essential amino acid and is active in the regulation of behavior and mental performance (Jiménez-Colmenero, 2013).

1.4 Amino acids

Natural proteins consist of 20 different amino acids, which are bonded by peptide bonds. The structure of these amino acids consists of an α-carbon atom covalently attached to a hydrogen atom, an amino group, a carboxyl group and a side-chain R-group (figure 1.6). Because of the carboxyl group (acidic) and the amino group (basic), in aqueous solutions amino acids are present, depending on pH, as cations, zwitterions or anions (Damodaran, Parkin, & Fennema, 2007).

Physicochemical properties, such as net charge, solubility, chemical reactivity and the hydrogen bonding potential of the amino acids depends on the chemical nature of the side chain. Therefore, these biomolecules are classified as a function of the interaction of the side chain with water into hydrophilic and hydrophobic. The hydrophilic can be sub-classified into charged and uncharged, while the hydrophobic can be also sub-classified into aliphatic and aromatic (Damodaran et al., 2007).
Hydrophobicity is defined as the excess free energy of a solute dissolved in water compared to that in an organic solvent in similar conditions. The most direct and simplest way to determine the hydrophobic scale of amino acid side chains is by determining the free energy changes of the amino acid side chains in water with respect to a solvent, like ethanol or octanol. In table 1.2, hydrophobicity values of amino acid side chains by this method are given, where positive values correspond to hydrophobic amino acid side chains and negative values correspond to hydrophilic ones (Damodaran et al., 2007).

Table 1.2 Hydrophobicity of amino acids side chains at 25°C (Damodaran et al., 2007).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Hydrophobicity (kcal/mol)(^a) ((\Delta G^0_{tr} ))</th>
<th>Amino acid</th>
<th>Hydrophobicity (kcal/mol)(^a) ((\Delta G^0_{tr} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>0.4</td>
<td>Leucine</td>
<td>2.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>-1.4</td>
<td>Lysine</td>
<td>-1.4</td>
</tr>
<tr>
<td>Asparagine</td>
<td>-0.8</td>
<td>Methionine</td>
<td>1.7</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>-1.1</td>
<td>Phenylalanine</td>
<td>2.4</td>
</tr>
<tr>
<td>Cysteine</td>
<td>2.1</td>
<td>Proline</td>
<td>1.0</td>
</tr>
<tr>
<td>Glutamine</td>
<td>-0.3</td>
<td>Serine</td>
<td>-0.1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>-0.9</td>
<td>Threonine</td>
<td>0.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>0</td>
<td>Tryptophan</td>
<td>3.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.2</td>
<td>Tyrosine</td>
<td>1.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.5</td>
<td>Valine</td>
<td>1.7</td>
</tr>
</tbody>
</table>

\(^a\) the \((\Delta G^0_{tr} )\) value are relative to glycine based on the side chain distribution coefficient \((k_{eq})\) between 1-octanol and water.

Bull & Breese (1974) stated that a hydrophobicity scale of amino acid residues could be also based on the effect of the amino acids on the surface tension in water, where the greater the decrease of the surface tension the greater the hydrophobicity. As dipolar ions, amino acids have an antagonistic effect that increases the surface tension, but the number and size of the organic groups in the side chain tend to lower it (Pappenheimer, Lepie, & Wyman, 1936). In figure 1.7 an example is shown of the effect of glycine, alanine, valine and leucine on the surface tension of water, leucine has a longer aliphatic chain, it significantly reduces the surface tension. Meanwhile, glycine has a hydrogen as side chain end acts as a dipolar ion which increases the surface tension.
1.4.2 Chemical reactivity

In figure 1.8 some standard reactions of amino acids are shown. Mainly these reactions are due to the presence of the carboxyl and amino groups. Moreover, depending on the amino acid, other reactions are due to the functional groups present in the side chain. Some of these reactions lead to the destruction of the amino acid or into derivates which are not metabolized, losing the nutritional value of these compounds (Belitz, Grosch, & Schieberle, 2009).

![Diagram of amino acid reactions](image-url)
1.4.3 Quantification

Some of the previous reactions can be used to quantify amino acids and specific amino acid residues in proteins. For example, the reaction of amino acids with ninhydrin, O-phthalaldehyde, or fluorescamine is regularly used in the quantification of amino acids (Damodaran et al., 2007). Satake et al. (1960) developed a spectrophotometric method, without showing any undesirable side reaction, by measuring the optical density at 340 nm of the TNP-lation of amino acids with trinitrobenzenesulfonic acid (TNBS) (figure 1.9).

![Figure 1.9 Reaction of amino group with trinitrobenzenesulfonic acid (Fields, 1972).](image)

1.4.4 Encapsulation

As mentioned before, double emulsions have been used to encapsulate amino acids. However, we are only aware of the encapsulation of L-tryptophan realized by Owusu, Zhu, & Dickinson (1992) and Weiss, Scherze, & Muschiolik (2005). Owusu et al. (1992) realized the encapsulation, varying different factors to determine that under the conditions of that study the amino acid was released by diffusion. The method to quantify the amount of amino acid was dialyzing the double emulsion. Then the equilibrium concentration was assayed from a spectrophotometric reading at 280 nm. Weiss et al. (2005) used L-tryptophan as a marker for measuring the release, because this amino acid has a small molecular weight compared to other potential markers. Therefore, it diffuses more rapidly than larger molecules used as potential markers. In this study, the released amino acid was determined by centrifugation to obtain the external phase and measuring the absorbance at 280 nm.

On the other hand, many of the applications of double emulsions regarding encapsulation of functional ingredients focus on peptides. Several studies focus on the encapsulation of these peptides to make them more orally bioavailable (Couvreur, Blanco-Prieto, Puisieux, Roques, & Fattal, 1997; Dogru, Çalis, & Öner, 2000), because most of them are susceptible to strong acidic environments and the proteolytic enzymes in the gastrointestinal tract (Dogru et al., 2000).

Furthermore, an additional potential use could be to mask undesirable sensory attributes, because all amino acids stimulate certain tastes (sweet, bitter, salt, sour, umami). Some amino acids have a low threshold value, so they will indeed stimulate a certain taste. Also, others with a higher threshold value can still have an effect on the taste because they might contribute to the final taste via synergistic effects (Kato, Rhue, & Nishimura, 1989).
Chapter 2
Materials and methods

2.1 Materials

The low HLB emulsifier polyglycerol polyricinoleate (Palsgaard® 4150, Palsgaard A/S, Denmark) and the high HLB emulsifier polysorbate 80 (Tween® 80, Sigma-Aldrich, St. Louis, USA) were used. The following amino acids were used: L-leucine (Acros Organics, Geel, Belgium), L-glutamine (Merck KgA, Darmstadt, Germany), L-serine (Serdary research laboratories, Ontario, Canada) and DL-alanine (UCB, Leuven, Belgium). Also sunflower oil (purchased from a local supermarket), high oleic sunflower oil (HOSO; Contined B.V., Bennekom, Netherlands), potassium chloride (AnalaR NORMAPUR®, VWR Chemicals, Leuven, Belgium), sodium azide (NaN₃) (Acros Organics, Geel, Belgium), picrylsulfonic acid solution 5% w/v in H₂O (Sigma-Aldrich, St. Louis, USA), NaHCO₃ (AnalaR NORMAPUR®, VWR Chemicals, Leuven, Belgium) and hydrochloric acid (HCl) 32% (VWR Chemicals, Fontenay-sous-Bois, France) were used in the following experiments.

2.2 Absorbance spectrum analysis

The protocol for the spectrophotometric determination of amines, amino acids and peptides by Satake et al. (1960) was evaluated to verify if it was applicable in this research. For this purpose, the absorption spectrum, between 250-500 nm, of a solution of 0.1 mM L-leucine and of the external phase containing 0.1 mM L-leucine (composition in table 2.1) were measured in quartz cuvettes, using a spectrophotometer (UV-1600PC UV-VIS, VWR International, Radnor, PA). All readings were subtracted with their corresponding blank, for the external phase with 0.1mM L-leucine the blank was a solution with the same composition but without the amino acid and for the solution of 0.1mM L-leucine the blank was distilled water. The obtained results were compared to the ones reported by Satake et al. (1960).

