ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

ENCAPSULATION AND RELEASE OF AMINO ACIDS IN DOUBLE EMULSIONS

Ph.D. THESIS

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AMİNO ASİTLERİN ÇOKLU EMÜLSİYONLARDA ENKAPSÜLASYONU VE SALIMI

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Esra KOCAMAN, a Ph.D. student of ITU Graduate School student ID 506162503, successfully defended the thesis/dissertation entitled "ENCAPSULATION AND RELEASE OF AMINO ACIDS IN DOUBLE EMULSIONS ", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To those who encouraged me to fly toward my dreams,

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March 2021

Esra KOCAMAN
(Food Engineer, M.Sc.)

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ABBREVIATIONS

C	: Concentration
CLSM	: Confocal Laser Scanning Microscopy
CMC	: Critical micelle concentration
Cryo-SEM	: Cryogenic Scanning Electron Microscopy
D	: Diffusion coefficient
DLS	: Dynamic light scattering
G	: Magnetic gradient strength (T/m)
HLB	: Hydrophilic lipophilic balance
HOSO	: High oleic sunflower oil
LCT	: Long Chain Triglyceride
MCT	: Medium chain triglyceride
MF	: Microfluidizer
MW	: Molecular weight
NMR	: Nuclear Magnetic Resonance
O/W/O	: Oil-in-water-in-oil emulsion
p	: Probability Value
PC	: Principal component
ppm	: Parts per million
PCA	: Principal component analysis
PGPR	: Polyglycerol polyricinoleate
SGF	: Simulated gastric fluid
SLS	: Static light scattering
SIF	: Simulated intestinal fluid
SDS	: Sodium dodecyl sulfate
TNBS	: 2,4,6-trinitrobenzenesulfonic acid
T2	: Transverse (spin-spin) relaxation time
UT	: Ultra-Turrax
XG	: Xanthan gum
W1	: Internal water phase
W2	: External water phase
W/O	: Water-in-oil
O/W	: Oil-in-water
W/O/W	: Water-in-oil-in-water

SYMBOLS

C_0	: Initial concentration in the external water phase
C_e	: Equilibrium concentration in external water phase
D_{43}	: Volume weighted average droplet diameter (m)
g	: Gravitational acceleration (9.81 m/s^2)
Log P_{ow}	: Partition coefficient (-)
t_a	: Kinetic constant (s)
pK	: Logarithm of ionization constant (-)
PI	: Isoelectric point (-)
T	: Temperature (K)
t	: Storage period (s)
r	: Correlation coefficient (-)
Δ	: Diffusion delay (s)
η	: Viscosity (Pa.s)
I	: Echo intensity
I_0	: Echo intensity without magnetic gradient
K	: Flow consistency index ($Pa \cdot s^n$)
k_b	: Boltzmann constant ($1.381 \cdot 10^{-23}$ J/K)
R_h	: Hydrodynamic radius (m)
$\Delta G°_{tr}$	: Free energy change (J/mol)

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ENCAPSULATION AND RELEASE OF AMINO ACIDS IN DOUBLE EMULSIONS

SUMMARY

Double emulsions have been studied for many years, given their potential as encapsulation systems. It is also possible to control the release of diverse bioactive components by means of double emulsions. As amino acids might be degraded to some extent due to environmental factors such as pH, temperature, light exposure as well as some reactions (i.e. oxidation, Maillard), their encapsulation may be advantageous to avoid these issues. Besides, encapsulation may enable to release of these compounds in a later stage of the gastro-intestinal tract.

The main research question of our research project was to what extent the release of encapsulated components from double emulsions can be controlled by the emulsification method, emulsion composition and environmental factors. Moreover, it was evaluated whether the release kinetics were substantially influenced by the molecular properties of the encapsulated compounds. Hence, this thesis studies the influence of some parameters on double emulsion stability as well as amino acid encapsulation and release in double emulsions. The current study consist of the evaluation of these parameters: solute characteristics (i.e hydrophobicity, molar mass) and concentration, pH of the aqueous phases, hydrophobic and hydrophilic emulsifier, homogenization and thickener. For the investigation of the effect of these parameters, the emulsion droplet size, and the entrapped water volume fraction were evaluated to characterize the double emulsions. Moreover, the release of amino acids was observed during storage using spectrophotometric and Nuclear Magnetic Resonance (NMR) techniques.

A modification of the original method was performed to enable the optimum conditions for amino acid quantification (**section 4.1**). Due to the high background absorbance of the reagent 2,4,6-trinitrobenzenesulfonic acid (TNBS) which was the case for many of the measured concentrations, different TNBS concentrations were evaluated in order to determine the optimum concentration. Hence, the solution containing 0.6 mM TNBS was choosen as it demonstrated the lowest absorbance among the studied concentrations as a blank and the TNBS solution reacted with leucine. As the absorbance was not substantially changed after 3 hours, it was used as the reaction time.

In **section 4.2**, the effect of solute characteristics on double emulsion stability and release of encapsulated compounds were presented. Different amino acids (i.e. hydrophilic and hydrophobic) were used to investigate the hydrophobicity effect at different temperatures. Also, di-peptides were used as encapsulated compound in order to evaluate the influence of molecular mass. The results showed that an increase was observed from 50 up to 90 μm in the average droplet size for the samples homogenized with Ultra-turrax at 17500 rpm within the 32 days time frame. The double emulsions at 4 °C indicated a higher increase in average droplet size as compared to 37 °C. To investigate the main instability mechanism in the emulsion, double emulsions were

diluted with sodium dodecyl sulfate (SDS) before laser diffraction measurement. The measurement of the droplet size in the presence of SDS showed that flocculation was the main instability mechanism, which caused an increase in droplet size. On the other hand, a constant enclosed water volume fraction was found in double emulsions during 16 days of storage, independent from the temperature and hydrophobicity studied in this thesis. The encapsulation efficiency of amino acids in the inner water droplets was found to be higher than 80% in all cases. From the release results, amino acid hydrophobicity and storage temperature were found to largely influence the release rate of the encapsulated amino acids. The amino acid release rates were fastest at 37 °C, which was the highest temperature examined in this section of the thesis. This can be explained by the higher solubility as well as increased diffusion rate of amino acids in the intermediate phase. Also, an increase was observed in the release rates of amino acids as a result of higher hydrophobicity. The significant effects of hydrophobicity and temperature, as well as the constant enclosed water volume revealed that the release of amino acids from the inner to the outer water phase was mainly governed by a direct diffusion mechanism. As the di-peptides released faster than the amino acids, it follows that the increased solubility overruled the effect from the decreased diffusion coefficient of the dissolved compound in the oil phase.

In **section 4.3**, the influence of solute concentration (i.e. 5, 10, 20 and 40 mM) on the release and double emulsion stability was investigated. The varying concentrations of amino acid did not cause a significant difference in the increase of volume weighed droplet size during 16 days. The entrapped water volume was stable for double emulsions that contained varying solute concentrations except from the double emulsion which contained 40 mM where a decrease was observed through 16 days of storage. This can be a result of the faster diffusion velocity of the amino acid across the oil phase to the external water phase as compared to the diffusion of potassium chloride (KCl) through the oil phase to the internal water phase. Hence, a fraction of the internal phase was expelled to the external water phase to equalize the osmotic pressure which resulted in a decrease in yield of entrapped water volume. Regarding the average residence time (t_a) values, the double emulsion that contained the highest solute concentration studied (i.e. 40 mM) in this thesis indicated a faster release as compared to the other samples at 37°C, whereas there was no significant difference among the samples at 4°C.

The pH effect of the aqueous phases on the release of amino acids and di-peptides was evaluated in **section 4.4**. Regarding the average droplet size, there was no significant difference between samples as a function of pH of the aqueous phases. Considering the release, the transport of the amino acids and di-peptides was faster at neutral pH as compared to acidic and basic pH values, which was thought to be due to the increased solute solubility in the oil phase for the zwitterionic (rather than ionic) form of the more hydrophobic molecules at neutral pH.

The oil type effect on amino acid release and double emulsion stability was demonstrated in **section 4.5** comparing long chain and middle chain triglycerides. The average droplet size of the long chain triglyceride (LCT) containing double emulsions were larger than of the medium chain triglyceride (MCT) containing samples. This can be due to the stronger aggregation of LCT containing samples as a consequence of the higher viscosity of the LCT oil. From the release results, much faster transport of L-leucine was observed through MCT oil as compared to LCT oil due to its higher solubility. Also, the lower viscosity of MCT oil gives rise to a higher diffusivity of dissolved compounds, which may also fasten molecular transport.

In **section 4.6**, the influence of the hydrophobic emulsifier concentration (from 1 to 5%) on the double emulsion stability and release of entrapped amino acids was demonstrated. The entrapped water volume fraction of the polyglycerol polyricinoleate (PGPR) stabilized samples remained around 100% during 32 days of storage, except from the one with only 1% PGPR which had a decreasing yield due to insufficient stabilisation of the internal water droplets. It follows that the use of higher concentrations of PGPR enabled the entrapped water volume to remain constant, whereas a PGPR concentration below the critical micelle concentration (CMC) caused a water flux from the internal to the external phase. The average residence time (t_a) of enclosed L-leucine among the PGPR stabilized double emulsions was lowest at the highest PGPR concentration, which indicates the faster release of L-leucine in the presence of an excess of reverse PGPR micelles in the oil phase.

The effect of partial replacement of PGPR by native and phosphatidylcholine (PC) depleted lecithin on double emulsion stability and amino acid release was shown in **section 4.7**. Although a droplet size increase was observed in the PGPR-stabilised double emulsions during storage, the use of 5% of a PGPR-native lecithin (1/1) mixture resulted in a constant droplet size during storage. The used PGPR and PC-depleted lecithin concentration influenced the droplet size of the double emulsions. The lowest droplet size was about 30 µm just after preparation and during storage in double emulsions containing 5% PC-depleted lecithin. This indicates that partial replacement of PGPR can be beneficial in terms of stability of the double emulsion droplet size. Considering the entrapped water volume, the inclusion of PC-depleted lecithin could not facilitate to overcome the instability at too low (i.e. less than 2% in this case) PGPR concentration. In fact, lecithin addition had a negative impact on the etrapped water volume fraction. The average residence time t_a, on the other hand, was much lower in PC-depleted lecithin-containing double emulsions as compared to the emulsions with only PGPR.

The effect of hydrophilic emulsifier concentration on amino acid release and double emulsion stability was investigated (**section 4.8**). It was found that the use of a higher Tween 80 concentration facilitated a less pronounced increase in average droplet size during storage. The use of less than 2% Tween 80 concentration seemed to be insufficient to cover the interface between oil and outer aqueous phase. A constant entrapped water volume fraction was obtained during storage regardless of the Tween 80 concentration. Differences in Tween 80 concentration, varying from 0.5 to 2.0%, did not change the release kinetics to a large extent.

In **section 4.9**, the influence of microfluidization (at 0.75 and 1.00 bar of driving compressed air pressure) and rotor stator homogenization treatment (at 17500, 21500 and 24000 rpm of Ultra-turrax) and the presence of xanthan gum were investigated. Considering the particle size distribution, multimodal and monomodal particle size distributions were observed for microfluidized double emulsions and those prepared by rotor stator homogenization treatment, respectively. The inclusion of xanthan gum decreased the size of the oil droplets, which resulted from the decreased viscosity ratio between the oil and the aqueous phase. Also, an increased homogenization intensity induced a decreased droplet size, resulting from the higher shear stress applied to the fluid. The entrapped water volume fraction was about 90% for all double emulsions prepared with rotor stator homogenization treatment and without xanthan gum. As the cream and serum layers of the double emulsions stabilized with xanthan gum were not separated during 2 hours of analytical centrifugation, the reliable estimation of the enclosed water volume fraction was troublesome. The release rate of L-leucine in

double emulsions prepared with rotor stator homogenization treatment was proportional with the homogenization level, which can be explained from the smaller droplet size: a faster release rate was observed at higher homogenization intensity as a result of a smaller droplet size. Xanthan gum addition remarkably increased the release rate of L-leucine, which was thought to be due to the smaller droplet size.

Preliminary gastrointestinal tests indicated that double emulsion encapsulation provided a gradual release of amino acids in the gastrointestinal environment (**section 4.10**). The release of amino acids might be governed by diffusion in the gastric environment, whereas the oil digestion can change this mechanism as well as the relase rate. The smaller droplets obtained after intestinal digestion was likely due to the triglycerides hydrolysis which resulted in the disruption of the oil phase and hence release of encapsulated amino acid.

In **section 4.11**, the release of L-phenylalanine was investigated by means of high resolution NMR diffusometry. As the first and last decay profile of water overlapped, it follows that the enclosed water volume fraction remained constant during incubation (at 30 and 50 °C). Moreover, a slower amino acid diffusion coefficient was obtained in the external water phase as compared to the internal water phase (i.e. before emulsification). This might be due to the presence of xanthan gum in the external (but not in the internal) water phase, which restricts the thermal motion of the amino acids, and hence the diffusion behaviour. The diffusion behaviour of L-phenylalanine in double emulsions exhibited a typical bi-exponential decay, which enabled to discriminate between encapsulated (slowly diffusing due to restriction in a spherical confinement) and released (fast diffusing due to the absence of confinements) amino acid. Whereas the main purpose of the experiment was to enable a more detailed investigation of the influence of the incubation temperature, a clear conclusion was hampered by the extensive release before the start of the NMR experiment.

This research enables a better insight to understand the influence of molecular properties and double emulsion composition on the release kinetics. From a practical point of view, our results provide guidance in the design of colloidal systems for the encapsulation and controlled release for nutritional applications. In order to extend this study, the double emulsions containing amino acids can be incorporated in the food matrix or drugs.

AMİNO ASİTLERİN ÇOKLU EMÜLSİYONLARDA ENKAPSÜLASYONU VE SALIMI

ÖZET

Çeşitli biyoaktif bileşiklerin enkapsülasyonu ve kontrollü salımını sağlamak amacıyla, çoklu emülsiyonlar uzun yıllardır incelenmektedir. Amino asitler, pH, sıcaklık, ışık gibi bazı çevresel faktörlerin yanı sıra oksidasyon ve Maillard reaksiyonu gibi bazı tepkimeler nedeniyle degradasyona uğrayabilmektedir. Enkapsülasyon işlemi ile bu problemlerin önüne geçmek mümkündür. Ayrıca enkapsülasyon biyoaktif bileşiklerin mide-bağırsak sisteminde daha geç salımını sağlayabilmektedir.

Bu tezin konusu; hedef bileşenlerin çoklu emülsiyonlardan salımına emülsiyon hazırlama tekniği ve parametreleri, emülsiyon bileşimi ve çevresel faktörler gibi etkilerin araştırılmasıdır. Ayrıca, salım kinetiğinin hedef bileşiklerin moleküler özelliklerinden ne ölçüde etkilendiği değerlendirilmiştir. Bu faktörlerin çoklu emülsiyon stabilitesini nasıl etkilediği de araştırılmıştır. Mevcut çalışmada; enkapsüle edilen maddenin özellikleri (hidrofobisite, moleküler ağırlık) ve konsantrasyonu, sulu fazların pH değeri, hidrofobik ve hidrofilik emülgatör türü ve konsantrasyonu, homojenizasyon türü/hızı ve koyulaştırıcı ajan etkileri değerlendirilmiştir. Bu parametrelerin etkilerinin araştırılmasında, emülsiyon damlacık boyutu ve enkapsüle edilen su hacmi fraksiyonu incelenmiştir. Yağ damlacıkları ayrıca ışık mikroskobu ile gözlenmiştir. Amino asitlerin salımı, spektrofotometrik ve Nükleer Manyetik Rezonans (NMR) teknikleri kullanılarak araştırılmıştır.

Amino asitlerin konsantrasyon ölçümü için deneydeki optimum koşulları sağlamak amacıyla orijinal spektrofotometrik yöntemde bazı modifikasyonlar yapılmıştır (bölüm 4.1). Örneklerin yüksek absorbansı nedeniyle, optimum 2,4,6-trinitrobenzensulfonik asit çözeltisi (TNBS) konsantrasyonunu belirlemek amacıyla farklı TNBS konsantrasyonları değerlendirilmiştir. Sonuç olarak 0.6 mM TNBS çözeltisi, incelenen konsantrasyonlar arasında hem kör örnek hem de amino asit ile reaksiyona giren TNBS çözeltisi olarak en düşük absorbans değerini gösterdiğinden, optimum TNBS konsantrasyonu olarak seçilmiştir. Reaksiyonun üçüncü saati sonrasında absorbans değeri büyük ölçüde sabit kaldığından, reaksiyon süresi 3 saat olarak belirlenmiştir.

Bölüm 4.2'de, enkapsüle edilen madde özelliklerinin çoklu emülsiyon stabilitesi ve salıma etkisi incelenmiştir. Farklı sıcaklıklar dikkate alınarak hidrofobisite etkisinin araştırılmasında çeşitli amino asitler (hidrofilik ve hidrofobik) kullanılmıştır. Ayrıca, moleküler kütle etkisini değerlendirmek için enkapsülasyon bileşiği olarak di-peptitler kullanılmıştır. Emülsiyonların hazırlanmasında 17500 rpm'de Ultra-turrax ile homojenize edilen numunelerde ortalama damlacık boyutu 32 gün depolama süresinde 50'den 90 μm'ye artış göstermiştir. Damlacık boyutundaki artış 4 °C depolama sıcaklığında, 37 °C'de depolananlara kıyasla daha fazla olarak bulunmuştur. Emülsiyondaki esas kararsızlık mekanizmasını araştırmak için çoklu emülsiyonlar, partikül boyutu ölçümünden önce sodyum dodesil sülfat (SDS) ile seyreltilmiştir. SDS varlığında damlacık boyutununda önemli bir azalma gözlenmiştir. Bu durum, çoklu

emülsiyonlardaki esas kararsızlık mekanizmasının geri dönüşümlü bir destabilizasyon olan flokülasyon olduğunu göstermiştir. Öte yandan, sıcaklık etkisinin (4-37 °C) enkapsüle edilen iç su faz hacminde 16 günlük depolama süresince önemli bir değişikliğe neden olmadığı gözlenmiştir. İç su fazı damlacıklarında enkapsüle edilen amino asitlerin verimi tüm koşullarda %80'den daha yüksek bulunmuştur. Amino asit hidrofobisitesinin ve depolama sıcaklığının enkapsüle edilmiş amino asitlerin salım hızını büyük ölçüde etkilediği bulunmuştur. Amino asit salım hızının bu tezde incelenen en yüksek sıcaklık olan 37 °C'de en hızlı olduğu gözlenmiştir. Bu durum, amino asitlerin yağ fazında yüksek çözünürlüğünün yanı sıra artan difüzyon hızıyla açıklanabilir. Ayrıca amino asidin hidrofobisitesi arttıkça, salım hızında bir artış gözlenmiştir. Hidrofobisite ve sıcaklığın önemli etkileri ve sabit iç su fazı hacmi, amino asitlerin iç fazdan dış su fazına salımının bir doğrudan difüzyon mekanizması olduğunu göstermiştir. Enkapsüle edilen bileşiğin hidrofobisitesi ve moleküler ağırlığının salım hızına etkisinin değerlendirilmesinde amino asit ve di-peptidler karşılaştırılmıştır. Di-peptidlerin daha yüksek moleküler ağırlıklarına rağmen amino asitlere göre daha hızlı salımı, hidrofobisitenin moleküler ağırlığa göre daha önemli bir etki olduğunu göstemiştir.