The solutions and blanks were submitted to reaction with TNBS, according to the method by Satake et al. (1960), prior to measuring absorbance spectrum. The conditions for this reaction are described in section 2.6.2.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>External phase</strong></td>
<td>2 wt% Tween 80</td>
</tr>
<tr>
<td></td>
<td>0.1 M KCl</td>
</tr>
<tr>
<td></td>
<td>0.02 wt% sodium azide</td>
</tr>
<tr>
<td></td>
<td>0.1 mM L-leucine</td>
</tr>
<tr>
<td><strong>L-leucine</strong></td>
<td>0.1 mM L-leucine</td>
</tr>
</tbody>
</table>
2.3 Composition of the water-in-oil-in-water (W$_1$/O/W$_2$) emulsions

Several experiments were performed with double emulsions with varying compositions of the internal, external and oil phases.

2.3.1 W$_1$/O/W$_2$ emulsions with low amino acid concentration

Table 2.2 shows the compositions of the three double emulsions prepared with low amino acid concentrations.

<table>
<thead>
<tr>
<th>Phase</th>
<th>L-leucine double emulsion</th>
<th>L-glutamine double emulsion</th>
<th>Blank double emulsion</th>
<th>Mass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal (W$_1$)</td>
<td>0.1 M KCl 0.02 wt% NaN$_3$ 1.5 mM L-leucine</td>
<td>0.1 M KCl 0.02 wt% NaN$_3$ 1.5 mM L-glutamine</td>
<td>0.1 M KCl 0.02% NaN$_3$</td>
<td>25</td>
</tr>
<tr>
<td>Oil (O)</td>
<td>5 wt% PGPR 95 wt% HOSO</td>
<td>5 wt% PGPR 95 wt% HOSO</td>
<td>5 wt% PGPR 95 wt% HOSO</td>
<td>25</td>
</tr>
<tr>
<td>External (W$_2$)</td>
<td>2 wt% Polysorbate 80 0.1 M KCl 0.02 wt% NaN$_3$</td>
<td>2 wt% Polysorbate 80 0.1 M KCl 0.02 wt% NaN$_3$</td>
<td>2 wt% Polysorbate 80 0.1 M KCl 0.02 wt% NaN$_3$</td>
<td>50</td>
</tr>
</tbody>
</table>

2.3.2 W$_1$/O/W$_2$ emulsions with high amino acid concentration

In order to have an osmotic equilibrium between the internal and external phases, the concentration of the electrolyte KCl was increased in the external phase, to counteract the osmotic contribution given by the amino acid in the internal phase. In table 2.3 the composition of each double emulsion is shown.

<table>
<thead>
<tr>
<th>Phase</th>
<th>L-leucine double emulsion</th>
<th>L-glutamine double emulsion</th>
<th>L-serine double emulsion</th>
<th>DL-alanine double emulsion</th>
<th>Blank double emulsion</th>
<th>Mass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal (W$_1$)</td>
<td>0.1 M KCl 0.02 wt% NaN$_3$ 15 mM L-leucine</td>
<td>0.1 M KCl 0.02 wt% NaN$_3$ 15 mM L-glutamine</td>
<td>0.1 M KCl 0.02 wt% NaN$_3$ 15 mM L-serine</td>
<td>0.1 M KCl 0.02 wt% NaN$_3$ 15 mM DL-alanine</td>
<td>0.1075 M KCl 0.02 wt% NaN$_3$</td>
<td>25</td>
</tr>
<tr>
<td>Oil (O)</td>
<td>5 wt% PGPR 95 wt% commercial sunflower oil</td>
<td>5 wt% PGPR 95 wt% commercial sunflower oil</td>
<td>5 wt% PGPR 95 wt% HOSO</td>
<td>5 wt% PGPR 95 wt% HOSO</td>
<td>5 wt% PGPR 95 wt% commercial sunflower oil</td>
<td>25</td>
</tr>
<tr>
<td>External (W$_2$)</td>
<td>2 wt% Polysorbate 80 0.1075 M KCl 0.02 wt% NaN$_3$</td>
<td>2 wt% Polysorbate 80 0.1075 M KCl 0.02 wt% NaN$_3$</td>
<td>2 wt% Polysorbate 80 0.1075 M KCl 0.02 wt% NaN$_3$</td>
<td>2 wt% Polysorbate 80 0.1075 M KCl 0.02 wt% NaN$_3$</td>
<td>2 wt% Polysorbate 80 0.1075 M KCl 0.02 wt% NaN$_3$</td>
<td>50</td>
</tr>
</tbody>
</table>
2.3.3 $W_1/O/W_2$ emulsions with varying hydrophilic surfactant concentration

Three double emulsions with L-leucine in the internal phase were prepared with the compositions shown in table 2.4. Hereby, the concentration of the hydrophilic surfactant in the external phase was varied (0.5, 1 and 2 wt%).

<table>
<thead>
<tr>
<th>Phase</th>
<th>L-leucine double emulsion</th>
<th>Mass (%)</th>
</tr>
</thead>
</table>
| Internal ($W_1$) | 0.1 M KCl  
0.02 wt% NaN$_3$  
15 mM L-leucine | 25 |
| Oil (O) | 5 wt% PGPR  
95 wt% HOSO | 25 |
| External ($W_2$) | Polysorbate 80*  
0.1075 M KCl  
0.02 wt% NaN$_3$ | 50 |

$*$0.5, 1 and 2 wt%

2.4 Preparation of $W_1/O/W_2$ emulsion

2.4.1 Water in oil ($W_1/O$) emulsions preparation

The internal phase and the oil phase were heated to 60 °C and stirred for 10 minutes. Then, the internal phase was slowly added (1 minute of addition) to the oil phase while mixing. The mixtures were homogenized with an Ultra-Turrax rotor-stator homogenizer (S25-10G, IKA®-Werke, Germany) at 24,000 rpm during 5 minutes (of which 1 minute of addition).

2.4.2 $W_1/O/W_2$ emulsions preparation

The external phase was mixed at room temperature with the freshly prepared $W_1/O$ emulsion with an Ultra-Turrax rotor-stator homogenizer (S25-10G, IKA®-Werke, Germany) at 17,500 rpm for 5 minutes. All double emulsions were stored at 4°C after production.

2.5 Characterization of $W_1/O/W_2$ emulsions

All double emulsions were characterized with the following tests after 0, 1, 2, 4 and 16 days of storage at 4 °C.

2.5.1 Determination of average droplet size

These tests were carried out using a Malvern particle size analyzer (Mastersizer 3000, Malvern Instruments Ltd., Malvern, Worcestershire, UK). The average volume-weighted mean diameter ($D[4,3]$) of a duplicate, each one consisting of five measurements, was considered for the droplet size analysis, where a solution of 0.1 or 0.1075 M KCl was used as a dilution liquid to prevent osmotic effects during the measurements. Parameters were adjusted as follows: refractive index of dispersant=...
1.33; material refractive index= 1.53, absorption index= 0.01, measurement duration of background= 10 s, measurement duration of sample= 10 s, number of measurements= 5; stirring speed= 1,500 RPM and obscuration limits= 10-20 %.

2.5.2 Water yield determination

The yield is defined as the proportion of the water originally present as dispersed phase in the primary W/O emulsion that is retained as internal water droplets in the W/O/W (Dickinson & Miller, 2001). For this determination, analytical photocentrifugation was performed according to the method of Balcaen et al. (2016). It was performed at room temperature, with air cooling of the LUMiFuge 116 (LUM GmbH; Berlin, Germany). Rectangular synthetic cells with 2.2 mm path length were used. The light intensity was set to 100%. The percentages of water yields were determined in duplicate after 3 hours of centrifugation at a speed of 3000 rpm. The position of the interfaces between air and cream, and cream and serum were determined using 30% of the transmission. Figure 2.1 shows an example of a transmission profile after 3 hours of centrifugation at 3000 rpm to determine the position of the interfaces.

\[
\text{VF}_{\text{cream}} = \frac{\text{Height of the cream layer (mm)}}{\text{Height of sample (mm)}} = \frac{\text{Position start cream} - \text{Position end cream}}{\text{Position start cream} - \text{Total height cell}} \quad (1)
\]

Using \( \text{VF}_{\text{cream}} \), the volume fraction of internal water (\( \text{VF}_{\text{internal water}} \)) is obtained with the equation 2, where \( X \) refers to the mass fraction, which was used during preparation of the double emulsion, and \( \rho \) to the density of the respective phases (g/ml).

\[
\text{VF}_{\text{internal water}} = \text{VF}_{\text{cream}} - \frac{X \rho_{\text{oil}}}{X \rho_{\text{oil}} + X \rho_{\text{internal water}} + X \rho_{\text{external water}}} \quad (2)
\]
Finally, the yield is obtained, dividing the volume fraction of internal water by the volume fraction of the maximum internal water (Equation 3); the latter value refers to the theoretical value of internal water corresponding to the composition of the W₁/O/W₂.

\[
Yield = \frac{VF_{\text{internal water}}}{VF_{\text{maximal internal water}}}
\]  (3)

2.5.3 Microscopy

Tenfold dilutions of the double emulsions were prepared using a solution of 0.1 mM KCl as diluent, and observed using an optical microscope (CX40RF200, Olympus optical, Japan), using the 40x objective. This was performed to verify that the samples were still double emulsions.