Bölüm 4.3'te, enkapsüle edilen madde konsantrasyonunun (5, 10, 20 ve 40 mM) emülsiyon stabilitesi ve salıma etkisi araştırılmıştır. Farklı amino asit konsantrasyonlarının, 16 gün boyunca damlacık boyutunda önemli bir artışa neden olmadığı gözlenmiştir. Enkapsüle edilmiş iç su hacminin, bu çalışmadaki en yüksek konsantrasyon olan 40 mM amino asit içeren çoklu emülsiyon haricinde, sabit olduğu gözlenmiştir. Yüksek amino asit konsantrasyonu kullanımında (40 mM) 16 günlük bir depolama süresinde iç su fazında azalma gözlenmiştir. Bunun durum amino asidin, ozmotik ajan olarak kullanılan potasyum klorür tuzuna (KCl) kıyasla daha hızlı difüze olmasıyla açıklanabilir. KCl'nin amino asit salımına karşın ozmotik basıncı dengelemede daha yavaş olması, iç su fazının bir kısmının dışarı akışına neden olmaktadır. Amino asitlerin iç su fazında ortalama kalma süresi (t_a) değerleri ile ilgili olarak, en yüksek amino asit konsantrasyonu içeren çoklu emülsiyon (40 mM), 37 ° C'de diğer numunelere kıyasla daha hızlı salım göstermiştir, 4 °C 'de ise örnekler arasında önemli bir farklılık gözlenmemiştir.

Bölüm 4.4'te sulu fazların pH'ının salım ve stabilite üzerine etkisi değerlendirilmiştir. Amino asit ve di-peptitlerin salımı, nötr pH'ta asidik ve bazik pH değerlerine kıyasla daha hızlı olarak bulunmuştur. Salımın nötr pH'ta daha hızlı olması, zwitteriyonik formdaki moleküllerin yağdaki çözünürlük ve difüzyon hızının iyonik formdaki moleküllere göre daha fazla olmasından kaynaklanmaktadır.

Yağ türünün amino asit salımı ve çoklu emülsiyon stabilitesine etkisinin incelenmesi amacıyla uzun ve orta zincirli trigliseritler kullanılmıştır (**bölüm 4.5**). Damlacık boyutunun uzun zincirli trigliseritler (LCT) ile stabilize edilen çoklu emülsiyonlarda, orta zincirli trigliseritler (MCT) ile stabilize edilenlere kıyasla daha büyük damlacık boyutu izlenmiştir. Bu durum LCT ile stabilize edilen emülsiyonlarda daha fazla agregasyon olmasıyla ilişkilendirilmiştir. Amino asidin MCT'deki çözünürlüğünün LCT'dekine kıyasla fazla olması, L-lösinin MCT'de çok daha hızlı taşınmasına neden olmuştur. Ayrıca, MCT türündeki yağın daha düşük viskozitesi nedeniyle, çözünmüş bileşiklerin bu yağ içerisinde daha yüksek bir yayılma ve taşınım gösterdiği düşünülmektedir.

Bölüm 4.6'da, hidrofobik emülgatör polygliserol polirisinoleat (PGPR) konsantrasyonunun (%1-5) stabilite ve salıma etkisi incelenmiştir. PGPR ile stabilize

edilmiş örneklerin enkapsüle edilmiş su fraksiyonu, %1 PGPR kullanılanlar hariç %100'e yakın olarak bulunmuştur. Çoklu emülsiyonlardan %1 PGPR içeren örnekler, yetersiz stabilizasyon nedeniyle 32 günlük bir depolama süresinde iç su fazı fraksiyonlarında %50'ye kadar azalma göstermiştir. Dış faz yönüne su akışına neden olan yetersiz su enkapsülasyonu, stabiliteyi sağlamak için gereken minimum emülgatör konsantrasyonunun (CMC) kullanılmamasıyla açıklanabilir. Ayrıca, PGPR ile stabilize edilmiş çoklu emülsiyonlar arasında en düşük kinetik salım sabiti (t_a) en yüksek PGPR konsantrasyonu kullanımında izlenmiştir. Bu durum yüksek emülgatör konsantrasyonu kullanımının yağ fazında ters PGPR misellerini oluşturması ve L-lösinin daha hızlı salımıyla açıklanabilir.

Hidrofobik emülgatör PGPR'a alternatif olarak doğal ve fosfotidilkolini (PC) azaltılmış lesitin kullanımın emülsiyon stabilitesi ve salıma etkisi **bölüm 4.7**'de incelenmiştir. PGPR ile stabilize edilmiş çoklu emülsiyonlarda depolama esnasında damlacık boyutunda artış gözlemlenmesine rağmen, %5 PGPR-doğal lesitin (1/1) karışımının kullanılması sabit damlacık boyutu elde edilmesini sağlamıştır. PGPR ve PC-azaltılmış lesitin kullanımı, çoklu emülsiyonların damlacık boyutunu önemli derecede etkilemiştir. En düşük damlacık boyutu, %5 PC azaltılmış lesitin içeren emülsiyonlarda bulunmuştur (30 μm). Bu sonuç PC azaltılmış lesitinin PGPR yerine kullanımın çoklu emülsiyon damlacık boyutunun stabilitesi açısından faydalı olabileceğini göstermektedir. Enkapsüle edilen iç su hacmi göz önüne alındığında, PC azaltılmış lesitinin çok düşük PGPR konsantrasyonları ile birlikte kullanımında (<%2) stabil bir emülsiyon elde edilememiştir. Lesitin ilavesi, enkapsüle edilmiş iç su hacmi fraksiyonunu olumsuz etkilemiştir. Ayrıca, amino asitlerin iç su fazında ortalama kalış süresi t_a, sadece PGPR ile stabilize edilmiş emülsiyonlarda, PC-azaltılmış lesitin içerenlere göre daha yüksek olarak bulunmuştur.

Hidrofilik emülgatör (Tween 80) konsantrasyonunun etkisi **bölüm 4.8**'de araştırılmıştır. Daha yüksek Tween 80 konsantrasyonu kullanımı (2%), depolama sırasında ortalama damlacık boyutunun daha stabil olmasını sağlamıştır. Daha düşük Tween 80 kullanımının daha düşük stabilite göstermesinin sebebi, yağ ve dış su fazı yüzeyinin emülgatör miselleri ile yeterince kaplanamamasıyla açıklanabilir. Depolama sırasında, Tween 80 konsantrasyonunun enkapsüle edilmiş iç su hacminde önemli bir değişikliğe neden olmadığı gözlenmiştir. % 0,5-% 2 arasında değişen Tween 80 konsantrasyonu, salım kinetiğini de önemli ölçüde etkilememiştir.

Bölüm 4.9'da, emülsiyon stabilitesi ve salıma mikro-akışkanlaştırma (0.75 ve 1.00 bar) ve rotor-stator homojenizasyon tekniklerinin (Ultra-turrax ile 17500, 21500 ve 24000 rpm) etkisi incelenmiştir. Ayrıca kıvam artırıcı bir ajan olan ksantan gam etkisi incelenmiştir. Partikül boyut dağılımı göz önüne alındığında, mikro-akışkanlaştırma ile homojenize edilmiş emülsiyonlar için çok modlu bir dağılım gözlenirken, rotor-stator homojenizasyon tekniği ile hazırlanmış olanlar için tek modlu bir dağılım gözlenmiştir. Ksantan gamın eklenmesi ile yağ damlacıklarının boyutunda önemli ölçüde bir azalma gözlenmiştir. Bu durumun ksantan gamın su ve yağ fazı arasındaki vizkozite farkını azaltması ile sağlandığı düşünülmektedir. Enkapsüle edilmiş su hacmi fraksiyonu, rotor-stator homojenizasyon tekniği ile hazırlanmış ve ksantan gam içermeyen tüm çoklu emülsiyonlar için yaklaşık % 90 olarak bulunmuştur. Ksantan gam ile stabilize edilen çoklu emülsiyonların krema ve serum katmanları, 2 saatlik analitik santrifüj sırasında ayrılmadığından, iç su hacmi fraksiyonu tam olarak bulunamamıştır. Rotor-stator homojenizasyon yöntemi ile hazırlanan çoklu emülsiyonlarda L-lösinin salım hızı, homojenizasyon seviyesi ile doğru orantılı olarak bulunmuştur. Yüksek homojenizasyon derecesinde daha küçük damlacık boyutu, daha

hızlı bir salıma neden olmuştur. Ksantan gam ilavesi, daha küçük damlacık boyutu elde edilmesi sebebiyle L-lösin salım hızını önemli ölçüde artırmıştır.

Gastrointestinal testler, çoklu emülsiyon ile enkapsülasyon yönteminin gastrointestinal ortamda aşamalı bir amino asit salımı sağladığını göstermiştir (**bölüm 4.10**). Amino asitlerin salımının mide ortamında büyük ölçüde difüzyonla sağlandığı ancak bağırsakta bu mekanizmanın yağların sindirimi ile değiştiği düşünülmektedir. Bağırsak sindirimi sonunda elde edilen damlacıkların boyutu, mide sindirimi sonrasında elde edilen damlacıklara göre daha küçük olduğu gözlenmiştir. Bu durum trigliseridlerin bağırsakta hidrolize olmasıyla açıklanabilir. Ayrıca bağırsak ortamında mideye göre daha hızlı gerçekleşen amino asit salımı, emülsiyondaki ara faz olan yağın sindirimi sonucunda enkapsüle edilmiş amino asitlerin dış faza dağılması ile açıklanabilir.

Bölüm 4.11'de, L-fenilalanin amino asidinin çoklu emülsiyon içindeki salımı yüksek çözünürlüklü NMR difüzometresi vasıtasıyla izlenmiştir. Suyun ilk ve son şiddet profillerinin örtüştüğü gözlenmiştir. Bu durum enkapsüle edilmiş iç su hacminin 30 ve 50 ° C inkübasyon sıcaklıklarında sabit kaldığını göstermiştir. Ayrıca dış su fazında, enkapsüle edilmemiş iç su fazına kıyasla daha düşük bir amino asit difüzyon katsayısı bulunmuştur. Bunun nedeninin dış su fazına eklenen ksantan gamın bu fazdaki difüzyonu yavaşlatması olarak düşünülmektedir. Çoklu emülsiyondaki L-fenilalaninin difüzyon davranışı, enkapsüle edilmiş (küresel bir sınırda kısıtlanmış) ve salınan (sınırlama olmaması sebebiyle daha hızlı difüzyon gösteren) fraksiyonlarından yararlanılarak bulunmuştur. L-fenilalaninin çoklu emülsiyondaki difüzyon davranışı tipik bir çift eksponansiyel azalma göstermiştir. Sıcaklığın salım hızına etkisi, amino asidin ölçüm öncesi büyük ölçüde salımı sebebiyle izlenememiştir.

Bu çalışma emülsiyon içeriği ve enkapsüle edilen maddenin moleküler özelliklerinin salım kinetiği üzerindeki etkilerini incelemektedir. Bu tezin, biyoaktif bileşenlerin enkapsülasyonu ve kontrollü salımı için geliştirilebilecek kolloidal sistemlerin dizaynında faydalı olabileceği düşünülmektedir. Bu çalışmayı genişletmek amacıyla, çoklu emülsiyonlarda enkapsüle edilen amino asitler ilaç ya da gıda matrisine dahil edilip salım davranışları incelenebilir.

1. INTRODUCTION

Double emulsions may be used for protection and controlled release of water soluble compounds (Tamnak *et al*., 2016; Jiménez-Colmenero, 2013). Various bioactive substances such as vitamins (Benichou *et al*., 2007; Muschiolik *et. al*., 2006), microorganisms, amino acids (Weiss *et al.,* 2005) and minerals (Bonnet *et al*., 2009) can be entrapped in double emulsions.

The oil phase has as role to seperate the water compartments in double emulsions. This barrier has a full or semipermeable character depending on the conditions (Garti and Lutz, 2004). Different applications have been suggested concerning the release of bioactive compounds. First, functional components can be entrapped to prevent chemical degradation as a result of environmental factors. Also, various bioactive compounds can be encapsulated to enable a controlled release in gastrointestinal conditions. Besides, incompatible compounds can be also included in a similar system to avoid their reaction. Finally, hydrophilic compounds with undesired flavor characteristics can be also encapsulated in double emulsions in order to mask these sensorial properties (Jiménez-Colmenero, 2013).

In this thesis, amino acids were encapsulated due to their sensitivity towards environmental effects such as temperature, light exposure and pH. Furthermore, amino acids may participate to some reactions, such as Maillard reactions or oxidation (Unger and Holzgrabe, 2018). Hence, their encapsulation in double emulsions may enable their protection againts undesired factors. The formation of double emulsions with high encapsulation efficiency and long term stability is still a difficult matter (Ding *et al*., 2019). Also, double emulsion stability as well as the release of the encapsulated compounds is dependent on the properties of the water soluble compound and on environmental factors such as pH and temperature (Magdassi and Garti, 1984; Fechner *et al*., 2007). Therefore, the influence of temperature and pH as well as of the hydrophobicity of the entrapped compound was investigated in this study. For the evaluation of the effect of hydrophobicity on the release rates, hydrophilic and hydrophobic amino acids were used.

Most small neutral and drug molecules are transported passively across the membrane. Passive transport is a type of transport in which solutes move along their respective concentration gradients, which means that the solutes tend to migrate from a zone of higher concentration to a zone of lower concentration (Ouyang and Smith, 2015). Passive diffusion depends to a large extent on three independent physicochemical properties, i.e. hydrophobicity, polarity and molecular size (Camenisch *et al.*, 1997). Hence, the effects of the concentration and molecular properties of the entrapped solute and pH of the aqueous phase on the permeation across the oil layer were examined in this study. For the evaluation of molecular properties of encapsulated compounds, di-peptides were used.

The most commonly used low-HLB emulsifier in the preparation of liquid W/O emulsions and subsequent W1/O/W2 emulsions for potential food applications is polyglycerol polyricinoleate (PGPR). However, the use of PGPR should be avoided because it can lead to off-tastes and is also subjected to legal restrictions (Akhtar and Dickinson, 2001). Hence, the effect of the hydrophobic emulsifier type/concentration on the retention and release kinetics of entrapped hydrophilic compounds in double emulsions was studied.

Besides the hydrophobic emulsifiers, the hydrophilic emulsifier also plays a significant role on the structure of the aqueous phase (Muschiolik and Dickinson, 2017). As synthetic hydrophilic emulsifiers, polysorbates are commonly used in food products such as whipped cream, non-dairy cream and ice cream. Additionally, Tween 80 is widely used in the fabrication of food grade nanocapsules (Esmaeili and Gholami, 2015). Due to increasing concerns about possible negative aspects of synthetic emulsifiers, their replacement or at least reduction is encouraged. Hydrocolloids (such as gum arabic, xanthan gum, or sodium alginate), on the other hand, are commonly used in the aqueous phase of emulsions to prevent or minimise creaming phenomena. Considering these issues, the effect of xanthan gum on the stability and release of entrapped amino acids were observed.

The stability of emulsions is strongly affected by the hydrophobicity of the oil phase and the molecular volume of the oil. Using PGPR as emulsifier, it was found that long chain triglycerides (soybean oil) formed better emulsions than medium chain triglycerides, whereas the opposite was true for PC-depleted lecithin (Benichou *et al.*,

2001). To evaluate the influence of the oil type, medium chain triglycerides (MCT) and long chain triglicerides (LCT) were compared.

The droplet size (distribution) is one of the most significant parameters of colloidal dispersions as it influences the stability, appearance, shelf life and texture of emulsions. The droplet size is critically affected by the emulsification conditions (i.e shear stress). The emulsion stability therefore can be influenced by various parameters such as the interfacial tension between the phases, the emulsification method, as well as the time and pressure of high-pressure emulsification.

Double emulsions has been suggested as an encapsulation technique in food and pharmaceutical industries. The release of the bioactive components in a controlled manner makes double emulsions a potential delivery system. The digestion studies can be performed to test the degree of the protection of the specific compounds against the gastric juices (Muschiolik and Dickinson, 2017). In order to evaluate whether the amino acid release can be delayed in gastrointestinal system, double emulsions contained amino acid was exposed to gastric and intestinal fluids.

NMR methods have been widely used to examine the molecular exchange and particle size of single and double emulsions (van Duynhoven *et al.*, 2002; Bernewitz *et al.*, 2011). In comparison with other methodologies, NMR is considered as a more user friendly technique. In addition, it is a nondestructive technique (Bernewitz *et al.*, 2011). By means of NMR diffusometry, the residence time of encapsulated molecules in the internal aqueous phase can be found while the bulk composition of double emulsions (i.e. relative contribution of internal and external aqueous phase as well as oil phase) can be determined using relaxometry. The relative contribution of the inner and outer aqueous phases was determined by the method of Vermeir *et al.* (2014) using T_2 relaxometry. Additionally, the NMR diffusometry technique is used for the investigation of the inner water droplet size. In this thesis, the diffusion behaviour of amino acids and enclosed water phase was observed using high resolution H-NMR.

Due to the suboptimal quality and/or quantity of foods, enhancing their nutritional and physiological value by enrichment with specific compounds has been a popular research topic during the last years. Some examples include foods fortified with vitamins, minerals, probiotics, or fiber. As some health promoting compounds may have an undesirable taste, their encapsulation is a hot topic. Besides, encapsulation of

functional ingredients is also investigated to enable a slower release within the gastro-intestinal tract, which may prevent undesired reactions (e.g. at the low pH in the stomach) or can also stimulate satiety, which is a desirable property to overcome obesity. The current research project focuses on the encapsulation (and subsequent release) of amino acids (and derived small peptides) within the internal aqueous phase of double emulsions. Amino acids were selected as they are essential nutrients as building blocks of proteins (Belitz et al., 2004). Especially the branched-chain amino acids such as valine, leucine, and isoleucine, might be interesting for functional food applications as many nutraceutical effects have been reported, such as fatigue reduction during physical activities and blood sugar level regulation. Thus, supplements and drinks containing amino acids have become popular for sportive consumers (Kimball and Jefferson, 2006; Shimomura et al., 2006). As the inclusion of free amino acids as well as peptides in foods is challenging due to their possible chemical degradation, the double emulsion encapsulation technique is a promising approach to protect them (e.g. against the strongly acidic pH in the stomach) and to control the release of the enclosed amino acids or peptides. Besides the above-mentioned nutritional and physiological reasons, amino acids and peptides were also chosen because the can easily be detected (by spectrophotometry upon chemical modification of the amino group). Last but not least, these solutes were selected as they enable structure-activity research, considering the variations in amino acid side chains, which can be electrically charged, polar uncharged or hydrophobic. In the latter case, differences in hydrophobicity can be explored by selecting amino acids with increasing hydrophobic side chain molar mass. In addition, the influence of the solute molar mas can be easily investigated by comparing amino acids to dipeptides. Hence, amino acids and peptides were thought to be ideally suited to systematically investigate the effect of the molecular properties of the solute on its encapsulation efficiency in and release characteristics from double emulsions.

The emulsions were characterized considering the oil droplet size (distribution) and entrapped water volume fraction of the double emulsions. To evaluate the oil droplet size and possible instability mechanisms in the double emulsions, laser diffraction was used. In order to investigate the water transport between the inner and outer aqueous phases, analytical centrifugation was performed. Furthermore, the double emulsion structure was confirmed with microscopic observations. These observations also

allowed to prove the presence of the internal water droplets. For the quantification of the release of entrapped amino acids, a spectrophotometric method was followed, which is based on the specific reaction of trinitrobenzenesulfonate with the free amino groups of amino acids. Also, the encapsulation efficiency and release kinetics of the entrapped amino acids in double emulsions was examined during storage using a mathematical model. The schematic outline of the study is demonstrated in Figure 1.1.

<table>
<tr><th colspan="6">Schematic overview of this thesis</th></tr>
<tr><th colspan="5">Production</th><th>Characterization</th></tr>
<tr><th colspan="2">Internal aqueous phase</th><th>Oil phase</th><th colspan="2">External aqueous phase</th><th>Other properties</th><th>Optimization of the colorimetric method</th></tr>
<tr><td>Solute hydrophobicity</td><td rowspan="3">pH</td><td>Oil type</td><td>Thickener</td><td rowspan="3">pH</td><td>Preparation: homogenization technique/intensity</td><td>Simulated gastrointestinal digestion test</td></tr>
<tr><td>Solute molar mass</td><td rowspan="2">Hydrophobic emulsifier (type/concentration)</td><td rowspan="2">Hydrophilic emulsifier (type/concentration)</td><td rowspan="2">Storage: temperature</td><td rowspan="2">NMR-diffusometry</td></tr>
<tr><td>Solute concentration</td></tr>
</table>

Figure 1.1 : Schematic outline of the Ph.D. thesis.