2.6 Determination of released amino acid

2.6.1 Collection of the external phase

Unless stated differently, samples of the double emulsions were centrifuged (Sigma 1-15P No. 10050, SIGMA Laborzentrifugen, Osterode am Harz, Germany) at 10,000 g for 5 minutes. After this, the serum phase was extracted using a syringe, then filtered using a filter (Pore size: 0.45 µm with nylon membrane; VWR International, USA) and stored at 4°C until analysis.

2.6.2 Reaction with TNBS

The determination was performed according to the method of Satake et al. (1960), whereby 1ml of the filtered sample, 1ml of a 4% NaHCO₃ solution and 1ml of 0.1% TNBS were mixed and kept in a dark place at 40°C for 2 hours. Then 1ml of 1M HCl was added to stop the reaction. Also, blank and standard curve solutions were submitted to this reaction.

For the determination of samples from double emulsions with high concentration of amino acid, these were diluted tenfold with distilled water after filtration and then followed the same method as mentioned in the previous paragraph.

2.6.3 Method of determination

2.6.3.1 Standard curve determination

This method consisted on following the procedure by Satake et al. (1960), where the absorbance of the samples was measured at 340 nm in a spectrophotometer (UV-1600PC UV-VIS, VWR International, Radnor, PA) and with a standard curve, the concentration of amino acid in the external phase was determined. The blank of these samples consisted of a solution of the external phase but without amino acid, this blank value was subtracted from the readings of the samples.

The standard curve was constructed by plotting the absorbance, at 340 nm, as a function of the concentration of the amino acid. The solutions were prepared by varying the amount of the internal phase (W₁) in a fixed amount of the external phase (W₂). The minimum concentration means the
absence of internal phase and the maximum is equivalent to the concentration in the case where all internal phase is released (table 2.5). The first dilution was taken as the zero point of the curve.

For the analysis of the double emulsions with high concentration of amino acids, the same procedure was followed for the standard curve, but the solutions of internal phase/external phase \((W_1/W_2)\) were diluted tenfold in distilled water.

### 2.5 Dilution series of \(W_1\) with \(W_2\) used for the construction of the standard curve.

<table>
<thead>
<tr>
<th>(W_1/W_2^*)</th>
<th>Amino acid concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/1</td>
<td>0.000</td>
</tr>
<tr>
<td>1/128</td>
<td>0.012</td>
</tr>
<tr>
<td>1/64</td>
<td>0.023</td>
</tr>
<tr>
<td>1/32</td>
<td>0.045</td>
</tr>
<tr>
<td>1/16</td>
<td>0.088</td>
</tr>
<tr>
<td>1/8</td>
<td>0.167</td>
</tr>
<tr>
<td>1/4</td>
<td>0.300</td>
</tr>
<tr>
<td>1/2</td>
<td>0.500</td>
</tr>
</tbody>
</table>

*For the standard curve of double emulsions with high concentration of amino acids, dilution series of \(W_1/W_2\) was diluted 10 times in distilled water ((\(W_1/W_2\))/10).

### 2.6.3.2 Determination considering turbidity effect

In the second method, due to a possible influence of turbidity in the absorbance spectrum of the samples, a mathematical model was adjusted considering the contribution of this effect. So, the absorption spectrum of the samples and of a standard solution of external phase with 0.5 mM of the amino acid, between 250-500 nm, was measured in a spectrophotometer (UV-1600PC UV-VIS, VWR International, Radnor, PA). All readings were subtracted with their corresponding blank. The blank of the samples consisted of a solution of external phase but without amino acid, but for the samples of blank double emulsions, the blank was distilled water without TNBS.

The mentioned model consists of two contributions: the first refers to the turbidity effect, which follows an exponential decay in the form of \(\text{Absorbance}=e^{-k\lambda}\) (Hiemenz & Rajagopalan, 1997). The second contribution refers to the absorbance spectrum of the standard solution dye at a known concentration. Combining both contributions in one model, equation 4 is obtained, where \(x\) refers to the contribution of the turbidity, \(k\) is a constant, \(\lambda\) is the wavelength in nm and \(y\) is the contribution of the standard dye.

\[
\text{Absorbance}(\lambda)_{\text{experimental}} = xe^{-k\lambda} + y \times \text{absorbance}(\lambda)_{\text{standard}}
\] (4)

The parameters \(y\), \(x\) and \(k\) were determined by adjusting the model to minimize the sum of the squared errors using the tool solver in Microsoft EXCEL. And to obtain the concentration of amino acid, the contribution \(y\) of the sample was multiplied with concentration of the standard amino acid solution (0.5mM).
2.7 Model of released amount of amino acids

A mathematical model was designed (equation 5) to fit the experimental data from the release profiles of the amino acids. The fitting was performed with least squared errors method using the tool solver in Microsoft EXCEL by varying the parameters $t_A$ and $C_0$. In this equation, $C =$ concentration in the external phase (mM), $C_{\text{equilibrium}} =$ Equilibrium concentration in the external phase (mM), $C_0 =$ initial concentration in the external phase (mM), $t =$ time (days) and $t_A =$ kinetic constant (days).

\[
C = C_{\text{equilibrium}} - (C_{\text{equilibrium}} - C_0)e^{-\frac{t}{t_A}}
\]  

(5)

2.8 Statistical analysis

The following statistical analysis were performed to evaluate the results of the two methods of determination, average particle size and released amount of amino acid. To confirm the assumption of normality and equality of variance in the ANOVA tests, the Kolmogorov-Smirnov and Levene tests were performed, respectively.

For the determining differences between the standard curve and the turbidity determination methods a non-parametric Wilcoxon signed rank test at a confidence value of 95% was performed. The test was based on the concentration of the amino acid difference between the two methods per day of measurement, verifying if all these differences were equal to zero, or at least one was different from zero.

The average droplet size and concentration of amino acids in the external phase as function of storage time of the double emulsions with high amino acid content were analyzed by a two-way ANOVA at a confidence value of 95%. The main effects were type of amino acid, with 5 levels (blank, L-leucine, L-glutamine, L-serine and DL-alanine) and days of storage, with 6 levels (Days 1, 2, 4, 8, 16). Also, a Tukey post-hoc test, at a confidence value of 95%, was performed to check the significance of the differences between the various levels of the main effects.

Additionally, the average droplet size was also analyzed by a three-way ANOVA at a confidence value of 95%. In this case, the same main effects with the same levels, as mentioned before, were considered, but with an additional main effect, which was preparation method, with two levels (high and low). Also, a Tukey post-hoc test was performed.

The influence of the hydrophilic surfactant concentration in the average droplet size and concentration of amino acids in the external phase as function of storage time of the double emulsions with L-leucine and polysorbate 80 at 2, 1 and 0.5 wt% was analyzed with the non-parametric Wilcoxon signed rank test at a confidence value of 95%. Each double emulsion was compared with the two others, based on the size or concentration difference between the double emulsions per day, verifying if the values of the differences for all days were equal to zero, or at least one was different from zero.

All these statistical tests were performed using the R software (version 3.2.2).
Chapter 3
Results and discussion

3.1 Method development for the determination of amino acids in the external phase of W₁/O/W₂ emulsions

As mentioned in chapter 1, the only published researches found on encapsulation of amino acids with double emulsions are those of Owusu et al. (1992) and Weiss et al. (2005). Both of them encapsulated L-tryptophan, so quantification was performed using spectrophotometry, because the indole side chain of L-tryptophan residues strongly absorbs light near 280 nm (Spande & Witkop, 1967). As this study aims to measure the release of different amino acids, without an aromatic ring in the side chain, the method by Satake et al. (1960) shows to be the more adequate for this purpose, because the TNBS reacts only with primary amino groups of amino acids and peptides and is able to quantify concentrations of 0.01-0.8 mM of these compounds.

3.1.1 Absorbance spectrum test of L-leucine in external phase

As the amino acid content that is measured is present in the external phase, the purpose of this assessment was to verify if the phase composition has an influence on the absorbance spectrum. For this, the absorbance spectrum of a solution of 0.1mM L-leucine and of the external phase with 0.1mM L-leucine were measured. The plotted values correspond to the solutions, minus their respective blanks. This concentration of the amino acid was chosen in order to avoid exceeding the absorbance limit of the equipment.