2. LITERATURE REVIEW

2.1 Encapsulation Technology

Encapsulation is defined as the application in which a material is involved by a surrounding material for an effective protection against undesired environmental agents. Encapsulation technology is used in various fields such as food chemistry, medicine, biotechnology, and agriculture. In food industry, bioactive substances are entrapped inside protective microcapsules or matrices against harsh environmental conditions during prosessing and storage in order to maintain the stability and hence to extend shelf life. Encapsulation technologies can be distinguished into three categories, i.e. macroencapsulation (>5000 μm), microencapsulation (0.1-5000 μm) and nanoencapsulation (10-1000 nm) depending on the droplet size. Enzymes, preservatives, organic acids, antioxidants, flavors, essential oils, colorants and enzymes are among the bioactive ingredients encapsulated in the food industry (Nedovic *et al*., 2011).

The most commonly used encapsulation techniques are fluid bed coating, spray drying, freeze drying, spray cooling and extrusion (Figure 2.1). Spray drying is a mechanical process based on the homogenization of an active ingredient in a carrier/wall material solution and spraying the solvent through heat process which results in dry particles ranged from 10 to 400 μm. Spray drying facilitates many advantages, such as low cost, high efficiency and quick solubilisation. Some shortcomings can be noted due to the use of very high temperatures for the drying process: the method can not be used for the encapsulation of volatile components, whereas micro cracks are formed on the surface (Celli *et al*., 2015). In the freeze drying method, on the other hand, the sample is frozen and water is sublimed under vacuum at low temperature to obtain a powder. Although this method is advantageous for the encapsulation of heat sensitive components, it is not economical as it requires a long process time.

Spray cooling, which is also indicated as chilling, is a technique in which an atomization source is used to produce particles following a cooling process. Since the

process is carried out at low temperature, labile components (i.e enzymes, flavors) can be entrapped. Thus, salts can be finely immobilized in solidified lipid as a wall material (Gouin, 2004). On the other hand, the fluid bed coating technique involves the coating of an active component, which is sprayed to form a powder sample. Whereas the good reproducibility, short process time and low energy consumption can be noted as advantages, the direct contact with high temperatures may cause degradation of the entrapped components (Coronel-Aguilera and Martín-González, 2015). In the extrusion method, the core material is dissolved in a biobased solution and subsequently transferred to a gelling bath through a syringe. This process allows to produce glassy and tough wall material in order to protect the compounds against oxidation. The commonly used encapsulating compunds are antioxidants, probiotics and seed oils (Zuidam and Shimoni, 2010).

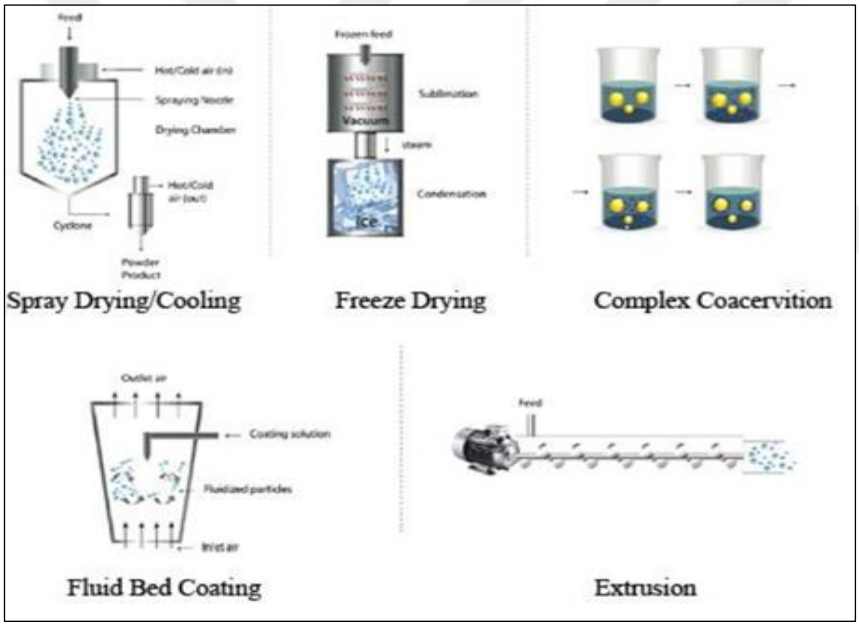

Figure 2.1 : Illustration of mostly used encapsulation technologies for industrial applications (de Souza Simões *et al*., 2017).

Besides these techniques, some other methodologies are applied by generating an intense energy to form encapsulation structure. For emulsion preparation, the devices such as membranes, high-pressure pumps, rotor–stator mixers, and ultrasonic systems are commonly used. High pressure homogenization involves the production of small droplets after the water and oil premix pass through the homogenization valve as a result of shear stress. The droplet diameter obtained with high pressure homogenization is typically smaller than 1 μm. Most commonly used high pressure homogenizers are emulsifying nozzles, jet dispersers and microfluidizers. In

microfluidization, a high pressure is applied to force the liquid through the microchannels which leads to a reduction in droplet size. This methodology is convenient, since toxic solvents are not used in the process. Rotor stator mixing equipment induces a high rotational speed from 1000 up to 25000 rpm to the liquid which is placed in a narrow gap between a constant disk and a rotor. This process enables a mechanical force against the fluid and forms droplets up to 1 μm. In the sonication technique, sound waves are applied to the liquid with high intensity. Water and oil phases are broken down by the high intensity which promotes smaller droplets. This technique is usually used in laboratory scale applications.

2.2 Colloidal Systems

A colloidal system is composed of dispersed and continuous phases. The dispersed phase indicates the particles, which range from 10 nm to 0.1 mm in colloidal systems, wherease the continuous phase refers to the medium in which the particles are dispersed. Colloidal systems can be categorized into two groups as lyophilic and lyophobic systems. Lyophobic colloidal systems require energy to disperse the hydrophobic particles in the continuous aqueous phase. This kind of colloidal system is not in thermodynamic equilibrium, and therefore it is not stable. The lyophilic type, on the other hand, occurs by dissolving hydrophilic particles in an appropriate continuous phase, which enables a thermodynamically stable system (Damodaran *et al*., 2017).

2.2.1 Emulsions

An emulsion can be defined as the mixture of two or more immiscible liquid phases. An emulsion is composed of an internal phase which is dispersed into a continuous phase (Chrisman *et al*., 2012). Emulsions have been widely used in many industrial branches such as food, agriculture, pharmacy and cosmetics (Ichikawa *et al*., 2007). In general, unstable emulsions containing hydrophilic and hydrophobic phases are stabilized by emulsifiers to provide a favourable contact between the immiscible phases (Akbari and Nour *et al*., 2018). Most often, emulsions can be classified into two groups as simple and double emulsions.

2.2.2 Simple emulsions

As two immiscible liquids can be included as an oil and an aqueous phase, two types of emulsions can be distinguished, i.e. water-in-oil (W/O) or oil-in-water (O/W emulsions (Figure 2.2). An emulsion is indicated as W/O in case the aqueous phase is dispersed in a continuous oil phase and as O/W if the oil phase exists as dispersed phase in an aqueous medium. Many food products such as mayonnaise, milk, cream, ice cream, as well as dressing sauces are oil-in-water emulsions. However, water-in-oil emulsions are less common in foods: examples are margarine and butter (McClements, 2007).

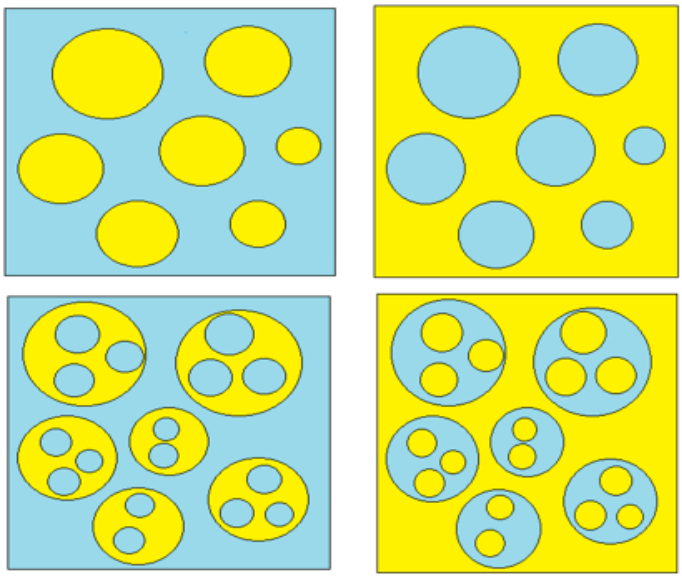

Figure 2.2 : Schematic illustration of the simple oil-in-water (O/W) emulsion on the upper left side and water-in-oil (W/O) emulsion on the upper right side and the corresponding water-in-oil-in-water (W/O/W) emulsion on the lower left side and oil-in-water-in-oil (O/W/O) emulsions on the lower right side; the oil phases are yellow and the aqueous phase are blue.

2.2.3 Double emulsions

Double emulsions, also referred to as multiple emulsions, are complex systems which involve the suspended small droplets in larger droplets that are dispersed in a continuous phase. Basically, there are two types of double emulsions: water-in-oil-in-water (W/O/W) and oil-in-water-in-oil (O/W/O) emulsions. The latter exist of an oil continuous system involving water droplets in which smaller oil droplets are dispersed. The more prevalent water-in-oil-in-water (W/O/W) type points out a water continuous system in which oil droplets are dispersed which themselves enclose smaller water droplets (Opperman *et al*, 2016; Kaimainen *et al.*, 2015). The illustrations of single and double emulsions are demonstrated in Figure 2.2.

In addition to the oil and aqueous phases, emulsifiers are also used in the preparation of the emulsions to support their stability. Although one emulsifier is sufficient for conventional emulsions, minimum two emulsifiers are mostly required for the stability of double emulsions: the hydrophobic emulsifier to stabilize the W/O emulsion, whereas the hydrophilic one to stabilize the oil droplets in the external water phase (i.e. prevention of coalescence of the oil droplets) (Leister and Karbstein, 2020).

Considering the preparation of double emulsions, the most known method is the two step emulsification procedure which was first described by Matsumoto *et al.* (1976). First, a W/O primary emulsion is produced as described in the previous part. Subsequently, the primary emulsion is dispersed in the external aqueous phase to obtain a double emulsion (Ding *et al.*, 2019). A schematic representation of the standard two step double emulsion preparation procedure is shown in Figure 2.3.

In order to convert an oil-water mixture into a stable emulsion, intense mechanical energy is applied using a homogenizer. Mostly used machines to homogenize a liquid mixture are high speed shearing devices (rotor-stator systems such as Ultra-turrax), membrane emulsification, high-pressure homogenizers, ultrasonic homogenizers, and microfluidic devices. For the homogenization process, convenient production conditions are determined to acquire the desirable emulsion characteristics (McClements, 2005).

Generally, conventional homogenization is applied using rotor stator equipment in the first step of homogenization. Afterwards, if required, an additional high pressure emulsification is carried out for the further decrease in the droplet size of the internal water droplets. For the second homogenization stage, rotor stator homogenizers are routinely used for more moderate shear forces to avoid the breakdown of the previously produced primary droplets, and hence destabilization of the W/O emulsion (Muschiolik and Dickinson, 2017).

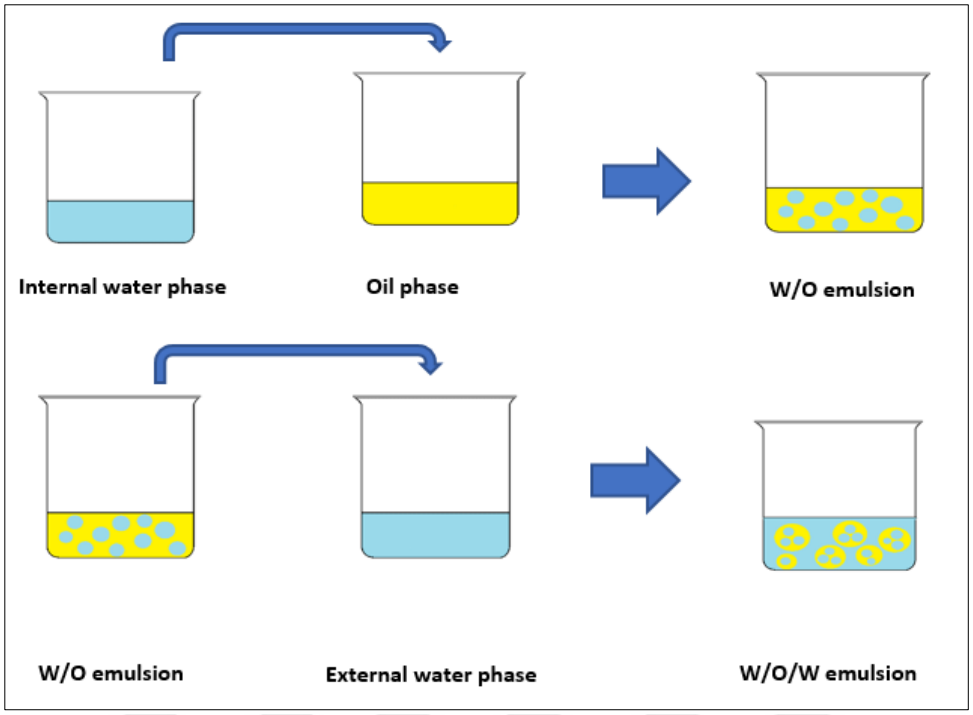

Figure 2.3 : Schematic illustration of the standard two step preparation method of water-in-oil-in-water (W/O/W) emulsions.

Double emulsions continue to gain a profound attention from researchers because of two main reasons. First, W/O/W type emulsions can be used instead of O/W emulsions to manufacture reduced fat products which have similar perceived characteristics, despite of the presence of the lower oil composition. The seond application is the encapsulation of sensitive active ingredients to protect them from deteriorative reactions, and to release these nutrients afterwards in the gastrointestinal tract (Muschiolik and Dickinson, 2017). Many bioactive compounds such as vitamins, minerals, enzymes, and microorganisms can be enclosed in double emulsions (Garti and Bisperink, 1998).

2.2.4 Emulsifiers and stabilizers

As previously mentioned, double emulsions are stabilized by two types of emulsifiers, i.e. both a hydrophobic and a hydrophilic emulsifier. Emulsifiers, also called a surface active substances, are molecules that prevent the aggregation of dispersed droplets by reducing the surface tension and by introducing repulsive interactions (Whitehurst, 2004). Emulsifiers have an amphiphilic nature due to their combined lipophilic and hydrophilic characteristics. The lipophilic part, named as tail, dissolves in oil and is termed oil-loving. The hydrophilic part, named as head, is soluble in water and called water-loving (Miller, 2016).

A crucial index regarding the solubility of an emulsifier is its hydrophilic–lipophilic balance (HLB) value. This value is also important as it shows the suitability of an emulsifier for the emulsion (Damodoran *et al.,* 2017). Emulsifiers with low HLB values refer to more lipophilic emulsifiers while the ones with high HLB values refer to more hydrophilic emulsifiers. Basically, for the stabilization of W/O emulsions, a lipophilic emulsifier (HLB value = 3-6) is suitable, whereas a hydrophilic emulsifier is required for O/W emulsions (HLB value = 10-18) (Miller, 2016).

The most known and effective lipophilic emulsifier for food applications is polyglycerol polyricinoleate (PGPR). The typical PGPR concentration used in double emulsions ranges from 1.25 to 6%. However, due to the undesirable off taste which is induced by PGPR, the authorities restrict its level in foods (Lee *et al.,* 2013). Thus, it is reported that one of the solutions might be the replacement of PGPR with a more natural emulsifier, such as lecithin. Another alternative was reported as the use of PC depleted lecithin owing to its more hydrophobic structure. Moreover, the combination of PGPR with natural emulsifiers is another strategy to reduce the PGPR level (Altuntas *et al.,* 2017).

As hydrophilic emulsifier, Tween 80 is widely used in double emulsions. However, several researchers have examined alternatives for Tween 80 due to its reported toxicity (Tamnak *et al.,* 2016). For the stabilization of the emulsions, stabilizers, which are also known as texture modifiers, can be used as well. A stabilizer is an agent which minimizes the movement of the particles by enhancing the viscosity or forming a gel network in the aqueous phase, and thus improves the textural properties of the emulsion (Cui, 2005; Doublier and Cuvelier, 2006). Stabilizers are not adsorbed to the interface like emulsifiers, but they can give a notable shelf life to the emulsions. A lot of proteins (gelatin, soy protein, caseinate, whey protein and egg protein) and polysaccharides (carrageenan, pectin, alginate, starch, modified starch, cellulose, gums) are used as stabilizers owing to their gelling or thickening attributes (Eliasson, 2006).

2.2.5 Stability of emulsions

Emulsion stability can be defined as the resistance capability of an emulsion to physicochemical changes during storage. Due to the two immiscible phases, emulsions tend to become unstable over time. Since double emulsion involve two interfaces, it is even more difficult to form a stable emulsion. It is crucial to identify the main

instability mechanism in the emulsion in order to promote the stability. Emulsion stability is associated with the type and concentration of emulsifiers which enhance the stability of the emulsion by the formation of an interface around the water droplets in water/oil interfaces. Factors such as emulsion composition, homogenization intensity, and temperature can affect the emulsion stability (Abd *et al.*, 2014). The most common instability phenomena are creaming, sedimentation, flocculation, coalescence, partial coalescence, Ostwald ripening and phase inversion as illustrated in Figure 2.4.

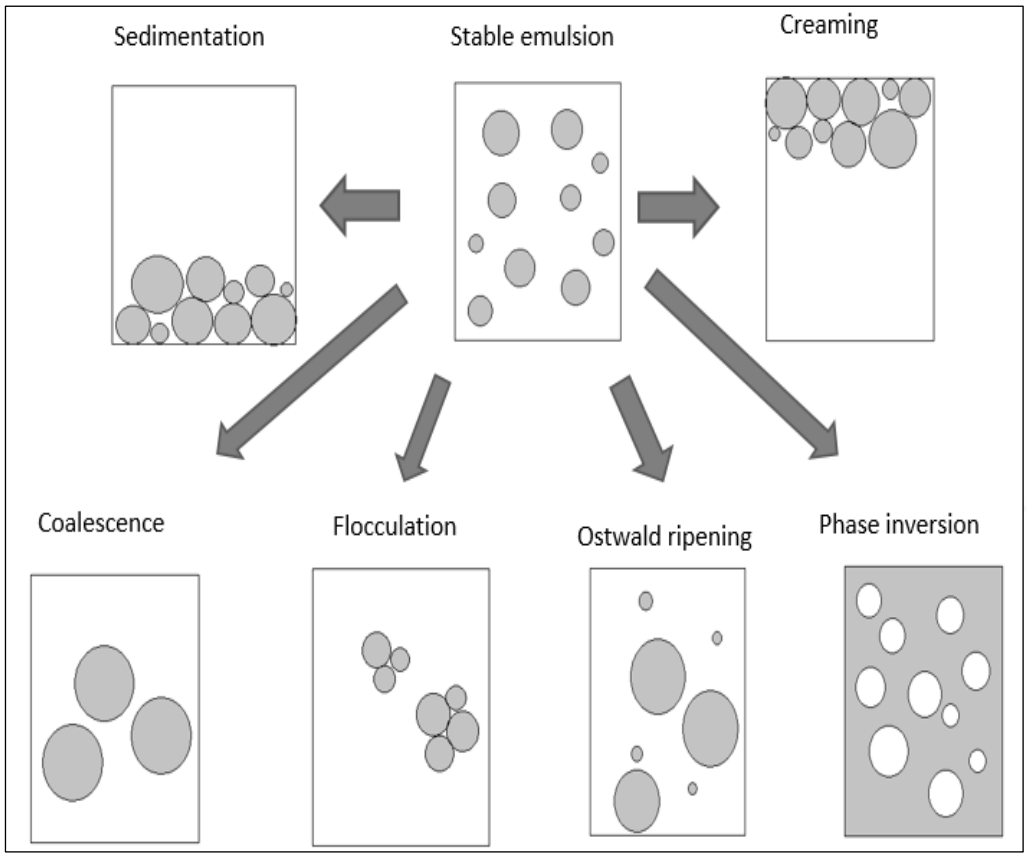

Figure 2.4 : Illustration of most common instability mechanisms of emulsions (Kontogeorgis and Kiil, 2016).