As can be observed from figure 3.1, there is a difference in the absorption spectrum between the solution 0.1mM L-leucine and the external phase with 0.1mM L-leucine. Taking into account that the external phase contains NaN₃, KCl and polysorbate 80, the difference should be related with these compounds. However, NaN₃ and KCl are present in very low concentrations, so the effects of these compounds can be neglected. So, the difference is most likely due to two factors, the first one could be related to the formation of micelles of polysorbate 80, because the critical micelle concentration is 0.01 mM (value obtained from the data sheet of the supplier) and the concentration in the external phase is 15 mM. The second factor could be the formation and retention of gas, formed by the reaction of the TNBS with the amino acid in the presence of the emulsifier. The TNBS reacts with the amino group, forming a complex, sulfite ion and hydrogen ion. When adding HCl to end the reaction, the excess acid reacts with the sulfite ion forming sulfur dioxide gas, according to the reaction $\text{SO}_3^{2-} \rightarrow \text{SO}_2 (g) + \text{H}_2\text{O}(l)$. In normal circumstances, the gas would diffuse rapidly. However, in this case, the presence of a hydrophilic emulsifier may retain these bubbles, which affects the absorbance readings.
Figure 3.1 Absorbance spectrum of 0.1 mM L-leucine and external phase with 0.1 mM L-leucine.

Wuelfing et al. (2006) described that polysorbate 80 can be oxidized under high temperatures and form a yellow-orange coloration that affects the absorbance at wavelengths greater than 400 nm. But the analytical interference of polysorbate 80 can be considered manageable at 3–5% (w/w) in UV/vis spectrophotometry at wavelengths greater than 300 nm, and in 0.1–1% solutions, at wavelengths higher than 260 nm, or in 0.05–0.01% solutions at all wavelengths with proper background subtractions.

As all the readings are corrected with their corresponding blank, the effect of the polysorbate 80 could be related more to an interaction effect with the other components of the double emulsion, as the bubble formation, than to the formation of micelles. However, it is observed that in the region below 300 nm, the pattern completely changes between both curves. This could be because the emulsifier has chromophores, C=C (λ<sub>max</sub>≈195nm), alkyl ester (λ<sub>max</sub>≈195-210nm) and poly ethylene glycol chains (λ<sub>max</sub>≈180-185nm) (Wuelfing et al., 2006). So, these chromophores affect the absorption as the wavelength approaches 200 nm, and as the surfactant is present in the sample and blank, the subtraction tends toward zero, as observed.

Despite the effect of the emulsifier, the solution of the external phase with L-leucine has a high absorbance in the region close to 340 nm, similar to the L-leucine and as Satake et al. (1960) reported (figure 3.2). Hence, they performed the determination of amino acids at this wavelength. The value reported by Satake et al. (1960) for the absorbance at 340nm of 0.13 mM L-leucine is 0.4, and the obtained values for 0.10 mM leucine in the external phase is 0.35. Therefore, this method can be considered acceptable for the scope of this work because the absorbance spectrum shows the highest values around 340 nm, and a similar absorbance value at this wavelength for 0.1mM as Satake et al. (1960) reported.
3.1.2 Amino acid quantification tests

Two trials were carried out to find the best methodology to quantify the amount of amino acid in the external phase. For these tests, double emulsions with L-leucine and L-glutamine were used as model amino acids. Also, a blank double emulsion was prepared.

3.1.2.1 Test with low amino acid concentration in the internal phase

For this trial, the amino acid quantification was done following the standard curve method protocol mentioned in section 2.6. While constructing the standard curves (figures 3.3 & 3.4), the most concentrated samples had more unstable readings, and the formation of gas increased as the concentration of amino acid also increased. This might be due to the effect of the hydrophilic emulsifier as mentioned in the previous section, and the concentration of amino acid seems to be directly proportional to the bubble formation. Therefore, the samples were vigorously shaken to try to eliminate the gas, in order to obtain a stable reading.
Figure 3.4 Standard curve for L-glutamine in external phase as obtained at a wavelength of 340 nm.

From figure 3.5, it can be observed that the quantification of the amino acids concentration in the external phase showed two different patterns of release for the two types of amino acids, but this will be discussed further on. For the blank series, it was expected that the readings would give readings of zero or close to it. However, the method does not give a constant measure, but the values vary around zero, though at the start shows a very high value. This inconsistency might due to the fact that there is no amino acid available to react with TNBS, so there is no formation of a dye product, therefore the readings come from the interference of background compounds present, causing some turbidity effects. Also, the sensitivity of the equipment might affect the quantification.

Figure 3.5 Amino acid concentration in the external phase as determined using the standard curve method as a function of storage time at 4 °C.
3.1.2.2 Test with high amino acid concentration in the internal phase

As the previous trial showed that bubble formation was an issue for the absorbance measurements of the standard curve, and the blank double emulsion samples showed a variation due to the possible presence of other compounds, it was decided to change the procedure and some parameters in order to improve the quantification. First, the concentration of the amino acid in the double emulsion was increased ten times in the internal phase, compared to the double emulsions of the previous trial. Second, a new step in the procedure was added, where once the sample was centrifuged, the serum phase extracted and filtered, it was diluted tenfold with distilled water. The quantification was performed using the two methods mentioned in section 2.6, the standard curve method and the method in which the turbidity effect is considered.

3.1.2.2.1 Standard curve method

For the standard curves of this trial (figure 3.6 & 3.7), as the solutions are also diluted tenfold in distilled water (as mentioned in section 2.6), the readings were more stable. It was not necessary to shake the samples with the low amino acid concentration, as bubble formation was practically absent. However, shaking was still necessary to apply to the samples with a high concentration (>0.2 mM) due to the presence of bubbles, but not as much as the one from the previous test.

Figure 3.6 Standard curve for L-glutamine in external phase ten times diluted in distilled water as obtained at a wavelength of 340 nm.

$y = 2.82x$

$R^2 = 0.99$
As in the previous test, the amino acid showed two different patterns of release for the two types of amino acids. Also, for the blank measurements, a fluctuation around zero is observed. Results of this method are shown at the end of this section.

3.1.2.2.2 Method considering turbidity effect

For this method, the absorbance spectrum, between 250 - 500 nm, of the samples of blank, L-leucine and L-glutamine double emulsions were measured to adjust the mathematical model described in section 2.6.3.2 and calculate the amino acid release based on the absorbance spectrum of a standard sample of external phase with 0.5 mM of the corresponding amino acid.

As can be observed from figure 3.8, the absorbance spectrum of the samples of the double emulsion containing L-glutamine doesn’t show the characteristic behavior of the curve of amino acid that reacted with the TNBS. This is an indication that there is a little or no release of this amino acid during 16 days of storage at 4°C. On the other hand, figure 3.9 shows that for the L-leucine double emulsion there is a considerable amount that is released during storage. Consequently, the absorption spectra of the samples of the latter amino acid resemble more to the standard as function of the storage time. These results are in accordance with the release patterns of the first trial of double emulsions with low amino acid content.
Figure 3.8 Absorbance spectrum of the external phase tenfold diluted with distilled water of the L-glutamine double emulsions and of an external phase with 0.5 mM L-glutamine.

Figure 3.9 Absorbance spectrum of the external phase tenfold diluted with distilled water of the L-leucine double emulsions and of an external phase with 0.5 mM L-leucine.

In the case of the blank double emulsion samples, the zero reading (blank) consisted of distilled water as mentioned in chapter 2. Therefore, the absorbance values obtained in these samples are not related to concentration of amino acids, but to the composition of the external phase of the blank double emulsion samples. From figure 3.10 it can be observed that the absorbance curves show a slight upward shift as function of storage time. This might be due to the fact that oil can be transferred from
an emulsion droplet to a micelle (Karaborni et al., 1993). And as explained before, micelles of the polysorbate 80 can be formed in the external phase, because the concentration of this emulsifier is above the cmc. So, the presence of this micelles with entrapped oil could affect the absorbance measurements. The shift over time could be explained by the fact that the number micelles containing oil increases as a function of time. Nevertheless, as said before the shift appears to be minimal, so it is highly recommended that this dilution step is done when measuring the absorbance of double emulsions with similar composition to those that were used in this research. It is important to keep in mind, that when diluting the samples, the final concentration of the amino acid is inside the range of quantification mentioned by Satake et al. (1960) of 0.01-0.8 mM.

![Absorbance spectrum of samples of the external phase tenfold diluted with distilled water of the blank double emulsions and of a solution of external phase.](image)

In order to obtain an adequate adjusted mathematical model, values of absorbance from 300 - 500 nm were used, because those below 300 nm were not constant for the samples of L-glutamine. This variation might be because the equipment becomes very sensible at low wavelengths, and as this amino acid seems not to be present or at a very low concentration, the readings are just measuring background components that give this variability.

As can be observed from figure 3.11, the absorbance spectrum of the L-glutamine samples displays mostly a linear behavior. This gives an indication that in the case of L-glutamine double emulsions, there is a small contribution by the turbidity term and basically there is no contribution of coloring by the reaction of the TNBS with the amino acid. That might be due to a low coloring intensity because of a low amino acid concentration released to the external phase.
Figure 3.11 Comparison of the adjusted models (dotted line) with experimental curves (solid line) for double emulsions of L-glutamine at day 0(a), 1(b), 2(c), 3(d), 4(e), 8(f) & 16(g).

On the other hand, the absorbance spectrum curves of the L-leucine double emulsion samples show a more visible contribution of the coloring agent (figure 3.12). This happens because it seems that the L-leucine is released faster into the external phase compared to L-glutamine. Therefore, there is more amino acid available to react with the TNBS, forming the coloring complex. In fact, the absorbance spectrum of L-leucine shows the two peaks a bit more clearly, as described by Satake et al. (1960) than the curves of L-glutamine samples, indicating that the former is present in the external phase in a higher concentration than the latter.
In table 3.1 are shown the values of the parameters of the model. As can be observed, for L-leucine the contribution of the dye product \( x \) increases over time as expected, because the amino acid is released into the external phase. It is important to remark that the turbidity contribution \( y \) also increases with time, for both amino acids, but as the constant \( k \) is small over time the whole exponential term is reduced nearly to zero. Moreover, the sum of squared residuals shows low values, so the discrepancy between the data and an estimation model is low. So, this model seems to be reliable for quantifying the amount of amino acid released. So, for the purpose of this research, this is an acceptable model.
Table 3.1 Contribution factors of the dye (y), turbidity (x) and decay constant (k) of the adjusted model of the external phase absorbance spectrum of the samples and the sum of squared residuals (SSR) of each day.

<table>
<thead>
<tr>
<th>Day</th>
<th>x</th>
<th>k</th>
<th>y</th>
<th>SSR</th>
<th>x</th>
<th>k</th>
<th>y</th>
<th>SSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>399.83</td>
<td>0.107</td>
<td>0.035</td>
<td>0.00</td>
<td>399.84</td>
<td>0.171</td>
<td>0.013</td>
</tr>
<tr>
<td>1</td>
<td>0.97</td>
<td>0.01</td>
<td>0.015</td>
<td>0.002</td>
<td>3.59</td>
<td>0.02</td>
<td>0.290</td>
<td>0.019</td>
</tr>
<tr>
<td>2</td>
<td>0.70</td>
<td>0.01</td>
<td>0.068</td>
<td>0.009</td>
<td>12.70</td>
<td>0.02</td>
<td>0.389</td>
<td>0.059</td>
</tr>
<tr>
<td>4</td>
<td>1.30</td>
<td>0.01</td>
<td>0.029</td>
<td>0.002</td>
<td>36.04</td>
<td>0.02</td>
<td>0.507</td>
<td>0.091</td>
</tr>
<tr>
<td>8</td>
<td>1.92</td>
<td>0.01</td>
<td>0.039</td>
<td>0.009</td>
<td>66.37</td>
<td>0.02</td>
<td>0.569</td>
<td>0.073</td>
</tr>
<tr>
<td>16</td>
<td>1.11</td>
<td>0.01</td>
<td>0.027</td>
<td>0.003</td>
<td>2.1x10^{12}</td>
<td>0.11</td>
<td>0.728</td>
<td>0.093</td>
</tr>
</tbody>
</table>

3.1.2.2.3 Differences between determination methods

![Figure 3.13 Concentration of the amino acids in the external water phase as function of storage time at 4°C.](image)

In figure 3.13, it can be observed that both methods which were used to determine the concentration of amino acid led to very similar values. From the non-parametric paired t-tests, there is not enough evidence to prove that there is a difference in the amount of L-leucine (p-value=0.06) and L-glutamine (p-value=0.06) released between the two determination methods. This means that both methods are suitable for measuring the concentration of amino acids.

Moreover, a difference between the L-leucine and L-glutamine released can be observed, as in the first trial, confirming that the release behavior of both amino acids is different. This is clearly shown
in the contribution factor $y$ of the adjusted model (table 3.1), where most of L-glutamine contributions are very small compared to those of L-leucine. That also means that there is a larger concentration of L-leucine than of L-glutamine in the external phase. The variation observed for the L-glutamine and blank samples might be due to the problems of variability at very low concentrations of amino acid, as mentioned previously.

Therefore, as both methods show to have similar values and the same tendency in the amount of quantified amino acid, either of the two can be used. For the following experiments, the determination was based on the standard curve method due to the ease of calculating the amount compared to the other method.

3.2 Encapsulation efficiency of amino acids in double emulsions

The release of amino acids in double emulsions was evaluated on two different fronts. The first one involved the study of the release as function of the amino acid properties. The amino acids evaluated were: L-leucine, L-glutamine, L-serine and DL-alanine. The second one considered the release as a function of the hydrophilic emulsifier concentration. In both approaches, the double emulsion droplet size and entrapped water volume fraction changes were followed during the period of evaluation.

For these experiments, double emulsions with high concentration of amino acid were prepared and the standard curve method was used to quantify the amount of amino acid in the external phase, as this methodology showed to be best and most practical as mentioned in the last section.

3.2.1 Release as function of amino acid properties

3.2.1.1 Double emulsion droplet size

![Figure 3.14 Mass-weighted mean double emulsion droplet size as a function of storage time at 4°C.](image)

From the statistical analysis, the normality assumption was confirmed by the Kolmogorov-Smirnov test, with a $p$-value $= 0.08$. The assumption of equal variances was confirmed by the Levene test for type of amino acid ($p$-value $= 0.36$) and days of storage ($p$-value $= 0.51$). The main effects (type of
amino acid encapsulated and days of storage) without interaction were tested, due to limited amount of data per experiment. Both main effects showed to have a significant effect (p-value < 0.001, for both effects). From the post-hoc analysis, day 16 shows a significant difference in droplet size with respect to day 1 (p-value < 0.001), day 2 (p-value < 0.01) and day 4 (p-value < 0.01). In table 3.2 it is shown which double emulsions have significantly different droplet sizes among each other.

Table 3.2 Differences in the droplet size between double emulsions

<table>
<thead>
<tr>
<th></th>
<th>L-glutamine</th>
<th>L-leucine</th>
<th>L-serine</th>
<th>DL-alanine</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamine</td>
<td>-</td>
<td>ND+</td>
<td>SD*</td>
<td>SD*</td>
<td>ND++</td>
</tr>
<tr>
<td>L-leucine</td>
<td>ND+</td>
<td>-</td>
<td>SD*</td>
<td>SD*</td>
<td>ND+++</td>
</tr>
<tr>
<td>L-serine</td>
<td>SD*</td>
<td>SD*</td>
<td>-</td>
<td>ND++++</td>
<td>SD*</td>
</tr>
<tr>
<td>DL-alanine</td>
<td>SD*</td>
<td>SD*</td>
<td>ND++++</td>
<td>-</td>
<td>SD*</td>
</tr>
<tr>
<td>Blank</td>
<td>ND++</td>
<td>ND+++</td>
<td>SD*</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

ND= not significantly different, SD= significantly different
p-values: * < 0.001, + = 0.95, ++ =0.18, +++ =0.51, ++++ =0.31.

As can be observed from figure 3.14, the change of droplet size over time is more noticeable for the L-serine and DL-alanine double emulsions, in which for the first days the increase is not significant but after day 4, the increase becomes significant compared to the initial size. This is in accordance with the information in table 3.2, where it is shown that this pair of double emulsions have a similar droplet size, but are significantly different from the other double emulsions.

Between L-leucine, L-glutamine and blank double emulsions, there are no significant droplet size differences. Moreover, they show a slower increase as a function of the day of storage. This might be because the initial droplet size is smaller for these double emulsions than the L-serine and DL-alanine, and usually smaller droplets extend the stability of the emulsion (Damodaran et al., 2007). So, the droplet size differences between double emulsions is not due to the type of amino acid that is entrapped, but related to the initial droplet size of the double emulsion. And the difference in the initial oil droplet sizes can be related with the preparation of the double emulsions.