Table 2.1 also demonstrates the definitions of these instability mechanisms. It can be also stated that some of the instability mechanisms in emulsions are related to each other and can take place in the emulsion simultaneously. For example, coalescence occurs as a further stage of flocculation and both mechanisms cause an increased droplet size. Subsequently, the density difference of oil and aqueous phase leads to gravitational separation instability such as sedimentation and creaming (McClements, 2015).

Table 2.1 : Instability mechanisms observed in water/oil and oil/water emulsions (Goodarzi and Zendehboudi, 2019).

Instability	Definition
Sedimentation	Downward movement of the droplets due to the density difference in oil and water.
Creaming	Upward movement of the droplets due to the density difference
Coalescence	Formation of bigger droplets by the process that droplets merge with each other
Flocculation	Formation of aggregates while the droplets retain their identities
Ostwald ripening	Enlargement of droplets due to the mass transport from smaller to larger droplets
Phase inversion	The process in which the system alters from a water-in-oil emulsion to an oil-in-water emulsion, or vice versa.

2.2.6 Stability of double emulsions

The main instability phenomena of double emulsions are known as coalescence and diffusion. These instabilities impact the texture and encapsulation efficiency of the double emulsions. Whereas some instabilities, such as flocculation, or creaming are reversible, coalescence and diffusion are irreversible. Considering coalescence, three types are possible: coalescence of oil droplets, coalescence of internal water droplets and coalescence of water droplets with the continuous phase (Figure 2.5).

Considering diffusion, two possibilities may occur depending on the osmotic gradient in the inner and outer water phases: water loss or water gain in the internal water droplets. In case the water transfer is from the inner to the outer phase, this will induce the shrinking of the internal water droplets. This will diminish the volume fraction of dispersed phase and hence the viscosity, which will decrease the effect of fat reduced products. Conversely, the water transfer to the inner water phase will cause the swelling of the internal water droplets. The oil droplets will grow as well as a result of external water transfer and the viscosity will increase simultaneously. It should be noted that although the net osmotic pressure difference does not absolutely need to be zero, it should be low enough to ensure the osmotic balance for the prevention of coalescence and rupture of the swollen inner water droplets (Muschiolik and Dickinson, 2017). Stability of the double emulsions can be supported by modification of the external aqueous phase. Besides the emulsifiers, the addition of polymers can be helpful as they limit the mobility of the droplets, which further will minimize the coalescence of oil droplets and prevent their gravitational separation (Hattrem *et al.,* 2014).

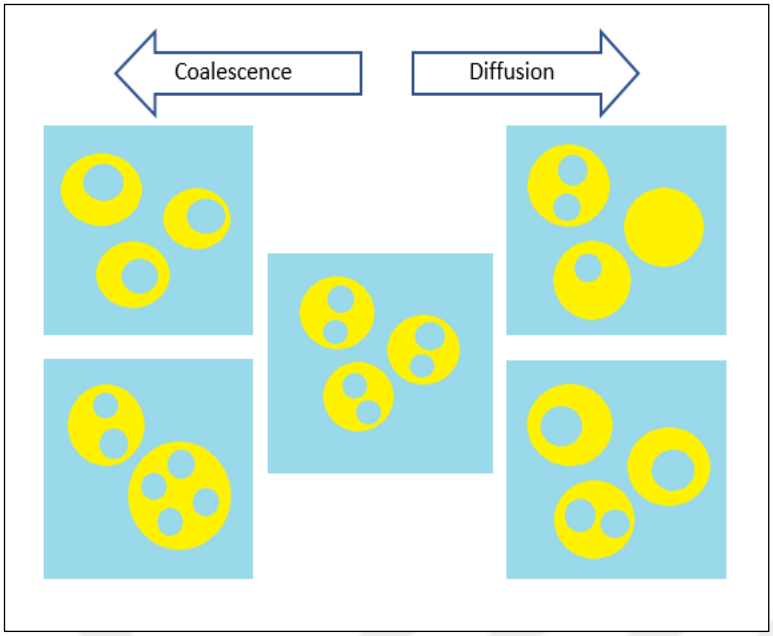

Figure 2.5 : Illustration of common coalescence (left) and diffusion (right) mechanisms taking place in W/O/W emulsions.

Additionally, food grade stabilizers such as polysaccharides and proteins can form complexes which enable a better stability than with only one protein owing to the thicker multilayered film in the interface (Garti and Lutz, 2004). According to Perez-Moral *et al.* (2014), the use of gelling agents in the internal water phase also decreases the droplet size and increases the stability. It was also stated that the use of surfactants with high molecular weight enables a better stability in comparison with those with lower molecular weight. This can be explained from the higher energy required to remove the larger molecules from an interface **(**Frasch-Melnik *et al.*, 2010).

2.2.7 Encapsulation of active compounds in double emulsions

As previously stated, double emulsions enable protection as well as controlled release of water soluble compounds. Besides water-soluble ingredients, hydrophobic substances can also be entrapped in the oil phase of the double emulsion which enables the simultaneous delivery of both hydrophobic and hydrophilic compounds.

Encapsulation of ingredients via double emulsions has been already used in many industries such as the food, pharmaceutical and agricultural industry (Jiménez-Colmenero, 2013). Recent studies indicated that enrichment with hydrophilic bioactive compounds can be used to improve the nutritional and physiological effects of foods. However, these hydrophilic active ingredients may be susceptible to chemical and physical effects within the complex food matrix. In this context, the encapsulation and

controlled release of the active ingredients may prevent degradation of these substances during processing, storage and/or in the gastro-intestinal system (McClements, 2015). The double emulsion encapsulation system was found to be efficient to prevent degradation of several substances in the gastro-intestinal track, such as anthocyanins (Frank *et al.,* 2012), betalain (Kaimainen *et al.,* 2015), vitamin B12 (Giroux *et al.,* 2013) and caffeine (Hernandez-Marın *et al.,* 2016). Moreover, Zhang *et al.* (2015) found that the viability of probiotics can be increased by encapsulation in double emulsions.

There is an increasing attention for bioactive peptides due to their beneficial activities such as antioxidant, antimicrobial, immunomodulatory and antihypertensive. However, these peptides are sensitive to be degraded by proteases (Mohan *et al.,* 2015). In order to prevent the chemical degradation of the peptides by hydrolysis, the double emulsion system may be used to succesfully encapsulate them. Hereby, the double emulsion system was found to be a more efficient encpsulation technique compared to liposomes since the latter may undergo lipid oxidation and hydrolysis (Aditya *et al.,* 2017). Giroux *et al.* (2016) reported that casein derived peptides encapsulated within double emulsions could be succesfully protected from chemical degradation during gastro-intestinal digestion.

Concerning the encapsulation of amino acids, several studies are available using single and double emulsions. Bhatti *et al.* (2016) stated that a mixture of amino acids could be retained by means of single W/O emulsions with 79.8% encapsulation efficiency. To the best of our knowledge, only two studies have been published so far regarding amino acid encapsulation by means of double emulsions (Owusu *et al.,* 1992; Weiss *et al.,* 2005). In both studies, the essential amino acid L-tryptophan was encapsulated in the internal water phase. Owusu *et al.* (1992) stated that the release of both vitamin B12 and L-tryptophan was higher at the isoelectric point in comparison to the release of ionized forms at pH conditions away from the iso-electric point. Furthermore, the encapsulation yield decreased after heating at 80 ^{o}C in both double emulsions that contained vitamin B12 and L-tryptophan. It was also reported by Weiss *et al.,* (2005) that the release rates of tryptophan in double emulsions decreased as a consequence of lower temperature. They also compared different oils with varying melting points and stated that the release was lowered using high melting point oils.

Although there is an increasing research interest about double emulsions, industrial applications are higly limited. In this respect, two main problems have been stated by Muschiolik and Dickinson (2017). First, there is a need for sophisticated equipment to be used for large scale processing for especially the second emulsification stage. Second, the absence of a food grade hydrophobic emulsifier (without inherent chemical connotation) to replace polyglycerol polyricinoleate (PGPR) is a major hurdle for the widespread application of double emulsions.

2.2.8 Release in double emulsions

In the literature, two main mechanisms have been reported concerning the release of encapsulated compounds. The first mechanism is the coalescence of internal water droplets with the oil globule surface. The second one is referred to as compositional ripening. This one does not involve film rupturing and it involves the diffusion of the chemical compounds to the oil phase. According to Pays *et al.* (2002), the release mechanism depends on the hydrophilic emulsifier concentration: when the hydrophilic emulsifier concentration is above CMC, the release occurs by coalescence of internal water droplets and oil globules. Conversely, in case the hydrophilic emulsifier concentration is below CMC, then the transfer is governed by diffusion without film rupturing.

Looking at the diffusion mechanisms, hydrophilic substances can permeate across the oil phase with three primary pathways as can be seen from Figure 2.6. The first mechanism is based on the permeation across the thin films of the surfactant between inner water droplets and the external water phase (Mezzenga *et al.*, 2004). In this case, the internal water droplets move across the oil droplet which induces a fluctuation in the oil layer thickness. The second one is indicated as micellar transport in which the hydrophobic emulsifier forms inverse micelles. It was reported that the attemps made by increasing the HLB of the hydrophobic emulsifier to promote the emulsion stability ended in lower stability and encapsulation efficiency due to the formation of more inverse micelles (Koroleva and Yurtov, 2006). The third mechanism is less prevalent and related to the transfer via hydrated surfactant. Wen and Papadopoulos (2000) stated that the permeation is governed by diffusion through hydrated surfactants when there is contact between the internal and external phase. Another mechanism reported by Bahtz *et al.* (2015) includes the transport by tiny droplets which permeate across the oil layer. Lutz *et al.* (2009) also reported that transport of water soluble compounds

can occur due to the breakdown of the globules as a result of swelling or intense shearing. In this mechanism, the rupture of globules occurs when there is an osmotic pressures gradient between the aqueous phases. In case the water flux is towards the inner phase, internal water drops enlarge and subsequently breakup. Yet, it was stated by Benichou *et al.* (2004) that transport of the active compounds can take place even if there is osmotic balance and stability of the droplets to coalescence. They observed enhanced release rates due to the reverse micelles formed in the oil phase, despite of the osmotic balance between the inner and outer water phases.

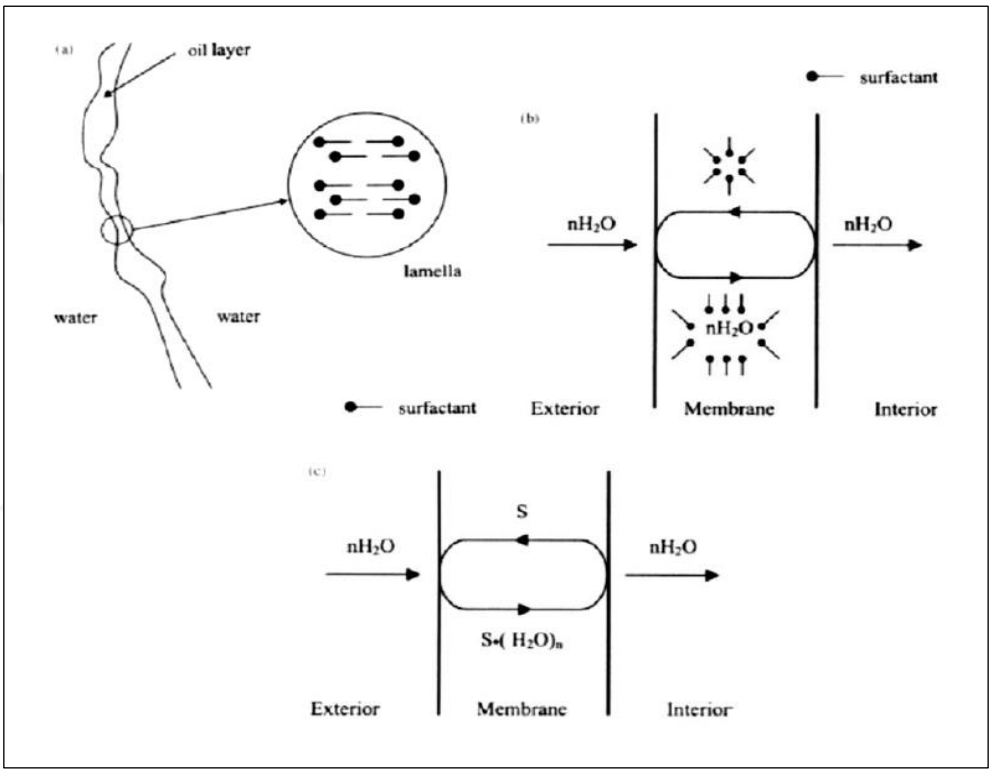

Figure 2.6 : Diffusion of water and water soluble substances via a) lamellar thinning of the surfactant layer, (b) reverse micelles, (c) hydrated surfactants (Garti and Lutz, 2004).

2.2.9 Characterization of emulsions

2.2.9.1 Droplet size distribution

The droplet size (distribution) of emulsions substantially affects their stability towards physicochemical changes such as texture, rheology and microbiological properties. Additionally, it helps to determine the optimum process conditions and the effect of the type and concentration of the emulsifiers. For example, the oil droplet size is affected in case of a loss of internal droplets during the emulsion preparation. Also, the droplet size may be influenced by the shrinkage or swelling of internal water

droplets (Mezzenga *et al.,* 2004). The coalescence of oil droplets is a reason for the increase in droplet size as well (Esfanjani *et al.,* 2017). For the characterization of double emulsions, the oil droplet size is investigated. Hereby, distilled water is usually used to dilute the samples for measurement. Because of the existence of internal water droplets, an osmotic pressure difference between the aqueous phases should be prevented to achieve a reliable size distribution prediction.

Static light scattering (SLS) is based on the measurement of the intensity of the light produced by the droplets when a light beam is directed through a diluted emulsion as a function of the scattering angle. The instruments include the software with mathematical model (i.e. Mie theory) which enables the prediction of scattering patterns of the emulsions using the properties of the particles (i.e. absorption coefficient, refractive index). Static light scattering is suitable for droplet size measurement of food emulsions ranged from 0.1 to 1000 μm (McClements, 2007).

Dynamic light scattering (DLS) is commonly used for oil droplet size determination. This technique is based on the detection of the diffusion coefficient of the particles which move in accordance with Brownian motion. Depending on the speed of the particles, slight fluctuations occur in the light intensity scattered by the particles (McClements, 2015). According to Brownian motion, small particles move faster than bigger ones. Therefore, higher fluctuations rates will be obtained in the scattered light of smaller particles. There is an inverse proportion between diffusion coefficient and particle size as can be seen in the Stokes–Einstein equation.

$$\mathbf{D} = \frac{k_B T}{6\pi\eta R_h} \tag{2.1}$$

In this equation, the diffusion coefficient is represented by *D*, k_B is the Boltzmann constant, T shows the absolute temperature, R_h is the hydrodynamic radius, while η is the viscosity of the medium. For the measurement of the sample, the refractive index of the continuous phase should be known as well (Ramos, 2017).

2.2.9.2 Microscopy

Optical techniques provide a useful insight about droplet size and possible instability mechanisms. Visualizing the oil droplets helps to prove the presence of double emulsions. Moreover, discharged oil droplets clearly indicate the loss of inner water droplets (Frank *et al.,* 2012; Schuch *et al.* 2014). Although the migration of the internal phase can be easily perceived, quantitative analysis is troublesome due to the low

amount of droplets that are used for evaluation. It is suggested that, for a reliable determination, the number of droplets should range from 2000 to 9000 (Schuster *et al.*, 2012). Furthermore, very large droplets can make the observation challenging if they do not fit under the cover slide of the microscope (Jiao *et al.*, 2002). Additionally, it was previously mentioned that coalescence and diffusion commonly take place in double emulsions. The use of microscopy can not easily help to distinguish these two mechanisms. To obtain a dependable outcome, oil droplets filled with inner droplets should be constantly monitored during storage. Whereas the oil droplets of double emulsions can be observed using light microscopy owing to their relatively large size, confocal laser scanning microscopy (CLSM) is usually used for the observation of internal water droplets of which the size is under 1 micron. This technique also enables a clear dictinction of water and oil phases. Moreover, the diffusion can be also tracked using CLSM (Bernewitz *et al.*, 2016).

Besides the light optical techniques, images can be viewed with high resolution using electron microscopy. However, these devices like scanning electron microscopy (SEM) and Cryo-SEM require more difficult sample preparation and complex data analysis (Leister and Karbstein, 2020).

2.2.9.3 Enclosed water volume fraction of double emulsions

The cream fraction of a double emulsion enables to evaluate the enclosed water volume fraction. A correlation was reported between the creaming speed and the density of the oil droplets (Pays *et al.*, 2002). Besides the creaming speed, the phase fraction also allows to evaluate the stability of the emulsion. In case an outward water flux exists through the oil phase, the height of the cream layer diminishes, whereas the fraction of the external aqueous phase becomes larger (Leal-Calderon *et al.*, 2012).

The phase fractions can be easily observed by analytical centrifugation considering the change in the boundaries of the phases. Although NMR is an alternative which is widely applied for the determination of the entrapped water fraction, it requires a complex measurement and interpretation (Vermeir *et al.*, 2014).

For the determination of the enclosed water fraction, some markers are also commonly used assuming the similar behaviour of the tracer and the inner water phase. In this method, the markers should be involved in the double emulsion during preparation. Observation of the released markers can be performed using spectroscopy,

conductivity and NMR. In this technique, the type of marker is also critical as their molecular structure can be different (Lamba *et al.*, 2015).

2.3 Amino Acids

2.3.1 Structure

Amino acids can be defined as the compounds composed of a basic nitrogen group, an acidic carboxyl group and a side chain attached to a central α-carbon. These amino acids are the basic elements of proteins. Despite of the existence of 100 amino acids in nature, most proteins involve only 20 of them (Damodoran *et al.*, 2017). The R group of the amino acid, also referred to as the side chain, identifies the amino acid and determines its biological functions and physicochemical nature such as solubility, net charge and hydrogen binding ability. Glycine is an amino acid which has only a hydrogen atom as an R group, and thus it is known as the simplest structure among all amino acids (Figure 2.7). The amino acid structure only varies by the chemical group of the side chain (Yu and Fukagawa, 2020).

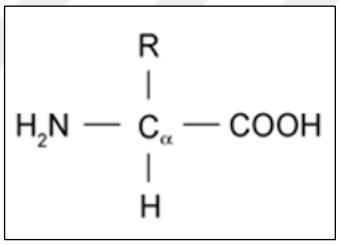

Figure 2.7 : Structure of amino acids.

Considering the classification of amino acids, several categories are possible. Based on the interaction properties of the functional group of the amino acid with the aqueous phase, two types can be distinguished, i.e. hydrophilic and hydrophobic. Hydrophobic ones involve aromatic and aliphatic side chains and display a low solubility in water. However, hydrophilic amino acids can be considerably dissolved in water.

Amino acids are classified into three groups according to their synthesis in the organisms. The first group are the essential ones which are not synthesized by the organisms and have to be consumed in the diet, whereas the second group contains non essential ones which can be synthesized by animal organisms. Finally, the third group involves the conditionally essential amino acids, which can be synthesized in some cases, such as pregnants and newborns (Ribarova, 2018).

2.3.2 Functions

Amino acids are crucial as they take part in diverse biological processes of humans. They are the subunits of many compounds such as enzymes, hormones and tissues. Also, they play various roles in the metabolism, intercellular signalling and neurotransmission (Dietzen 2018). In case of metabolic problems or malnutrition, amino acids can be widely consumed by supplementation to support the metabolism and tissue building. After surgeries and traumas or during the medical care of patients, instant amino acid formulations are increasingly demanded. It was proved that branched chain amino acids such as leucine, valine and isoleucine are effective to cure hepatic encephalopathy. Glycine formulations were found to be beneficial to treat severe stress conditions while arginin and cystein are essential for pediatric care (Unger and Holzgrabe, 2018).

2.3.3 Polarity of amino acids

Amino acids are amphoteric compounds as they act as an acid and a base owing to the carboxyl and amino groups in their structure, respectively. Based on the different pH values, amino acids exist in three ionized states, i.e. as zwitterion, conjugate base and conjugate acid. In case the amino acid is dissolved at neutral pH conditions (around pH 7), both the amino and carboxyl groups are ionized. Therefore, the molecules exist as dipolar ion at neutral pH, which is also termed as zwitterion. The net charge is 0 at the zwitterionic state. The pH at which the molecules carry no net charge is called the isoelectric point. At acidic pH, the COO^- group will be protonated and the net charge of the amino acid will be +1. At basic pH, the NH_3^+ group will be deprotonated, and hence the net charge will be -1. The different ionization states of Gly are demonstrated in Figure 2.8 (Damodaran *et al.*, 2017).

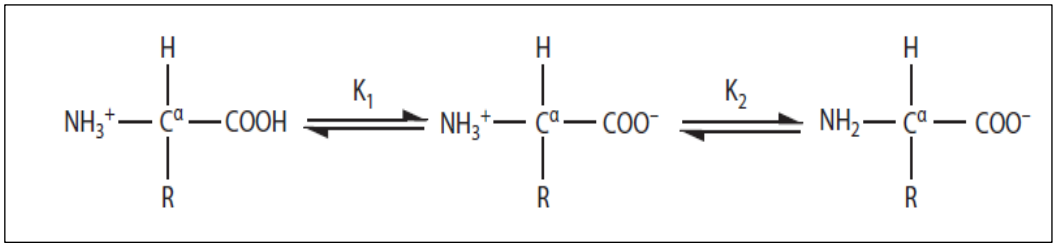

Figure 2.8 : Different ionization states of glycine amino acid (pK1 = 2.34; pK2 = 9.60).

2.3.4 Hydrophobicity of amino acids

Hydrophobicity, also termed as lipophilicity, is an indicator of the tendency of a solute to prefer an organic solvent over an aqueous environment. Hydrophobicity is indicated quantitatively with the free energy difference upon transfer of amino acids from a non-aqueous solvent to an aqueous solution. As an organic solvent, chemicals such as ethanol, methanol and octanol can be used. The free energy changes of the amino acids from octanol to water can be seen in Table 2.2. Amino acids with positive values refer to hydrophobic ones which tend to be dissolved in organic solvents, while the ones with negative values points out the hydrophilic amino acids, which exhibit a tendency to be dissolved in an aqueous phase (Damodaran *et al.,* 2017).

Table 2.2 : Hydrophobicity of amino acids measured by the free energy change (ΔG°tr) of the amino acid side chain upon transfer from octanol to water, relative to glycine (Fauchere and Pliska, 1983).

Hydrophobic Amino Acids	**Hydrophobicity (kJ/mol) (ΔG°)**	**Hydrophilic Amino Acids**	**Hydrophobicity (kJ/mol) (ΔG°)**
Tryptophan	12.9	Glycine	0
Isoleucine	10.4	Serine	-0.48
Phenylalanine	10.0	Glutamine	-1.25
Leucine	9.62	Asparagine	-3.34
Cysteine	8.78	Glutamic acid	-3.76
Valine	7.11	Aspartic Acid	-4.60
Methionine	7.11	Lysine	-5.85
Tyrosine	5.43	Arginine	-5.85
Proline	4.18		
Threonine	1.67		
Alanine	1.67		
Alanine	1.67		

2.3.5 Chemical reactivity of amino acids

Amino acids can take part in some reactions due to their reactive groups such as carboxyl, amino, hydroxyl, phenolic and sulfhydryl groups. Some reactions occuring due to the amino group; these involve deamination, reductive alkylation, acetylation and arylation. The expected reactions take place in the carboxyl group can be noted as esterification, reduction and decarboxylation. Although some of these reactions are used as a quantification method of amino acids, some are not desirable in terms of the quality of food products. Some of these reactions cause the formation of toxic compounds at higher temperatures. For example, some undesirable side products may occur at high temperatures due to Maillard reaction. In this reaction, amino acids react

with reducing sugars which further facilitate the formation of side products such as aldehydes. As these products can provide aroma development in foods, their toxicity has been reported (Van Nguyen, 2006).

Food processing at higher temperatures and in alkaline media allows the formation of some modified amino acids, such as the isopeptide lysinoalanine (LAL), and lanthionine β-aminoalanine. LAL is regarded as a toxic compound. Besides, it was reported that the formation of LAL decreased the biological value of proteins due to the lysine loss. Also, some amino acids such as Cys, Trp, Met, and His are sensitive to oxidation. Upon exposure to thermal processes, the release of a sulfur compound may occur after cysteine decomposition. The oxidation products of Trp were observed to display a mutagenic activity in mammalian cells (Ribarova, 2018).

2.3.6 Quantification of amino acids

In the literature, many methods have been described concerning the detection of amino acids. Characterization and quantification techniques of amino acids consist of approaches such as derivatization, separation of amino acids using chromatography and detection. In the previous periods of amino acid determination, paper chromatographic techniques have been used. These methods have been evaluated to be good in resolution, but low in reproducibility. Gas chromatography is an alternative technique which requires preliminary derivatization to obtain volatile compounds. Besides this effort, the good reproducibility is not guaranteed in this technique. These difficulties make the implementation of gas chromatography less prevalent in comparison with other methods (Bos *et al.*, 1983).

The use of low or high pressure column liquid chromatography as well as ion exchange chromatography has become popular. However, the presence of derivatization makes the technique time-consuming and makes the sensitivity unsatisfactory. One of the most effective techniques was shown to be high-performance liquid chromatography (HPLC) owing to its sensitive and fast detection (Williams, 1986). Especially, the implementation of reversed phase HLPC enables a proper separation. Another option for amino acid detection is Nuclear Magnetic Resonance (NMR). This techniques provides some advantages since it does not require derivatization and separation. Additionally, the high speed sample preparation and the necessity of very small amounts of sample make this methodology promising (Kaspar *et al.*, 2009).

In addition to these techniques, methods based on the reaction between reactive groups of amino acids and specific compunds can often enable the quantification of amino acids. For example, the reaction between ninhydrin and free amino groups forms a purple color product. Based on the color change, a colorimetric determination is carried out by reading the absorbance at 570 and 440 nm. Also, the reaction of O-phthaldialdehyde with free amino groups enables the formation of a fluorescent compound of which the emission is measured at 450 nm (Damodaran *et al.*, 2017).

Another technique, which was also used in the current study for the determination of the amino acid concentration, is the spectrophotometric method of Satake *et al.* (1960). In the method, 2,4,6-trinitrobenzenesulfonic acid (TNBS) reacts with free amino groups allowing the formation of a yellow compound. Hence, the optical density is measured at 340 nm using a spectrophotometer.

3. MATERIALS AND METHODS

3.1 Materials

Polyglycerol polyricinoleate (PGPR 4150) (Palsgaard A/S Juelsminde, Denmark), Native sunflower lecithin (Cargill, Topcithin®;15.4% PC, 12.6% PI, 6.8% PE, 1.7% PA and 62.5% AI, Belgium) and PC-depleted lecithin (DP 1017; 5.0% PC, 15.2% PI, 10.6% PE, 6.4% PA and 63.5% AI, Belgium) were used as hydrophobic emulsifiers, while Polysorbate 80 (Tween 80) was used as hydrophilic emulsifier (Sigma-Aldrich, St. Louis, USA).

As an agent to balance the osmotic pressure between the inner and outer aqueous phases, a potassium chloride (AnalaR NORMAPUR, VWR Chemicals, Leuven, Belgium) solution with sodium azide was used (Sigma-Aldrich, Steinheim, Germany).

The amino acids used in this thesis were L-glutamine (Merck KgaA, Darmstadt, Germany), glycine (Sigma-Aldrich, St. Louis, USA), DL-alanine (UCB, Leuven, Belgium), L-valine (Sigma-Aldrich, St. Louis, USA), L-leucine (Acros Organics, Geel, Belgium), L-phenylalanine (Acros Organics, Geel, Belgium) in order to obtain a range of varying hydrophobicities. L-alanine-L-leucine (Sigma-Aldrich, St. Louis) and L-leucine- L-leucine (Sigma-Aldrich, St. Louis) were used in order to evaluate the molar mass effect. The chemical properties of amino acids and dipeptides used in this thesis are shown in Table 3.1.

As oil phase, high oleic sunflower oil (HOSO; Contined B.V., Bennekom, Netherlands) and MCT-oil 152 (Miglyol 812N) with approximately 58% C8:0 and 41% C10:0 (IMCD, Mechelen, Belgium) were used. For NMR measurements, glyceryltrioctanoate oil (Sigma-Aldrich, Belgium) was used. Also, xanthan gum (food grade, FF) was used as a thickening agent (Jungbunzlauer, Vienna, Austria). Additional reagents, such as SDS (Sigma-Aldrich, St. Louis, USA), hydrochloric acid 35% (HCl; VWR Chemicals, Fontenay-sous-Bois, France), sodium hydroxide (NaOH) (Merck KgaA, Darmstadt, Germany), sodium hydrogen carbonate (AnalaR

NORMAPUR®, VWR Chemicals, Leuven, Belgium), and picrylsulfonic acid solution 5% w/v in H_2O (Sigma-Aldrich, St. Louis, USA) were used in the experiments.

For gastrointestinal release experiments, pancreatin (from porcine pancreas, ≥3×USP specifications), pepsin powder (from porcine gastric mucosa, ≥ 400 U/mg protein) and lipase (Type II from porcine pancreas, 100-500 U/mg) were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Sodium chloride and calcium chloride was provided by VWR (PROLABO Chemicals, Belgium).

Table 3.1 : Chemical properties of the amino acids and dipeptides used: besides the molecular weight (MW), the pK_a of the acidic and basic group and the iso-electric point (pI), the hydrophobicity is indicated, which is quantified by the octanol-water partition coefficient (Log P_{ow}) of the amino acids and dipeptides.

Amino acid	Formula	MW (Da)	$pK_{a,1}$[a]	$pK_{a,2}$[a]	pI[a]	Log P_{ow}
Leu	$C_6H_{13}NO_2$	131.2	2.30	9.60	5.98	-1.61[a]
Val	$C_5H_{11}NO_2$	117.1	2.32	9.62	5.96	-2.08[a]
Ala	$C_3H_7NO_2$	89.1	2.34	9.69	6.00	-2.89[a]
Gly	$C_2H_5NO_2$	75.1	2.34	9.60	5.98	-3.15[a]
Glu	$C_5H_{10}N_2O_3$	147.1	2.17	9.13	5.65	-3.25[a]
Ala-Leu	$C_9H_{18}N_2O_3$	202.3	2.34	9.60	5.98	-2.35[b]
Leu-Leu	$C_{12}H_{24}N_2O_3$	244.3	2.30	9.60	5.98	-1.46[b]

[a]Pliska, Schmidt, & Fauchere (1981)

[b]Akamatsu, Nakamura, Iwamura, & Fujita (1989)

3.2 Methods

The experimental flow chart used in the major experiments of the thesis is presented schematically in Figure 3.1.

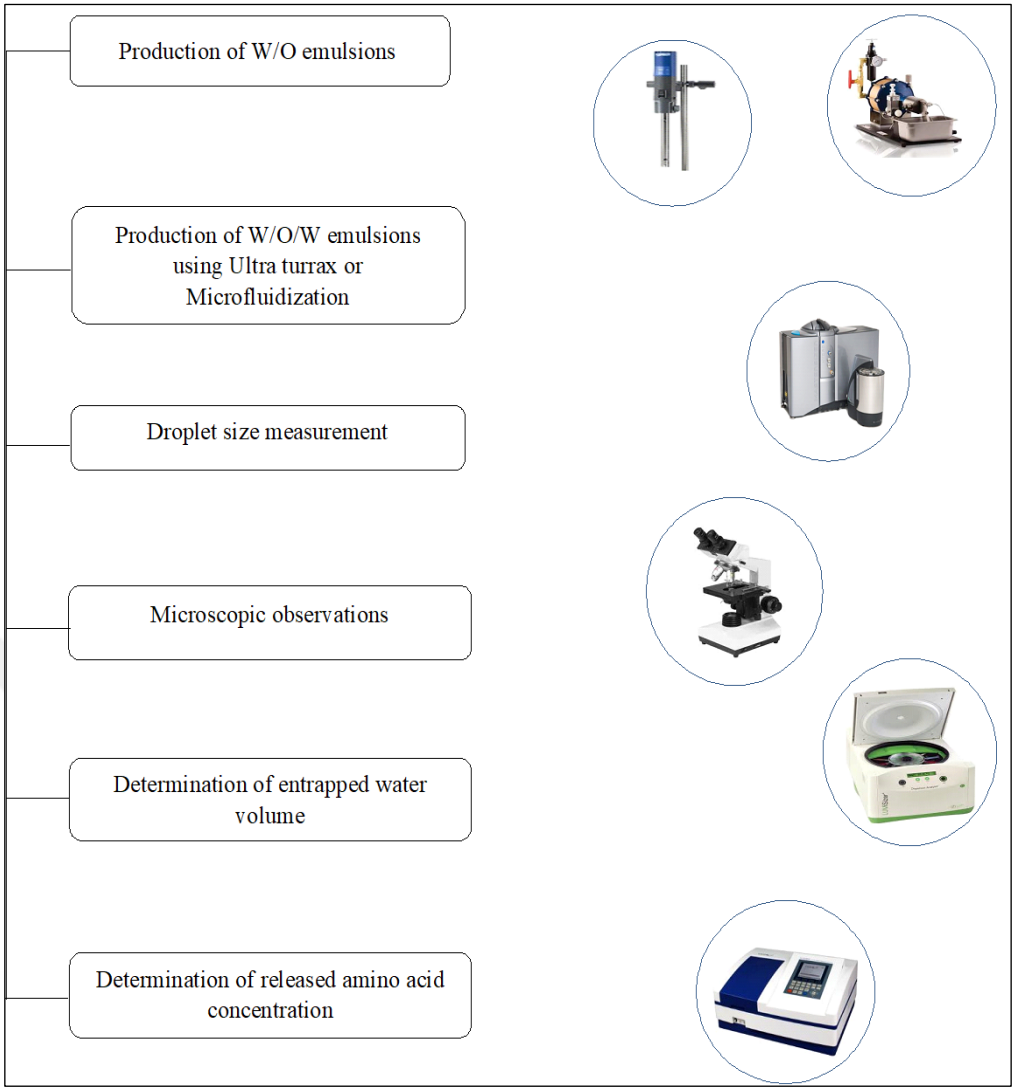

Figure 3.1 : Experimental flow chart of the main experiments performed in this thesis.

3.2.1 Emulsion preparation

3.2.1.1 W/O-emulsion preparation

The oil phase was prepared with 95% HOSO and 5 wt% of the hydrophobic emulsifier PGPR 4150 unless stated differently. To study the effect of the oil phase, MCT oil was used as well, whereas for the NMR experiments glyceryltrioctanoate was used instead of HOSO. In the experiments in which the effect of the hydrophobic emulsifier type and concentration was examined, 1-5 wt % PGPR, 5% of a PGPR-native sunflower lecithin mixture (1/1 mass ratio), 5% native sunflower lecithin, and 5% of a PGPR-PC depleted lecithin mixture (1/4-2/3-3/2-2/3-1/4-0/5 mass ratio) were prepared.

The internal water phase was prepared by dissolving 15 mM amino acid/di-peptide in distilled water that contained 0.02 % wt NaN_3 and 0.1 M KCl, unless stated differently. In the experiment in which the effect of the concentration of the amino acid was studied, the internal water contained also 30, 60 and 120 mM amino acid. Considering the 25/25/50 mass ratio of the W/O/W, the equilibrium concentration (i.e. the concentration reached upon homogeneous distribution of the solute over the internal and external aqueous phases) was expected to be 1/3 of the concentration that was used in the internal water phase (W1). Unless stated otherwise, the pH of the internal phase was about 6.8-7.0. For different pH values, the pH of the solution was adjusted using 0.1, 0.5 and 1 N NaOH and HCl. The primary emulsion (W_1/O) was prepared in a 50:50 w/w ratio. The internal water phase (W_1) was added gradually to the oil phase (O) after both phases were heated to 60°C. The phases were mixed with an Ultra-turrax (S25-10G, IKA-Werke, Germany) for 5 minutes at 24000 rpm.

3.2.1.2 W/O/W-emulsion preparation

The external water phase was prepared by dissolving 0.02 % wt NaN_3, 0.1075 M KCl and 2wt % Tween 80 in distilled water, unless specified otherwise. Depending on the entrapped amino acid concentration, the KCl concentration was adjusted to maintain osmotic balance. For the experiment in which the effect of the hydrophilic emulsifier concentration was investigated, 0.5 and 1% Tween was also used. To investigate the effect of xanthan gum, 0.3% of this thickener was also included in the external phase. Unless stated otherwise, the pH of the internal and external aqueous phase was about 6.8-7.0. For different pH values, the pH of the solution was adjusted using 0.1, 0.5 and 1 N NaOH and HCl.

Freshly prepared primary emulsion was mixed with the external water phase in a 50:50 w/w ratio. Subsequently, this mixture was homogenized using an Ultra-Turrax S25-10G (IKA®Werke, Germany) for 5 minutes at 17500 rpm, unless stated differently. To investigate the effect of homogenization method and intensity, a Microfluidizer (type M110S, Cobra Engineering NL) and Ultra-Turrax S25-10G (IKA®Werke, Germany) were used in the second step of the preparation procedure. For the samples homogenized with an Ultra-turrax, rotational speeds of 17500, 21500 or 24000 rpm were evaluated. The microfluidized double emulsions were emulsified using 0.75 or

1.00 bar of driving air pressure, which corresponded to 105 or 140 bar of fluid pressure, respectively. After preparation, the emulsions were stored at 4, 20 or 37 ºC.

3.2.2 Emulsion characterization

3.2.2.1 Determination of the W/O/W average droplet size

For the oil droplet size determination of the double emulsions, a Malvern Mastersizer 3000 (Malvern Instruments, Worcestershire, UK) linked to a Hydro MV sample unit was used. The real refractive indices for the continious and dispersed phases were 1.33 and 1.53, respectively. For the oil phase, the imaginary refractive index was set to 0.01. The sample was diluted in the dispersing unit with 0.1075 M KCl, unless stated otherwise. For the samples that contained a different KCl concentration (than 0.1075 M), the dilution solution was adjusted to the same KCl concentration of the external aqueous phase to prevent osmotic inbalance. In order to examine the primary instability mechanism in double emulsions, the samples were diluted with 0.1% SDS to enable better solubilization. The sample was added into the sample dispersion unit until an obscuration of 10-20% was obtained. Subsequently, the stirring speed was fixed at 2500 rpm and measurements were performed.

3.2.2.2 Light microscopy

In order to prove the double emulsion structure, the samples were diluted ten times with a 0.1 mM KCl solution. The microscopic images were taken by an optical microscope (CX40RF200, Olympus optical, Japan) linked to a digital camera (AxioCam ERc 5S). As objectives, 100-fold and 40-fold objectives were used for primary and double emulsions, recpectively.

3.2.2.3 Entrapped water fraction

Dickinson and Miller (2001) defined the entrapped water volume fraction as the ratio of the water that initially existed in primary emulsions to the one present in the internal water droplets of double emulsions. The entrapped water volume was determined using analytical photocentrifugation with the method reported by Balcaen *et al.* (2016). About 0.4 mL of the samples was filled in 2.2 mm path length rectangular cells in duplicates and the measurement was performed using of a LUMiSizer® (LUM GmbH; Berlin, Germany) at room temperature for 2 hours at 3000 rpm cenrifugation speed. The light intensity was fixed at 100% prior to measurement. The entrapped water

volume fraction was calculated considering the positions of cream-serum as well as cream-air interfaces at 30% transmission.