The difference in droplet size between double emulsions could be explained by the fact that during the preparation of these double emulsions the user has a lot of influence, because the equipment used (Ultra-Turrax rotor-stator homogenizer) is not completely automatized, particularly, when setting the speed of mixing is done using a scroll instead of a button. Thus, if the user does not perform the preparation in the same way, with the same mixing times and additions, the droplet size can vary.

In this case, all double emulsions were produced following the protocol mentioned in chapter 2. Nevertheless, achieving the same addition times every time was not possible. Also, different containers were used between double emulsions containing L-glutamine, L-leucine and blank with those containing L-serine and DL-alanine. So, this preparation factor might also have influenced the mixing of the phases, thus influencing the initial droplet size.

Therefore, another ANOVA was carried out but now taking into consideration the preparation as a main effect, with two levels, in order to differentiate between the preparation of the double emulsions.
containing L-glutamine, L-leucine and blank with those containing L-serine and DL-alanine. Also in this case, the main effects without interaction were tested and the preparation effect showed to have inequality of variance (p-value=0.02). The effects preparation and day of storage showed to have a significant effect (p-value< 0.001, for both effects), and show a trend towards statistical significance for the type of amino acid effect (p-value=0.05). But from the post-hoc analysis, there are no significant differences in the double emulsion droplet size between the double emulsions (p-values > 0.2 for all the possible combinations).

From this information, it is demonstrated that the amino acid encapsulated does not affect the double emulsion droplet size. This is in agreement with the fact that the double emulsions are in an iso-osmotic condition, so there is no net gain or loss of water in the internal phase. Hence, the increase in the droplet size might be due to coalescence or flocculation, and these types of instability are independent of the amino acid that is encapsulated in the double emulsion.

3.2.1.2 Yield of entrapped water

With respect to entrapped water, the calculation of the yield (see section 2.5) shows a fluctuation around 100% of the encapsulated water (figure 3.15) for all double emulsions. So, there is no clear change in the entrapped water volume of the double emulsion oil droplets. The fact that there is no tendency of water gain or loss in the internal phase and there is an increase of the droplet size, leads to confirm that coalescence or flocculation of oil droplets is occurring. Additionally, this also gives an indication that amino acids might be released by one of the different mechanisms of diffusion across the oil phase, which will be discussed in further sections.

![Figure 3.15 Yield of entrapped water (%) at different days of storage at 4°C.](image)
3.2.1.3 Standard curves

Standard curves of DL-alanine and L-serine are shown figures 3.16 and 3.17. The standard curves of L-glutamine and L-leucine were shown in figure 3.6 and 3.7.

![Graph showing standard curve of DL-alanine](image1)

**Figure 3.16 Standard curve of DL-alanine in external phase ten times diluted in distilled water obtained at a wavelength of 340 nm.**

![Graph showing standard curve of L-serine](image2)

**Figure 3.17 Standard curve of L-serine in external phase ten times diluted in distilled water obtained at a wavelength of 340 nm.**
3.2.1.4 Amino acids release profiles

![Figure 3.18 Released amino acid content of double emulsions as function of storage time at 4°C.](image)

From the statistical analysis, the assumption of normality was confirmed by the Kolmogorov-Smirnov test (p-value = 0.58). The assumption of equal variances was confirmed by the Levene test for the effect of days of storage (p-value = 0.96), but not for the type of amino acid (p-value < 0.001). Despite the assumption of equality of variances not being fulfilled for all the factors, if the factor in which significances are observed shows a large difference (very low p-values), the unfulfilled assumption does not affect the results in a substantial manner.

Only the main effects (type of amino acid encapsulated and days of storage) without interaction were tested, due to the limited amount of data per experiment. The type of amino acid (p-value < 0.001) encapsulated shows a significant effect in the release and there was not enough evidence to prove that the release was affected by day of storage (p-value = 0.23). From the post-hoc analysis, the only amino acid that presents a significant difference in the release compared to the others is L-leucine (L-leucine--L-glutamine, L-leucine--DL-alanine, L-leucine--L-serine, L-leucine-Blank: p-value < 0.001).

The fact that day of storage does not show a significant effect on the release, is because four of the double emulsions have a minimal or null release in the period of evaluation. So, the contribution of these values weighs enough on the statistical test to make this factor insignificant. Nevertheless, it is clear that for L-leucine release, this effect has a clear contribution, so the time of storage is significant for those amino acids that are released into the external phase.

For the extraction of the external phase of L-glutamine and L-leucine a different type of filter was used. This consisted of a polyethersulfon membrane with a pore size of 0.20 μm (Macherey Nagel,
The difference between the filters used, beside pore size, is that this one has a lower absorption of proteins, which can affect the measurements. Nevertheless, considering that the values of L-serine and DL-alanine do not change as a function of time, it is clear that the release over time is minimal. So, this factor does not have a considerable impact on the results.

The mathematical model mentioned in section 2.7 was constructed from the data of the release of amino acid (figure 3.19). From table 3.3, it can be observed that the model shows low discrepancy with the experimental results because of the low SSR obtained. In this model the parameter $C_0$ (initial concentration) is not zero because there is some amino acid release to the external phase during the preparation of the double emulsion, how this happens will be explained further on. The parameter $t_A$ is a value related to the kinetics of the release, which describes the time that it takes to have 63% of the equilibrium concentration of the amino acid in the external phase, plus the amount released during the preparation ($C_0$). As L-leucine is the one that is released faster, it has the smaller $t_A$, and as there is a considerable release during the preparation ($C_0=0.13\text{mM}$), thus, at day 12 more than 63% is already released. For the rest of the amino acids, the $t_A$ values are significantly large, hence, in the time frame of 16 days there is a minimal or not release.

Table 3.3 Parameters ($C_0$ and $t_A$) and sum of square residual (SSR) of the mathematical models of the release of amino acids.

<table>
<thead>
<tr>
<th></th>
<th>L-leucine</th>
<th>DL-alanine</th>
<th>L-serine</th>
<th>L-glutamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_0$ (mM)</td>
<td>0.133</td>
<td>0.016</td>
<td>0.017</td>
<td>0.040</td>
</tr>
<tr>
<td>$t_A$ (days)</td>
<td>11.600</td>
<td>277.418</td>
<td>855.358</td>
<td>1402.561</td>
</tr>
<tr>
<td>SSR TOTAL</td>
<td>$5.72\times10^{-3}$</td>
<td>$4.61\times10^{-5}$</td>
<td>$3.70\times10^{-5}$</td>
<td>$4.40\times10^{-4}$</td>
</tr>
</tbody>
</table>

Figure 3.19 Comparison of the adjusted model (dotted line) with experimental curves (solid line) for the concentration of L-leucine (a), L-glutamine (b), L-serine (c) and DL-alanine(d) in the external phase.
3.2.1.5 Influence of the type of amino acid

For L-leucine, from day 0, there is already a significant amount released to the external phase. In the case of L-glutamine, L-serine and DL-alanine, it is observed that the concentration in the external phase is very low at the beginning and during storage. From section 3.2.1.2, it was demonstrated that there was no net gain or loss of water in the internal phase through time. Hence, release of amino acids by rupture of the double emulsion droplets can be neglected. So, the amino acid is released by diffusion through the oil phase. This diffusion occurs despite there is an osmotic equilibrium between the internal and external phases, because in this case the release is driven by the chemical potential difference between the two water compartments (Pawlik, Cox, & Norton, 2010).

As mentioned in chapter 1, the reverse micellar transport, diffusion across a very thin lamella and hydrated surfactant transport are possible ways to explain the release of amino acids. Nevertheless, if one or more of these mechanisms were spontaneously happening, it would be expected also the release of L-glutamine, L-serine, and DL-alanine to the external phase, in a slower or at the same rate as L-leucine, depending on the pathway. So, it seems that these mechanisms are not happening during the release of L-leucine.

However, Pawlik et al. (2010) suggested that compounds that decrease the interfacial tension in double emulsions, facilitate the micellar transport of hydrated salt ions across the oil layer. And as mentioned in section 1.4, L-leucine has a significant effect on lowering the surface tension compared to the other amino acids used. So, this amino acid adsorbs at the interface of the water in oil emulsion, in order to decrease the interfacial tension. This adsorption might enable spontaneous emulsification by surfactants in the oil phase, facilitating the reverse micelle transport.

Although, it is possible that L-leucine could be released by the mechanism previously mentioned. It seems more probable that the main mechanism of release is through direct diffusion, because L-leucine have special characteristics that allows it to be released at a faster rate than the other amino acids.