3.2.3 Determination of released amino acid

The determination of the released amino acid concentration was carried out according to the method of Satake *et al.* (1960). This method is based on the color formation as a result of the reaction between primary amino groups and TNBS. Hence, the amino acid and di-peptide concentrations were quantified using spectrophotometry.

3.2.3.1 Collection of the external phase

The serum phase of the double emulsions was taken by means of a syringe after centrifugation (Sigma 1-15P, SIGMA Laborzentrifugen, Osterode am Harz, Germany) of the samples for 10 minutes at 17000 g. Subsequently, the extracted solution was filtered over a nylon membrane with 0.20 µm pore size (CHROMAFILE® Xtra PET 20/25, VWR International, USA). The filtered samples were stored in the fridge until the release analyses were performed.

3.2.3.2 Reaction with TNBS

Prior to spectrophotometric analysis, the samples were diluted 10, 20, 40 or 80 times for initial concentrations of 15, 30, 60 and 120 mM entrapped compound, respectively. For the amino acid and TNBS reaction, the reagents 0.6 mM TNBS and 0.48 M $NaHCO_3$ were added to a diluted sample in the same volume (each 1 mL). Then, the reaction proceeded in a waterbath placed in a dark place during 3 hours at 40 ^{o}C. To terminate the reaction, 1 ml of 1M HCl solution was added to the sample tubes. A spectrophotometer (UV-1600PC UV-VIS, VWR International, Radnor, PA) was used to read the absorbance values of the samples at 340 nm. As blank solution, the external water phase solution which does not contain amino acids or di-peptides was used. The standard solutions were prepared by diluting the internal water phase containing varying amino acid concentrations with the external water phase without amino acids or di-peptides. Hence, a standard curve of amino acids or di-peptides with concentrations ranging from 0 to 5 mM was established.

3.2.3.3 Release kinetics

The estimated release parameters were deduced from a mathematical model using the experimental release data of amino acids and di-peptides.

$$C = C_{eq} - (C_{eq} - C_0)\, e^{-t/t_a} \quad (3.1)$$

The inital, released and equilibrium concentrations in the external water phase were shown by C_0, C and C_e (mM), respectively. The incubation time is indicated by *t* (days), whereas the kinetic constant is represented by t_a (days). The average residence time (t_a) indicates the time required to reach 63% of the equilibrium concentration of the amino acid/di-peptide and hence enables to quantify the kinetics of the release. The experimental release results were fitted to equation (3.1) using Matlab R2018b to obtain the estimated kinetic parameters. Based on the mass ratio of the double emulsions (25/25/50), 5 mM of amino acid/peptide should be present in the external water phase after the complete release of a 15 mM enclosed component in the internal water phase. For the other concentrations enclosed in the internal phase, the released solute concentration was expected to be 1/3 of the initial enclosed concentration. The C_e was fixed at 5 mM when it was found below 5 mM or above 10 mM for emulsions whose primary aqueous phase contained 15 mM of solute. Using the jacobian matrix, the standard errors of the calculated parameters were also obtained in Matlab.

3.2.4 Release of amino acids in simulated gastrointestinal system

The release of the amino acids in a simulated gastrointestinal system has been examined according to the method of Nasrabadi *et al.* (2020) with slight modifications. In the experiment, 5 mM glycine and L-leucine solutions as well as double emulsions containing 5 mM of these amino acids were prepared. The background altered as a function of digestion time because the digestion modified some compounds (i.e lipids, enzymes). Therefore, the absorbance of the amino acid solution and of the amino acid-containing emulsion was found by subtracting the background measured on the external water phase (blank solution) and external water phase of a double emulsion without amino acids (blank emulsion), respectively. Samples and blank samples were added into simulated gastric fluid (SGF) and then into simulated intestinal fluid (SIF).

3.2.4.1 Gastric conditions

Emulsions and amino acid solutions (10 mL) were adjusted to pH 2 and mixed with 10 ml SGF (NaCl and pepsin) at pH 2. The pH of the mixture was adjusted to 2 and the mixture was placed in an incubator at 37 °C. It was agitated using an orbital shaker at 20 rpm. Samples were taken during the gastric release after 0, 30, 60, 90, and 120 min.

3.2.4.2 Intestinal conditions

The digestate (10 mL) from the gastric media after 2 hours of gastric digestion was adjusted to pH 7. Subsequently, the digestate was mixed with 10 ml SIF, which contained pancreatin (trypsin-based), 0.4 mg/ml lipase, 0.3 mM $CaCl_2$ and 47 mM NaCl in 50 mM phosphate buffer. Bile extract was not used in our experiments as the orange color of the bile solution affected the absorbance of the samples and hence interfered with the spectrophotometric amino acid quantification method. As bile extract is known to have a significant impact on the digestion of fat-containing matrices, it is important to consider the obtained results with due care. For future research, it is advised to use another amino acid quantification technique that does not suffer from interference of bile extract to obtain more meaningful results. The digestate pH was adjusted to 7 and incubated during 2 hours at 37 °C. The samples after the digestion step were centrifuged at 17000 g for 10 min and the outer water phase was filtered through a 0.2 µm syringe filter. The spectroscopic measurements were performed at 340 nm after digestion (Satake *et al.,* 1960). The released concentration of the amino acid was calculated from the absorbance considering the linear relationship of the standard curve of amino acid solutions as it was explained in section 3.2.3.

3.2.5 High resolution NMR

For NMR measurements, 60 mM of L-phenylalanine was encapsulated in double emulsion. This amino acid was selected as it provides typical contributions in the NMR spectrum that do not overlap with the triglyceride signals due to the aromatic ring. As oil phase, glyceryl trioctanoate oil was used due to its high saturation level and liquid state up to 11 ^{o}C; due to the absence of double bonds, a more simple NMR spectrum is obtained with less chance for interference with other compounds. A waterbath was used at 11 ^{o}C during the second step of the preparation procedure to avoid initial release

during preparation. NMR measurements were performed for freshly prepared samples at different temperatures for different incubation times (i.e. 20°C for 16h, 30°C for 16h, 40°C for 4 h, 50°C for 2h and 60°C for 1h).

3.2.6 Statistical analysis

Linear regression analysis was used to analyze the changes in the droplet size and entrapped water volume fraction of the double emulsions. Statistical analysis was performed considering the 95 % confidence intervals to check significant differences between the samples. Also, a Tukey post-hoc test, at a confidence value of 95%, was performed in SPSS to check the significance of the differences in droplet size and entrapped water fraction of double emulsions. The differences in the release kinetics of amino acids and di-peptides were evaluated in SPSS using a paired *t*-test or Wilcoxon test considering the distribution at a confidence value of 95%. In order to visualize the release of amino acids and di-peptides, principal component analysis (PCA) was carried out in SPSS.

4. RESULTS AND DISCUSSION

4.1 Optimisation of the Spectrophotometric Method for Released Amino Acid Quantification

When following the method as described by Satake *et al.* (1960), it was not possible to measure leucine concentrations over 0.5 mM (included) as the recorded absorbance value exceeded the measurement range of the spectrophotometer. Therefore, a modification of the original method was necessary. Another issue was identified due to the high background absorbance of the reagent TNBS which for many of the measured concentrations was higher than the signal from the free amino groups. The original Satake method consists of adding 1 mL of a 4% w/v $NaHCO_3$ buffer to 1 mL of sample. Subsequently, 1 mL of 3.4 mM (0.1% w/v) TNBS is added and the reaction terminated with 1 mL of 1N HCl (Satake *et al.,* 1960). However, different concentrations and volumes of reagents have been reported in literature. For instance, Obi (1980) used 1 mL of 2.4 mM TNBS and 3 mL of HCl to terminate the reaction therefore diluting the final concentration of both TNBS and reaction product as compared to Satake. Table 4.1 indicates that the absorbance value (without blank) of a 0.5 mM leucine solution decreased as the TNBS concentration used for the reaction decreased, keeping the same incubation time (3h). Furthermore, with all the TNBS concentrations used, except from 3.4 mM, it was possible to measure the absorbance of a 0.5 mM leucine solution. L-leucine was used in this optimisation study since a high absorbance was expected for the external aqueous phase of leucine containing double emulsions due its pronounced release as a further consequence of the relatively hydrophobic character of this amino acid.

Table 4.1 : Absorbance values of blanks and 0.5 mM leucine solutions measured using different TNBS concentrations. N.D. stands for not detectable because the measurement exceeded the instrumental range (Abs > 3).

TNBS (mM)	Absorbance (Blank)	Absorbance (0.5 mM Leu – Blank)
0.6	0.431 ± 0.001	1.247 ± 0.114
0.85	0.523 ± 0.001	1.528 ± 0.012
1.7	0.963	1.706 ± 0.048
2.4	1.368	1.822
3.4	1.926 ± 0.018	N.D.

Figure 4.1 shows that the absorbance of a 0.5 mM leucine solution reacted with a 0.85 mM TNBS solution increased as a function of increasing incubation time from 1 to 17 hours. Considering this result, together with the results reported in Table 4.1, it is safe to say that the reaction after 3 h of incubation at 40°C is not completed when using TNBS concentrations lower than 2.4 mM, whereas the reaction was already completed after 2 hours when using 3.4 mM TNBS, when carried out at the same incubation temperature and within the same optimal pH range (7.5 - 8.5) (Satake *et al.,* 1960).

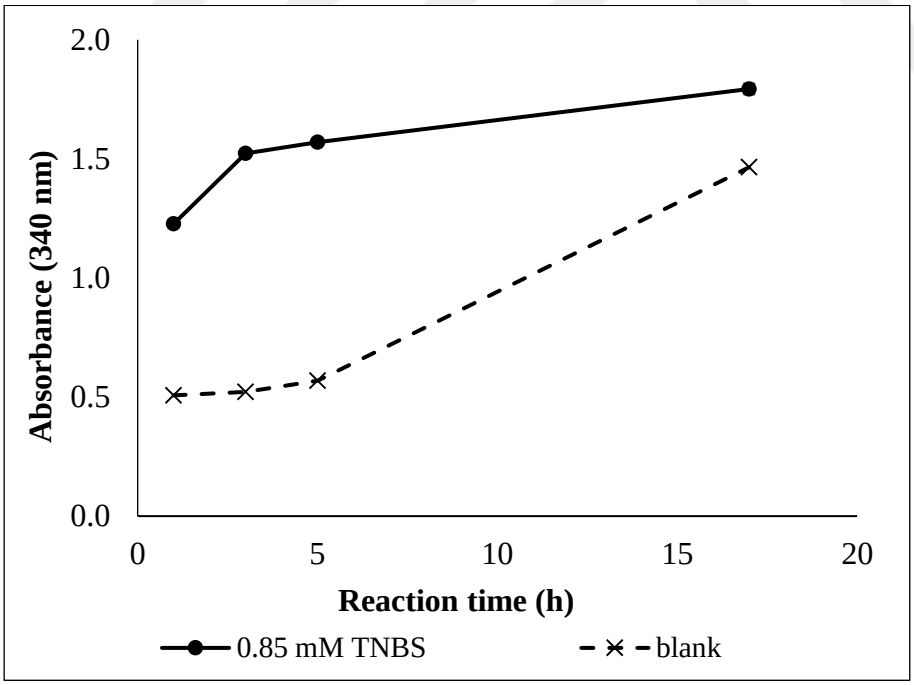

Figure 4.1 : Absorbance values as a function of reaction time of 0.5 mM L-leucine solution reacted with 0.85 mM TNBS and 4% NaHCO3 (continuous line) and a distilled water blank (dashed line).

Because the reaction is still going on after 3 h when such low TNBS concentrations are used, experimental errors could occur due to a delay of the time to start (and stop) the reaction between the first and last sample. However, Figure 4.2 indicates that the absorbance of a 0.5 mM leucine solution measured at 30 minutes intervals (2h 30, 3h and 3h 30) showed only a slight increase. Moreover, also the absorbance of the blanks containing only TNBS increased with the incubation time, as is also shown in Figure 4.2. According to Fields (1971) the increase of the blank absorbance with incubation time is due to the reaction of TNBS with hydroxyl ions at basic pH values.

It is generally known that the linearity between absorbance and concentration of the absorbing species (as predicted by the Lambert-Beer law) is limited at too high concentrations. Considering Figure 4.2, the TNBS concentration of 2.4 mM was not choosen as it displayed a high absorbance even for the blank solution. The solution that contained 0.6 mM TNBS demonstrated the lowest absorbance among the studied concentrations for both the blank and the leucine solution reacted with TNBS. Also, the increase in the absorbance with increasing incubation time was very limited. Hence, 0.6 mM TNBS was used in this thesis for the quantification of the amino acids.

Satake *et al.* (1960) reported that the pH has a significant effect on the absorbance. They reported that the optimum pH was around 7.5-8.5 which was obtained by the preparation of 4% $NaHCO_3$ solution. In this study, 0.48 mM $NaHCO_3$ was used instead of 4% as the optimum pH was obtained with this concentration (pH=8) wherease the latter showed slightly lower pH (pH=7.5).

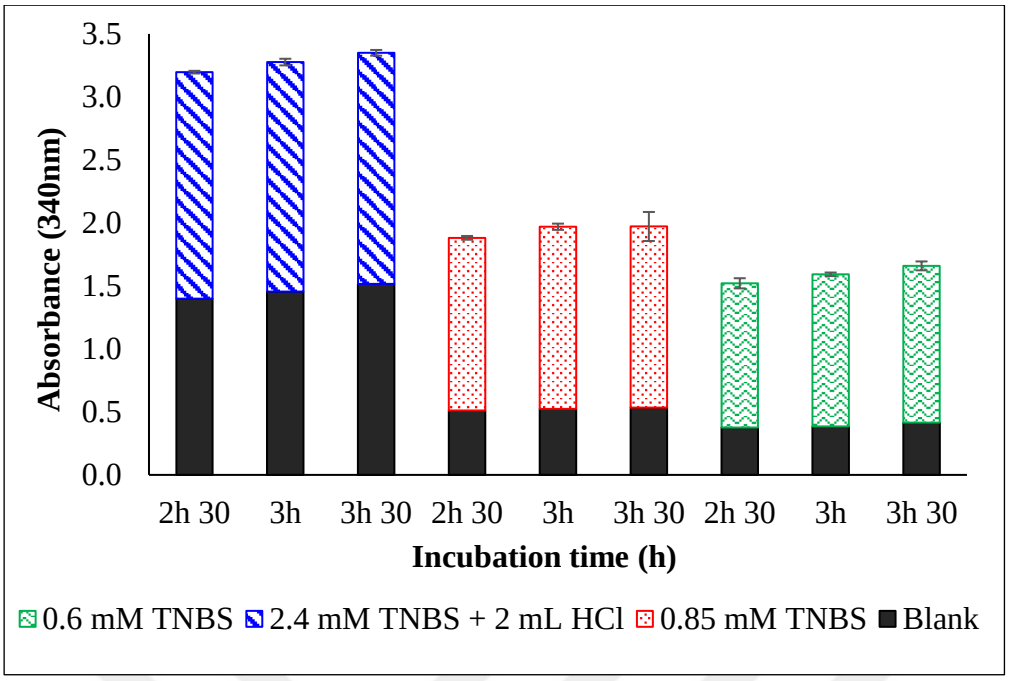

Figure 4.2 : Comparison of the absorbance values of a 0.5 mM leucine solution measured at intervals of 30 minutes with 0.6 mM TNBS (green stripes), 0.85 mM TNBS (red dots) and 2.4 mM TNBS (blue diagonal stripes) when the reaction is stopped with 2 mL of 1N HCl; blanks were prepared with distilled water (black).

4.2 Effect of Solute Characteristics

4.2.1 Effect of amino acid hydrophobicity

4.2.1.1 Particle size

The size distribution of the double emulsion without amino acid ranged from 20 to 200 µm immediately after preparation (Figure 4.3). This wide size distribution resulted into pronounced creaming which was clearly visible in the samples since a thickener (i.e xanthan gum) was not involved in the double emulsions. A bimodal distribution, which was observed for the samples stored at 4 °C for 16 days, is usually obtained for weakly aggregated dispersions. In this kind of system, in addition to the peak of the original particles, another peak is also located to the right of the original mode indicating the aggregated particles. The microscopic observations also proved the double emulsion structure as well as the presence of enclosed internal water droplets during 16 days of storage. A microscopic image of the double emulsion without amino acid was inserted in Figure 4.3.

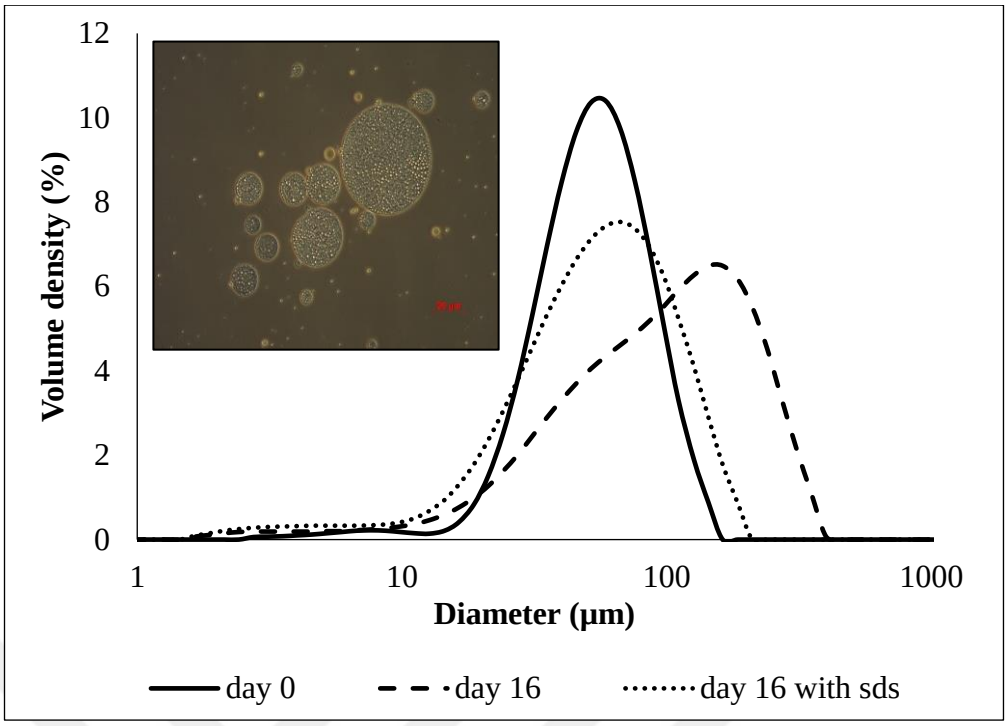

Figure 4.3 : Volume-weighted droplet size distribution of a double emulsion without amino acid directly after preparation and after 16 days of storage at 4 °C, diluted in either water (dashed line) or in 0.1% SDS (dotted line) in water. The insert indicates a microscopic image of the double emulsion after 16 days of storage.

In order to investigate the main instability mechanism of the double emulsions, the droplet size was measured in the presence and absence of SDS after 16 days of storage. The droplet size distribution of double emulsions after 16 days in the presence of SDS was similar to the result observed immediately after preparation. This clearly confirmed that the increase in droplet size of the double emulsions resulted from flocculation as it is a reversible mechanism. If the primary mechanism was coalescence, the droplets would not become separated despite of the presence of SDS. The existence of large droplets is thought to be a further consequence of creaming, as this forces the droplets to come closer in the cream layer.

From the particle size analysis determined by laser diffraction, the volume weighted average droplet size was around 50 µm following the preparation regardless of the amino acid type enclosed in the double emulsions (Figure 4.4). Morever, the volume weighted average droplet size ($d_{4,3}$) increased during 16 days of storage. Considering the temperature effect on the oil droplet size, the increase in double emulsions stored at 4 °C was higher in comparison with 37 °C. The most significant difference was found in the double emulsion contained alanine, in which the average droplet size reached to about 90 µm at 4 °C.

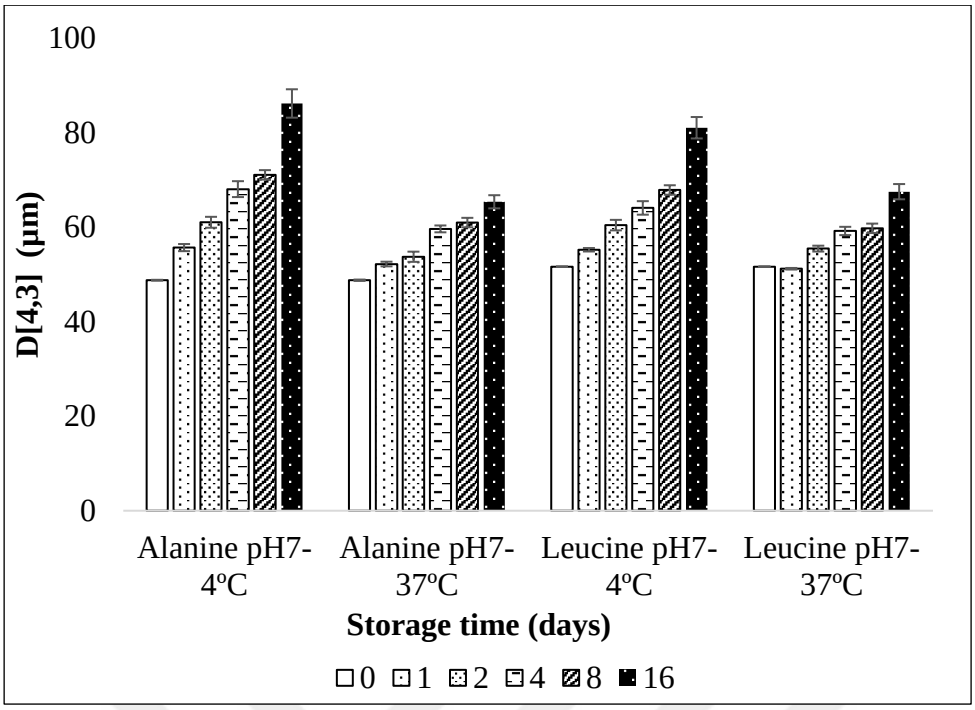

Figure 4.4 : Volume-weighted average droplet size (D[4,3]) of L-leucine and DL-alanine containing double emulsions at pH 7 upon storage for 0 up to 16 days at either 4 or 37 °C.

Linear regression analysis of the average droplet size data was performed with 95% confidence interval. The slope (corresponding to the average rate of change) and R^2 values were presented in Table 4.2. Regarding the amino acid type, Table 4.2 obviously presents that it does not have an effect on the oil droplet size of the double emulsions.

Table 4.2 : Average rate of change of the volume-weighted average droplet size during 16 days of storage at 4 or 37 ºC (expressed in µm/day) of L-leucine (L) and DL-alanine (A) containing double emulsions at pH 7.

Amino acid	T (°C)	Coefficient of determination (R^2)	Rate of change of D43 (µm/day)
A	4	0.92	2.09±0.84[a]
	37	0.84	0.95±0.58[a]
L	4	0.95	1.71±0.52[a]
	37	0.92	0.97±0.40[a]

4.2.1.2 Entrapped water volume

By means of analytical centrifugation, the external water phase and cream layer, in which the internal water droplets were involved, were completely separated after 2 hours. Creaming, which occured as the primary destabilization mechanism in the

double emulsions, can be seen in Figure 4.5. The cream layer occupied about 50% of the sample while 26.9% of this layer was occupied by the oil fraction. Hence, the enclosed water droplets constituted 23% of this sample which corresponded to 92% of entrapped water volume fraction. This indicates that 92% of the internal water phase was effectively encapsulated within the oil droplets in the final double emulsion, whereas 8% was released in the external water phase. This entrapped water volume fraction is sometimes also indicated as the water yield.

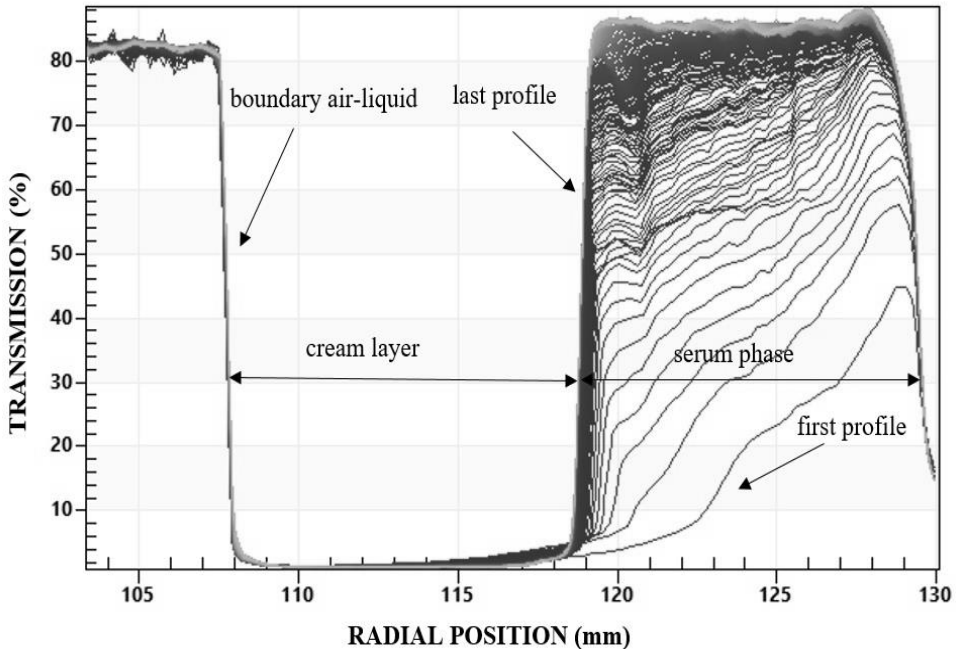

Figure 4.5 : Transmission profiles of an L-leucine containing double emulsion, upon 2 hours of centrifugation at 3000 rpm.

From Figure 4.6, the enclosed water volume fraction of all double emulsions fluctuated about 100% regardless of the amino acid used and storage temperature. This outcome clearly proves the iso-osmotic conditions in the double emulsions. Therefore, no loss or gain was observed in the internal water volume during storage.

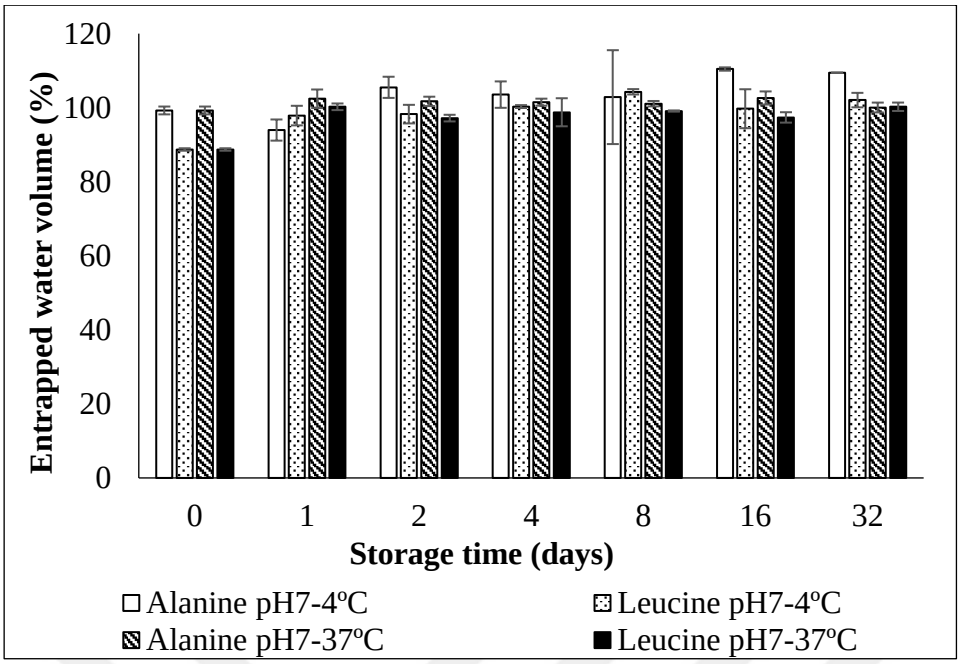

Figure 4.6 : Yield of entrapped water in double emulsions containing either L-leucine or DL-alanine (at pH 7) during 32 days of storage at 4 or 37 °C.

4.2.1.3 Amino acid release

The influence of amino acid type and temperature on the release was determined. In the tests, interactions between these effects were not considered. The released amino acid concentration was quantified in the external water phase. Due to the possibility of internal water phase transport to the external water phase, the absorbance spectra of these phases were measured to check the composition effect.

The internal and external water phases exhibited a similar absorbance spectrum although Tween 80 was found only in external water phase. For released amino acid quantification in the external water phase, the method of Satake *et al.* (1960) was used. They reported an absorbance of about 0.4 for a 0.13 mM L-leucine solution, while it was found as 0.35 for 0.10 mM released leucine concentration in the current research. Therefore, this quantification method was followed as identical absorbance values were observed at 340 nm as stated by Satake *et al.* (1960).

4.2.1.3.1 Temperature effect on the release of amino acids

Figure 4.7 indicates that the fastest release was measured in the L-valine containing double emulsions stored at 37 ºC. The release of amino acid was fastest at 37 ºC, while the slowest release was measured at 4 ºC. Looking at the initial release of amino acids immediately after preparation, the released concentration was below 1 mM in all

double emulsions. It follows that amino acid release was limited during the emulsification. The equilibrium concentration was achieved after about 8 days for L-valine containing double emulsion stored at 37 ^{o}C.

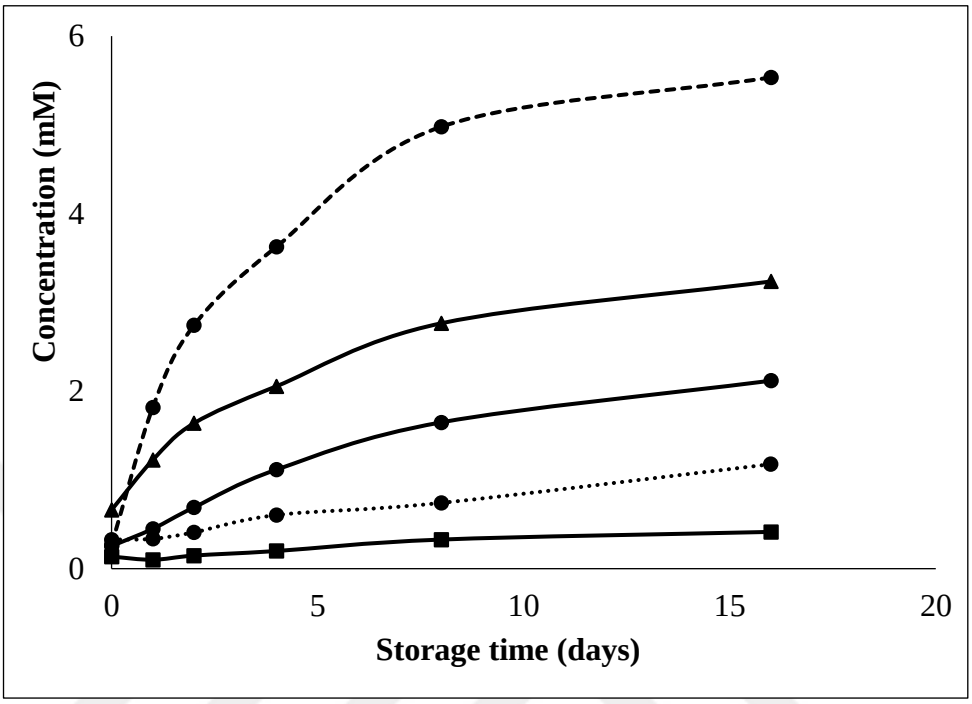

Figure 4.7 : Released L-valine (circles), L-leucine (triangles), and DL-alanine (squares) concentrations in double emulsions upon storage at pH 7 at either 4 °C (dotted line), 20 °C (full lines) or 37 °C (dashed line).

A paired *t* test was performed to analyze the effect of the amino acid type and temperature as well as their interactions. The *p* values are demonstrated in Table 4.3. The normality assumption was checked using R studio ($p \geq 0.05$). Concerning the amino acid effect (presented by rectangles), significant differences were observed in all 9 possible combinations (Table 4.3). Considering the influence of the storage temperature (presented by ovals), a remarkably slower release was observed for all double emulsions at lower temperature with an exception of L-leucine at 4 versus 37 ^{o}C; $p=0.06$).

Table 4.3 : Significant differences indicated with *p* values in release kinetics of amino acids in double emulsions concerning the influence of amino acid type (squares) and temperature (ovals) at the 95% confidence level.

	A7 T4	A7 T20	A7 T37	L7 T4	L7 T20	L7 T37	V7 T 4	V7 T20
A7 T4	-							
A7 T20	**(0.03)**	-						
A7 T37	**(0.02)**	**(0.02)**	-					
L7 T4	**(0.01)**	**(<0.01)**	**(0.03)**	-				
L7 T20	**(<0.01)**	**(<0.01)**	(0.27)	**(0.01)**	-			
L7 T37	**(<0.01)**	**(<0.01)**	**(<0.01)**	(0.06*)	**(0.03)**	-		
V7 T4	**(0.02)**	**(<0.01)**	**(0.025)**	**(<0.01)**	**(<0.01)**	**(<0.01)**	-	
V7 T20	**(0.03)**	**(0.02)**	**(0.02)**	**(<0.01)**	**(<0.01)**	**0.03***	**(0.045)**	-
V7 T37	**(<0.01)**	**(0.01)**	**(0.01)**	0.05	**(0.03)**	**(<0.01)**	**(0.01)**	**(0.01)**

In order to quantify the amino acid release by means of a limited set of parameters, a mathematical model was used. The release kinetics were evaluated by the average residence time t_a which can be defined as the time required to reach 63% of the equilibrium concentration of the amino acid. The initial amino acid concentration was indicated by the parameter C_0 which was not zero due to some amino acid release during the preparation.

Table 4.4 indicates the estimated C_0 and t_a parameters of the double emulsions which contained different amino acids at pH 7, stored at 4, 20, or 37 °C. As the release of amino acids was too slow at 4 °C, reliable estimated parameters could not be obtained as can be seen from the large confidence interval. However, a nice comparison could be performed concerning the amino acid effect for the samples stored at 37 °C. When the kinetic constants (t_a) of the amino acids were compared at 37 °C, the lowest t_a was found for leucine, whereas the highest one was observed for glutamine (Table 4.4). Looking at the hydrophobicity values in Table 3.1, the kinetics of the amino acid release are clearly related to their hydrophobicity.

Table 4.4 : Optimized average residence time t_a and original amino acid concentration in the external phase C_0 (with their 95% confidence interval) of L-leucine release in double emulsions at pH 7 during storage at 4, 20, and 37 °C.

Amino Acid	T (°C)	t_a (d)	C_0 (mM)
Leucine	4	5.2±0.7	0.92±0.11
	20	5.2±0.6	0.80±0.07
	37	1.6±0.4	0.95±0.64
Valine	4	62±107	0.34±0.04
	20	23±2	0.40±0.10
	37	3.6±0.4	0.45±0.20
Alanine	4	166±52	0.23 ±0.06
	20	206±26	0.13±0.02
	37	7.5±2.3	0.43±0.20
Glycine	4	1732±1344	0.17±0.01
	20	210±23	0.15±0.02
	37	7.4±1.8	0.07±0.06
Glutamine	4	1363±619	0.05±0.01
	20	1135±378	0.03±0.01
	37	292±80	0.10±0.03

The lowest estimated t_a parameter was obtained at 37 °C for all amino acids, which means the fastest release occurs at this temperature. These findings are supported by the results of Weiss *et al.* (2005) as they found a faster tryptophan release at 23 °C compared to 7 °C. Faster release kinetics at higher temperatures are the results of increased solubility and a higher diffusion coefficient of the amino acids in the oil phase. According to the thermal agitation theory, there is a proportional relation between the square root of the absolute temperature and diffusion speed. Therefore, the molecules gain a higher kinetic energy at higher temperatures. Another possible reason might be the less viscous oil phase at a higher temperature which enables an easier migration behaviour of the enclosed amino acids to the external phase. These data indicate solution-diffusion transport as the main release mechanism of the entrapped amino acids.

4.2.1.3.2 Hydrophobicity effect on the release of amino acids

Figure 4.7 demonstrates that L-leucine release in double emulsions was faster in comparison with L-valine and DL-alanine during 16 days of storage at 20 °C. L-valine, on the other hand, exhibited a more significant tendency to be transported as compared to DL-alanine at 20 °C. The released DL-alanine concentration was relatively low during 16 days of storage at 20 °C.

Based on the statistical tests in Table 4.3, significant differences were found among all amino acids (shown by squares) stored at 4, 20 and 37 ^{o}C (p<0.02). This is a clear effect of hydrophobicity which substantially determines the release kinetics of amino acids. As L-leucine has the largest hydrocarbon chain and hence the highest hydrophobicity in the current study, its kinetic constant (t_a) was lowest indicating the fastest release among all amino acids studied. Moreover, remarkable inital release (C_0) concentrations were estimated for L-leucine, while it was below 0.5 mM for the other amino acids. Considering the inital release concentration of L-leucine, at least 15% of the entrapped L-leucine was released just after preparation (Table 4.4).

Although DL-alanine and DL-valine are relatively less hydrophobic than L-leucine, they are also categorized as hydrophobic components. The estimated t_a value of L-valine was for 3.6±0.4 days, while it was only 1.6±0.4 days for L-leucine at 37 °C (Table 4.4). The much higher t_a of DL-alanine was due to its less hydrophobic character which enables it to be dissolved in the aqueous phase. For the hydrophilic amino acids, the estimated kinetic constant was found larger than the storage period, except at 37 °C. For the case of glutamine, a very limited release was observed during 16 days, and thus the estimated t_a parameters were larger than the storage time at 4, 20 and 37 °C. This was due to the much lower hydrophobicity of glutamine in comparison with the other amino acids used in the present study.

Principal Component Analysis was performed to illustrate the amino acid release rates of amino acids using only the first component with 92.24% variation (Figure 4.8). The amino acids were ordered from higher to lower hydrophobicity, and the temperature from the higher to lower value from left to right according to increasing predicted release. Larger principal component values indicated faster release, whereas lower values indicated slower release to the external phase. It can be clearly seen that the hydrophobicity had the most significant impact on the release rates of the amino acids studied. Also, decreasing the temperature caused a slower release of entrapped compounds, as can be seen seen from the decreasing trend within each of the ovals in Figure 4.8. Furthermore, positive values were obtained for L-leucine release at each temperature while a positive value was observed only at 37 °C for valine and alanine. For glycine and glutamine, all principal component values were negative, indicating slow release at all conditions studied.