As mentioned in chapter 1 (table 1.2), L-leucine is the third most hydrophobic of the amino acids that naturally occur in proteins, just below isoleucine and tryptophan. DL-alanine, L-serine and L-glutamine are less hydrophobic. In fact, the last two are considered hydrophilic compounds. In figure 3.20 the structures of the mentioned amino acids can be observed. The reason why L-leucine is the most hydrophobic is because it has the largest carbon side chain of these amino acids. DL-alanine has only a methyl group in the side chain, which provides a slight hydrophobic character. For L-serine and L-glutamine, it can be observed that they are hydrophilic because of the hydroxyl group for the former, and carbonyl and amino group for the latter, in the side chain.
Figure 3.20 Molecular structure of (a) L-leucine, (b) L-glutamine, (c) L-serine and (d) L-alanine.

Table 3.4 shows the partition coefficients of amino acids in n-octanol-water. From this information, quantitative values of the distribution of the amino acids in organic solvent and water are obtained. These values can be used as a reference of lipophilicity. For example, the value of L-leucine represents that approximately 2.5% of the amino acid will be in the organic solvent and the rest in the water phase, and in the same way 0.13%, 0.05% and 0.01% for L-alanine, L-serine and L-glutamine, respectively.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Log $P_{ow}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-leucine</td>
<td>-1.61</td>
</tr>
<tr>
<td>L-alanine</td>
<td>-2.89</td>
</tr>
<tr>
<td>L-serine</td>
<td>-3.30</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>-4.19</td>
</tr>
</tbody>
</table>

So, the hydrophobic character of L-leucine seems to be a critical characteristic for the release, because it is more soluble in oil than the rest of the amino acids. However, the degree of solubility in oil depends on the state of ionization of the amino acid, which depends on the pH of the medium.

The charge density of each amino acid in the internal phase was determined with the Henderson Hasselbalch equation, using the measured pH of the internal phases and the pKₐ values of the amino acids, that are shown in tables 3.5 and 3.6. As observed from these tables, the pH of all the internal phases is above the isoelectric point. Therefore, all amino acids are present in the ionized state. And L-leucine is shown to be the amino acid with the lowest net charge.
Table 3.5 Amino acids dissociation constants and isoelectric points at 25°C (Belitz et al., 2009).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>pK₁</th>
<th>pK₂</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-leucine</td>
<td>2.4</td>
<td>9.6</td>
<td>6.0</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>2.2</td>
<td>9.1</td>
<td>5.7</td>
</tr>
<tr>
<td>L-alanine</td>
<td>2.3</td>
<td>9.7</td>
<td>6.0</td>
</tr>
<tr>
<td>L-serine</td>
<td>2.2</td>
<td>9.2</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Table 3.6 Experimental pH values of the internal phases of the double emulsions and calculated charge density of amino acids in the internal phases

<table>
<thead>
<tr>
<th>Double emulsion</th>
<th>pH</th>
<th>Charge density (C/kg of amino acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-leucine</td>
<td>6.2</td>
<td>-176</td>
</tr>
<tr>
<td>L-alanine</td>
<td>6.4</td>
<td>-457</td>
</tr>
<tr>
<td>DL-serine</td>
<td>6.4</td>
<td>-1395</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>6.6</td>
<td>-2055</td>
</tr>
</tbody>
</table>

As amino acids are zwitterions, at the pH of the internal phase, there is an equilibrium between the anion and zwitterion. And as uncharged entrapped materials are released by simple diffusion (Florence & Whitehill, 1982), the zwitterion fraction can be released by this mechanism through the oil phase. But this means that also the uncharged fractions of the other amino acids could diffuse. Nevertheless, this is not observed in the release profile, probably because the fraction of uncharged L-leucine is much larger than the uncharged fraction of other amino acids, as the pH of the internal phase of the double emulsion with L-leucine is the one closest to the pI.

As hydrophobicity and state of ionization seem to be two important characteristics for the release of amino acids, it is also important the factors that can influence them. Carta (1998) explained that amino acids interact with salts, influencing the solubility in two possible ways. First, salt binds water molecules so there is less free water for amino acids to dissolve. Second, highly charged amino acids contribute to the solvation, competing with water and increasing their solubility. Also, Carta (1998) demonstrated that sodium chloride has a salting out effect on L-leucine. This happens because this amino acid has a low electric charge, therefore, it is incorporated in a lesser way to the solvation sheath of the salt. Furthermore, the type of salt also has an effect, because the ion interactions with water molecules, hydrocarbon backbones, side chain groups, charged amino and carboxyl groups of the amino acids are different for each electrolyte (Khoshkbarchi & Vera, 1997).

So, as the double emulsions have KCl to equilibrate the osmotic pressure, it seems that the hydrophobic character increases for L-leucine, which might promote the diffusion through the oil phase. And for the other amino acids, as they have a greater charge density, it is probable that their solubility in water increases, which might promote a negative adsorption making the release slower, in such a way that in 16 days it is difficult to observe. Therefore, it seems that the components that are used to equilibrate the osmotic pressure between the aqueous phases, can have an effect during the release of amino acids.
These results are in agreement with the ones reported by Magdassi & Garti (1984), who demonstrated that the release pattern of the electrolytes ephedrine hydrochloride and sodium chloride in double emulsions are different, where the former was released faster than the latter. Concluding that the release of entrapped electrolytes in a double emulsion is dependent on the hydrophobicity and state of hydration.

It is also important to mention, that the reason why L-leucine begins with a significantly higher concentration at the starting point and the other amino acids do not, is related with the lipophilicity, as explained previously. But also, the surface-active properties might play a role, because as L-leucine has a significant effect on lowering the surface tension compared to the other amino acids, the molecules of L-leucine will be located mainly in the interface of the W/O. So, in the primary emulsion, because of the hydrophobic properties, there will be more L-leucine in the oil phase than the other amino acids. But also, there will be a higher amount of L-leucine adsorbed in the interface, compared to the other amino acids. So, when the second homogenization is performed, the L-leucine in the oil phase and some of the adsorbed at the interface might migrate easily to the external phase due to the energy applied to the system by mechanical force.

3.2.2 Release as function of the high HLB emulsifier concentration

3.2.2.1 Double emulsion droplet size

![Figure 3.21 Mass-weighted mean double emulsion droplet size as a function of storage time at 4°C.](image)

From the statistical analysis, the non-parametric paired t-tests showed that all the oil droplet sizes of the double emulsions demonstrate a significant difference among each other (2%-1% polysorbate 80...
(p-value= 0.03), 2%-0.5% polysorbate 80 (p-value= 0.03), and 1%-0.5% polysorbate 80 (p-value= 0.03))

From figure 3.21, it is observed that the hydrophilic emulsifier has an effect on the droplet size as expected. There is a clear tendency of the droplet size to increase as the amount of emulsifier decreases. This happens because there is not enough surfactant to cover the surface of the oil droplets. Thus, the oil droplets can move closer to each other and then coalescence or flocculation can probably occur.

3.2.2.2 Yield of entrapped water

With respect to the entrapped water, the yield shows a fluctuation around 100% of the encapsulated water (figure 3.22) for all double emulsions. So, in these double emulsions as well, there is not a clear change in water content of the double emulsion droplets during storage. Hence, coalescence or flocculation of oil droplets is also happening and that is why there is an increase in the droplet size. Therefore, amino acids might be released by one of the different mechanisms of diffusion across the oil phase, similarly as in the previous section.

![Figure 3.22 Yield of entrapped water (%) at different days of storage at 4°C.](image)
3.2.2.3 Standard curve

Figure 3.23 Standard curve of L-leucine in external phase ten times diluted in distilled water obtained at a wavelength of 340 nm.

3.2.2.4 Polysorbate 80 concentration influence in the release of L-leucine

Figure 3.24 Released L-leucine content of double emulsions with different amount of polysorbate 80 as function of storage time at 4°C
From the non-parametric paired t-tests, the double emulsion with 2% polysorbate 80 series shows a significant difference with respect to the double emulsion with 1% polysorbate 80 series (p-value=0.03) and with the double emulsion with 0.5% polysorbate 80 series (p-value=0.03). There is not enough evidence to prove that the double emulsion with 1% polysorbate 80 series is different from the double emulsion with 0.5% polysorbate 80 series (p-value=0.56).

From figure 3.24, the 1% and 0.5% polysorbate 80 release curves are similar from day 0 until day 8, but on day 16 there is a large difference. As the statistical test considers the differences of all the days, the difference of the first 5 values contributes more than the difference of the last day. Therefore, the test concludes that there is no significant difference between the series of 1% and 0.5%. However, with the graphical support, this statistical result cannot be considered for making a conclusion.

As can be observed, the initial values are not the same for the three double emulsions. This might be because the level of mixing during the second homogenization was not the same for the three double emulsions, probably due to the user handling influence. So, it seems that for the double emulsion with 2% polysorbate, the level of mixing was more intense, leading to a greater release of amino acid from the beginning.

Furthermore, the release of double emulsion with 1% of polysorbate shows to have a slower rate of release compared to the others. And the last point of the series of polysorbate 0.5% is higher than expected, considering that there is no evidence of disruption of the oil droplets from the droplet size and entrapped amount of water. These two unexpected results indicate that more test should be done to verify and determine why the release patterns change in these ways.

Nevertheless, in the previous section, it was suggested that L-leucine can be released by a facilitated micellar transport and direct diffusion through the oil phase. The fact that the concentration of the high HLB influences the amino acid release, leads to the assumption that one or both transport mechanisms could be occurring and are influenced by the amount of hydrophilic emulsifier.

In the simple diffusion, as the concentration of surfactant influences the droplet size of the double emulsion, as described before, it means that also the exchange surface area of the oil droplet depends of the concentration of the hydrophilic emulsifier. Therefore, as the droplet size increases, the total exchange surface area decreases, so diffusion of the amino acid decreases. This assumption explains the difference in release profile between the double emulsion with 2 and 1% of polysorbate, but not for the one with 0.5%.

In the other hand, there is a probable formation of reverse micelles by the hydrophobic and hydrophilic surfactants in the oil phase, because both are present in a concentration above the cmc. For polysorbate 80, the cmc in water is 0.01mM (value obtained from the data sheet of the supplier), so in the oil phase this value should be lower, and for PGPR is 1.8% (Bahtz et al., 2016). So, by increasing the concentration of the hydrophilic emulsifier, the probability of forming micelles increase, therefore also the release might increase.
Nevertheless, when the PGPR concentration is above the cmc, an excessive number of molecules of this surfactant are present in the oil phase, forming micellar structures and increasing the oil phase viscosity. This increase in viscosity limits the kinetics of emulsification of the entrapped components, reducing the diffusion by micellar transport (Bahtz et al., 2016). Therefore, simple diffusion seems to be the most probable mechanism of release of L-leucine. However, depending of the amount and type of emulsifiers this mechanism could shift to the other types of release. So, further experiments are required to determine exactly which is the mechanism of release of L-leucine and what role the emulsifiers have on it.

3.2.3 Microscopy

From the microscopic observation, it was verified that all the samples were double emulsions during the period that they were evaluated, in figure 3.25 some examples can be observed. In some cases, physical changes were observed in the oil droplets or the internal droplets, like increased size, but these changes are most probably caused by the pressure created with the application of the coverslip, which led to the deformation or disruption of the globule structure (Jiao & Burgess, 2007). Therefore, the method used is not adequate to detect droplet growth, shrinkage or deformation. For further research, in order to eliminate the pressure exerted by the coverslip, the method developed by Matsumoto et al. (1976) could be applied, where they used a glass slide with a small depression at its center to contain the liquid dispersion for the microscopic observation.

Nonetheless, all the systems were double emulsions and no phase inversion occurred in the samples. Also, all double emulsions presented a high amount of internal water droplets through time. In some figures, a slight increase in the internal droplet size can be observed, but this might also be due to the pressure created by the coverslip. Therefore, it seems that the coalescence between internal droplets is minimal. Thus, the internal structure seems to be very stable, and this might explain why the amount of entrapped water remains constant through time. Also, by assuming a highly stable internal phase, there should be minimal coalescence of the internal droplets with the globules interface. That is why there is no release of L-glutamine, L-serine and DL-alanine.
Figure 3.25 Photographs of double emulsions under 40x objective lens: (a) DL-alanine double emulsions at day 0, (b) L-leucine double emulsions at day 16, (c) blank double emulsions at day 0, (d) L-glutamine double emulsion at day 0, (e) L-leucine double emulsions with 1% polysorbate 80 at day 0, (f) L-leucine double emulsions with 0.5% polysorbate 80 at days 16, (g), L-serine double emulsions at day 0 and (h) blank double emulsions at day 16.
General Conclusions

The determination of amino acids by the methodology described by Satake, et al. (1960) proved to be suitable for measuring the amount of released amino acid in W/O/W emulsions. However, the samples needed to be pre-treated, since the external phase of the double emulsions contained compounds that could affect the results, such as the hydrophilic emulsifier. So, in order to study the release of amino acids in double emulsions, a procedure was developed to reduce the effect of the surfactant for the spectrophotometry analysis. This technique consisted of centrifugation of the double emulsion, extraction of the serum phase, filtration, dilution and subsequent spectrophotometric determination.

The quantification of the amino acid concentration using the standard curve method and by the adjustment of a mathematical model, showed no significant differences between each other. For this research, the standard curve method was chosen to evaluate the release of the double emulsions, due to being more practical, as less time is required and it is easier to calculate the results with this method.

As the entrapped water volume fraction was constant during storage, the release of amino acids should be via diffusion through the oil phase. Of the five amino acids encapsulated, only L-leucine showed a clear tendency to be released to the external phase of the double emulsion during 16 days of storage at 4°C. These results indicate that in the release of amino acids, the properties of these biomolecules have an important role.

It seems that the principal mechanism of release of L-leucine is direct diffusion, because this amino acid is by far the most hydrophobic of the amino acid evaluated in this research, therefore, a much larger fraction of this amino acid is soluble in the oil phase, compared to the others. This is possible because the pH of the internal phase is close to the pI, so L-leucine is mostly in its zwitterionic form, thus, release through direct diffusion can take place. Also, due to these factors, there is an amount of release to the external phase during the preparation of the double emulsion. The amount released during this process seems to depend on the intensity of mixing during the second homogenization.

Other mechanism of release that could occur is the reverse micellar transport, but catalyzed by the surface-active properties of the amino acid. As L-leucine has a large hydrocarbon side chain, it acts as a kind of a surfactant, so the amino acid adsorbs at the interface of the water in oil emulsion. This adsorption of the amino acid might enable spontaneous emulsification by surfactants in the oil phase, facilitating the reverse micellar transport.

The concentration of polysorbate 80 in the double emulsions showed to have a significant effect in the release of amino acids. At concentrations of 2, 1 and 0.5 wt% of polysorbate 80, the measurements of entrapped water and oil droplet size did not show evidence of oil droplet disruption. So, also in these double emulsions the release of L-leucine should be through direct diffusion and/or facilitated reverse micellar transport.
The concentration of the hydrophilic emulsifier seems to have an influence on direct diffusion, because the surfactant has an effect on the droplet size, so it also affects the exchange surface area. Therefore, if the concentration of emulsifier increases, the droplet size decreases and the total exchange surface area increases, allowing a faster release rate. Nevertheless, this assumption does not explain the release profile of L-leucine for the double emulsion with the lowest concentration of polysorbate 80.

In the case of the micellar transport, as the concentrations of polysorbate 80 and PGPR are higher than the cmc, micelles can be formed easily. But also, due to the high concentration of PGPR, the viscosity in the oil increases, so the transport through reverse micelles is limited. Therefore, simple diffusion seems to be the most predominant mechanism of release of L-leucine. But depending on the composition of the double emulsion, especially on the concentration and type of emulsifiers, the mechanism of release might change.

Finally, in order to extend this research and verify that the release of amino acids in double emulsions depends on their lipophilicity, it is recommended to evaluate the release of amino acids which are more hydrophobic than leucine like phenylalanine and isoleucine, which should have faster rates of release. Also, amino acids with a lower hydrophobicity than leucine, but higher than alanine, could be considered, like valine and methionine, which should have a release profile similar to leucine. Regarding hydrophilic amino acids, the time of evaluation should be increased enough to be able to detect a release. Alternatively, the exchange kinetics could also be improved by selecting a higher temperature. Furthermore, it is important to determine which mechanism of release predominates in the release of hydrophobic and hydrophilic amino acids, in order to establish possible effects on the composition of the double emulsion with these biomolecules and how the release is affected. Last but not least, the effect of pH or of the emulsifiers used on the exchange kinetics could be evaluated in future experiments. As far as pH is concerned, exchange is thought to be favoured when the pH is close to the isoelectric point, whereas slower exchange is expected as the amino acid groups become more charged. When using protein as an emulsifier, interfacial cross-linking (e.g. by transglutaminase) might help to slow down the exchange kinetics.
References