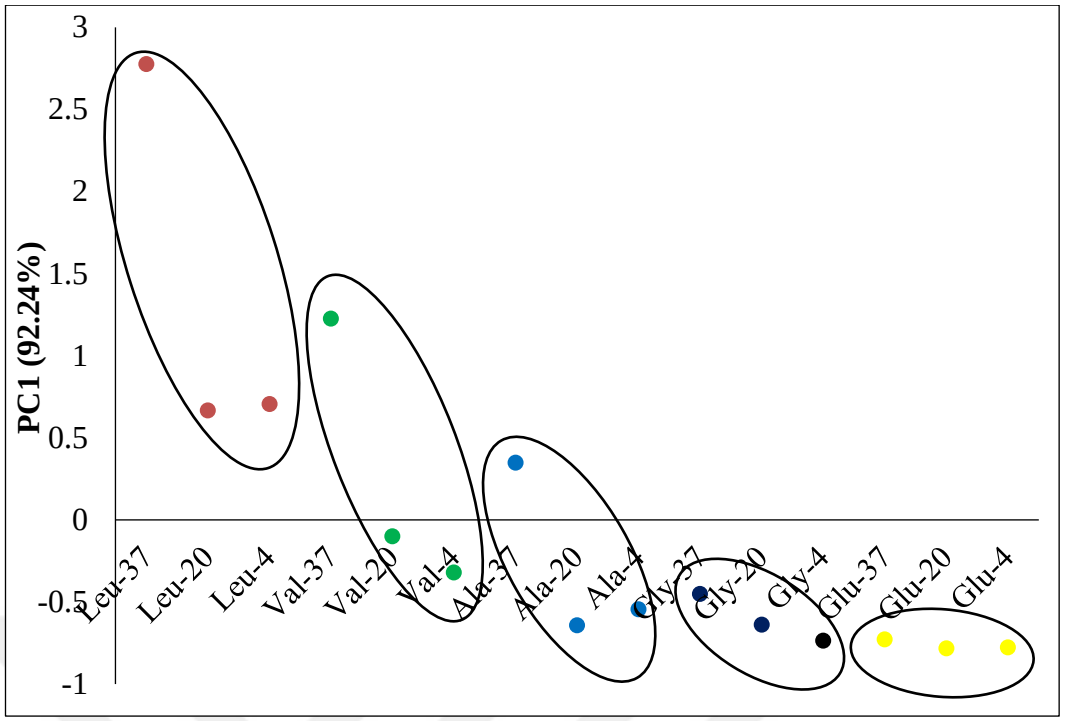

Figure 4.8 : Principal Component Analysis of the released amino acid concentration of 15 double emulsions containing 5% PGPR in the oil phase, as a function of storage time at 4, 20, or 37 °C. The codes along the X-axis indicate the entrapped amino acid (Leucine, Valine, Alanine, Glycine and Glutamine, indicated in brown, green, blue, black and yellow, resp.).

4.2.1.3.3 Release mechanism of the amino acids

In the literature, some diffusion mechanisms were reported concerning the transport of water and water soluble components across the oil phase from double emulsions. Three machanisms have been described as diffusion across a very thin lamella, hydrated surfactant transport and reverse micellar transport (Garti and Lutz, 2004).

It was reported that small solutes can be transported by means of reverse micelles from the inner to the outer aqueous phase of double emulsions. When there is no osmotic balance between the water compartments, water and water soluble compounds can be transported from the inner to the outer phase or vice versa. It was stated that these components can be carried via reverse micelles even in case of an osmotic balance (Garti and Lutz, 2004). Reverse micelles occur when the hydrophobic surfactant is present in a great excess. In this case, the enclosed components are solubilized in the association structures of the reverse micelles which results in the transfer of these compounds to the external phase. A reduction of the hydrophobic surfactant concentration limits the reverse micelles formation, as well as the release kinetics. The

CMC of PGPR was reported as 1% in vegetable oil by Nollet *et al.* (2018), while it was found around 1.8% by Bahtz *et al.* (2016). Due to the high (i.e. 5%) PGPR concentration used to form our double emulsions, the presence of reverse micelles is expected in this study. If the main release mechanism was by reverse micellar transport, similar release rates would be expected in all emulsions. However, this was not the case since the release kinetics were found to strongly depend on the temperature and hydrophobicity effects.

The water transport mechanism varies depending on the contact of the W1/O and O/W2 interfaces. It was reported that the diffusion occurs due to hydrated surfactants in case of contact between the water compartments. Water migration occurs via reverse micelles in case there is no contact between these phases. The release rate due to hydrated surfactants is faster than by reverse micelles and emulsified droplets. Thus, hydrated surfactants might be the primary reason of water migration (Wen and Papadopoulos, 2000).

In order to investigate the role of the emulsifier on the release of valine, double emulsions were stabilized with 2% or 5% PGPR under similar conditions (Figure 4.9). It was found that the inclusion of a lower PGPR concentration did not enable a significantly slower release (p=0.11). However, a PGPR concentration effect can not be ruled out as the t_a increased from 23±2 to 30±4 days when using 5% and 2% PGPR, respectively. This effect is, however, much smaller in comparison with the temperature or hydrophobicity effects. Thus, transport by reverse micelles or hydrated surfactants can not be regarded as the primary transport mechanisms concerning amino acid release.

The thin lamellar diffusion mechanism is based on the migration of the water and water soluble substances through the thin lamella of the surfactant in O/W2 interface (Cheng *et al.*, 2007). In this mechanism, the solutes migrate without coalescence. This mechanism does not seem to happen in our system since L-glutamine and glycine displayed a much slower release compared to L-leucine. However, similar release rates would be expected as a reason of thin lamellar diffusion.

Concerning the release in double emulsions, two pathways were described by Pays *et al.* (2002). The first one is film rupturing which involves the formation of a small hole in the surface of oil and internal water as a result of coalescence. Since the enclosed

water volume fraction was confirmed to remain constant (i.e. no loss or gain of internal water phase), film rupturing does not seem to be a possible reason in our double emulsions. The second one is the compositional ripening in which the entrapped compounds migrate to the oil phase by permeation or diffusion.

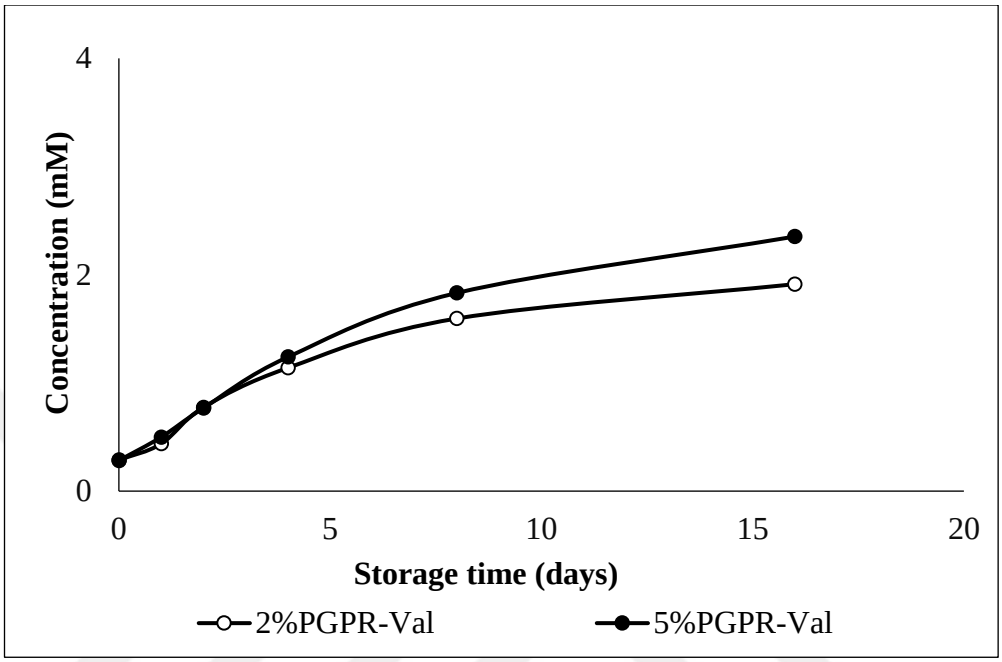

Figure 4.9 : Released L-valine content of double emulsions containing either 2% (open symbols) or 5% PGPR (closed symbols) in the oil phase as a function of storage time at pH 7 and 20 °C.

The data of this study suggest that the amino acid release might be governed by diffusion as a consequence of the chemical potential difference between the water compartments (Pawlik *et al.,* 2010). In this mechanism, the release rates were determined by the solubility and diffusion coefficient of the dissolved compounds in the intermediate phase, with strongly depend on the temperature and hydrophobicity effects. Thus, the primary amino acid release mechanism is thought to be direct diffusion. Moreover, some initial release was observed during preparation. The initial release amount can be related to the second homogenization step which is known as a factor affecting the distruption of the internal water droplets. Nevertheless, this amount was not higher than 2% of the entrapped concentration for slowly releasing amino acids (i.e. glycine and glutamine). Thus, the inital amino acid release was controlled by diffusion as can be seen from the strong correlation between C_0 and t_a: the lowest initial concentration was observed for glutamine, whereas the highest concentration was found for leucine. In fact, a power law fit yielded a determination coefficient of

0.57. Furthermore, no obvious temperature effect was found. In case the power law fit considered t_a at a fixed temperature (e.g. 37 °C), a higher determination coefficient was found (i.e. 0.74). Therefore, the released amount in the second emulsification step was associated with the solution-diffusion mechanism which might be also affected by the increased temperature due to the applied energy for emulsification.

4.2.2 Effect of molecular weight: amino acids versus di-peptides

4.2.2.1 Particle size

The average droplet size of the double emulsions containing amino acids and di-peptides was about 55 µm immediately after preparation wherease it increased to around 65 µm after 16 days timeframe (Figure 4.10). The increase in the average oil droplet size during 16 days of storage was confirmed for all double emulsions (p<0.05). Linear regression of the volume-weighed average droplet diameter versus storage time indicated that no significant differences could be found in the droplet size increase of the double emulsions over time (which is equal to the slope), regardless molecular size of the entrapped compound. The average daily change of the volume weighted average oil droplet size with 95% confidence interval for L-leucine, alanine-leucine and leucine-leucine were 0.75±0.27, 0.66±0.19 and 0.87±0.38 µm/day, respectively. On the other hand, the coefficients of determination (R^2) corresponding to the linear regressions was higher than 0.91 for all double emulsions.

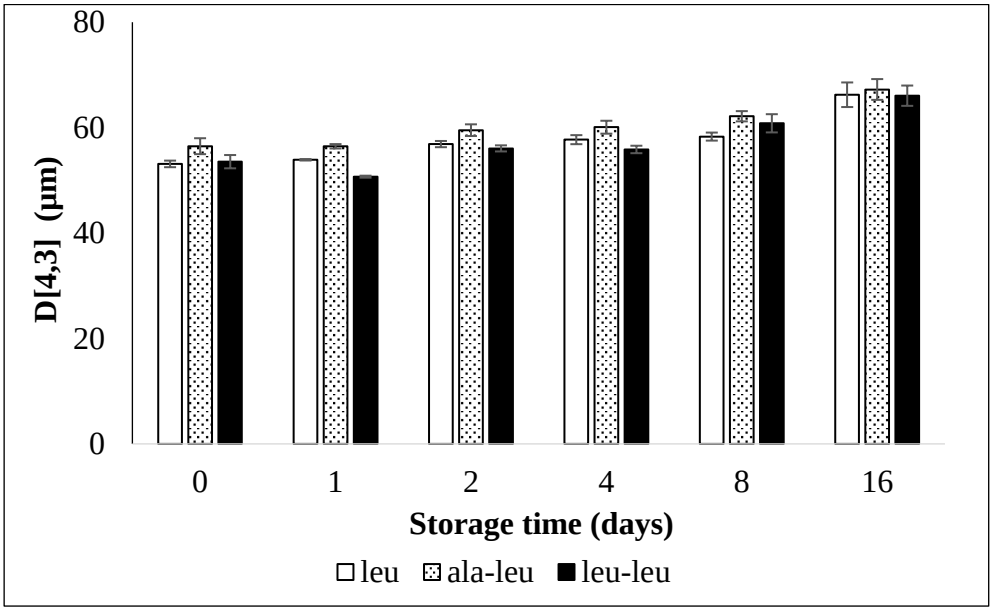

Figure 4.10 : Volume-weighted average droplet size (D[4,3]) of L-leucine, alanine-leucine and leucine-leucine containing double emulsions at pH 7 upon storage up to 16 days at 37 °C.

4.2.2.2 Entrapped water volume

From Figure 4.11, the entrapped water volume of the doube emulsions fluctuated around 80% during 32 days timeframe. The entrapped water fraction stability was achieved as a consequence of the iso-osmotic conditions as it was discussed in section 4.2.1.2.

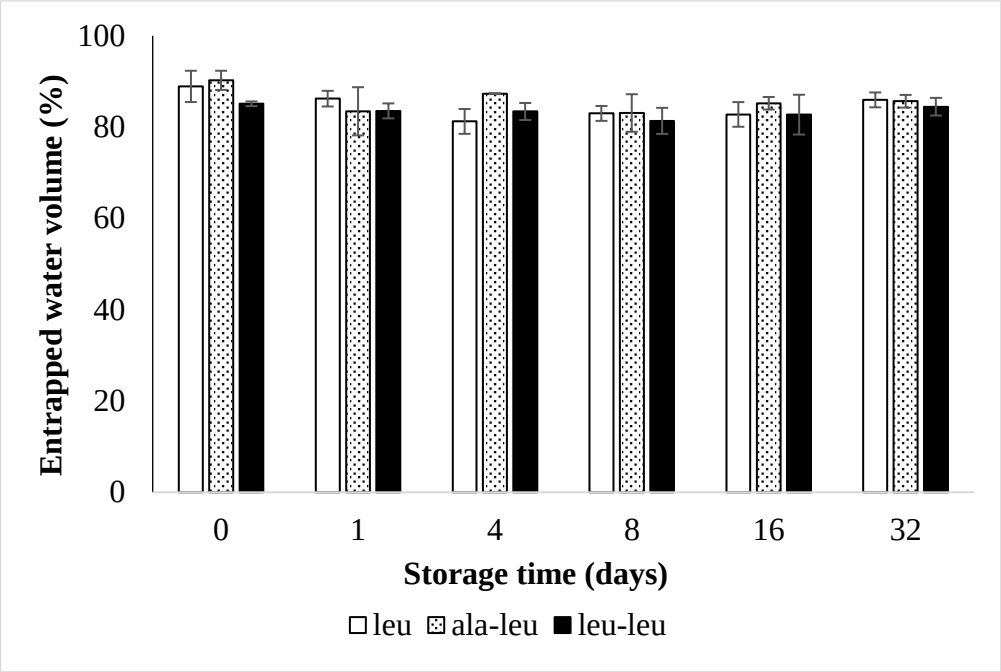

Figure 4.11 : Entrapped water volume fraction of L-leucine, alanine-leucine and leucine-leucine containing double emulsions at pH 7 upon storage up to 32 days at 37 °C.

4.2.2.3 Amino acid and di-peptide release

Amino acids and di-peptides were used to evaluate the effect of the molecular properties of the entrapped compounds on their release kinetics. Figure 4.12 indicates that L-leucine-L-leucine moved fastest to the outer phase whereas DL-alanine released slowest during 32 days of storage at 37 ºC. Statistical tests of the released entrapped compound were significantly different from each other in different days, except from L-leucine and L-leucine-L-leucine at 37 ºC (p=0.09). As it can be seen from Table 3.1, L-leucine-L-leucine had the highest hydrophobicity among the compounds entrapped in this study. Hence, it exhibited the highest tendency for permeation. The slowest release was observed in the double emulsion containing DL-alanine as it has the shortest hydrocarbon side. Considering Table 4.5, these data clearly show that the solute solubility in the oil phase is much more decisive than the solute diffusivity; as the latter is inversely proportional to molecular size, the largest value is expected for

alanine and the smallest for L-leucine-L-leucine. Moreover, the estimated equilibrium concentration (C_{eq}) in the external phase was close to the expected value for all emulsions. The amino acid or di-peptide concentration approached an equilibrium after only 1 day for leucine and L-leucine-L-leucine, whereas it took about 16 days for L-alanine-L-leucine at 37 ^{o}C. On the other hand, the release of alanine was slower since the equilibrium concentration was observed after one month of storage. It was also observed that the initial amino acid concentration in the external water phase was close to 0 for the double emulsion that contained alanine, which shows very limited release during preparation.

In the present work, it is clearly seen that the release of entrapped compounds significantly depends on the hydrophobicity of the enclosed solute. The permeability of small molecules across the intermediate oil phase separating the two aqueous phases can be explained by the solution-diffusion model. According to Overton's rule, the lipid membrane permeability of a molecule increases with its hydrophobicity. Our data indicate that the same holds for the permeability of an oil phase. Based on the permeation coefficients of the solutes, Owusu *et al.* (1992) reported that the rate of transfer across the oil layer in W/O/W emulsion of the hydrophobic L-tryptophan was greater than the rate of the hydrophilic vitamin B. However, Aditya *et al.* (2015) reported that the rate of release in W/O/W was higher for hydrophilic catechin as compared to hydrophobic curcumin in the gastrointestinal environment due to the fact that curcumin tends to remain within the lipid phase, whereas catechin diffuses readily to the hydrophilic releasing media.

Concerning the peptide permeation, Argudo *et al.* (2019) found that an increase in the hydrophobicity of di-peptides led to an enhanced interaction with phospholipid membranes, whereas less hydrophobic dipeptide molecules were expelled from the surface. Wei *et al.* (2017) mentioned that the hydrophilic serine-serine dipeptide was found to desorb from the interface to the aqueous phases, whereas hydrophobic phenylalanine-leucine and amphiphilic serine-leucine tended to accumulate at the interface. This indicates that the structure of the encapsulated compound largely influences the amino acid and di-peptide release.

According to the solution-diffusion transport model, the release kinetics of entrapped hydrophilic compounds in double emulsions during storage depends on both the solute solubility and diffusivity in the oil phase. This explains both the temperature and

hydrophobicity effects: hydrophobic amino acids and di-peptides released faster because of their higher solubility in the intermediate phase. Considering the data of Figure 4.12, it is clear that the solubility effect largely overrules the diffusivity as the release kinetics are proportional to the molecular weight, i.e. slower for smaller molecules.

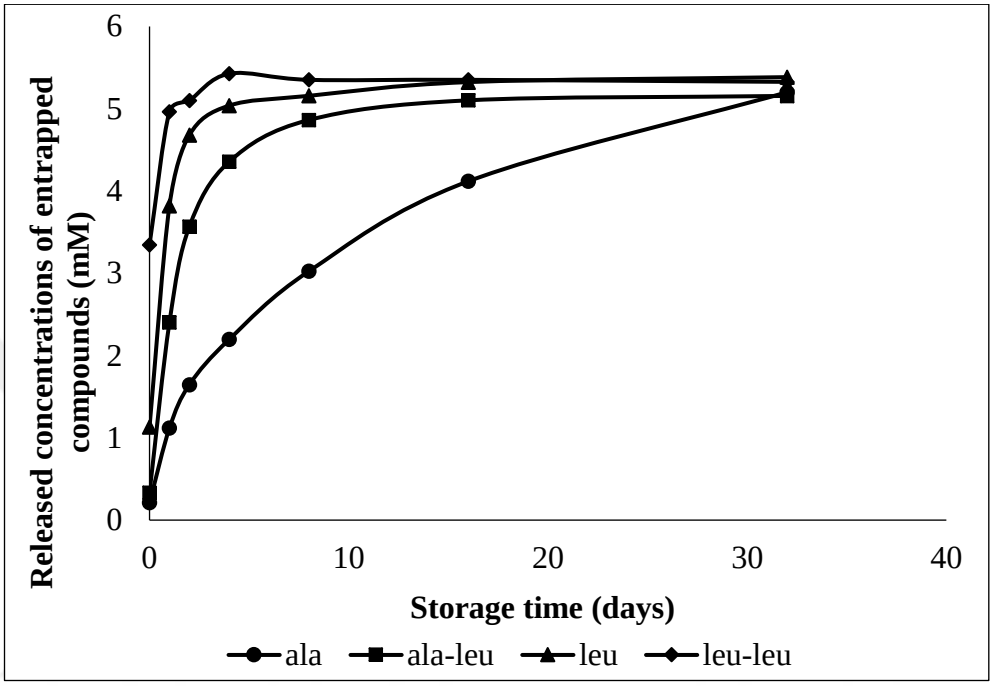

Figure 4.12 : Released concentration in the external phase of double emulsions containing alanine (circles), L-alanine-L-leucine (squares), leucine (triangles) and L-leucine-L-leucine (diamonds) at pH 7 as a function of storage time at 37 °C.

On the other hand, the release of entrapped compounds to the external aqueous phase during the production of double emulsions depends on the second homogenization step. It is well known that a higher speed of mixing may cause disruption of internal droplets, and therefore leads to solute migration together with internal phase. Nevertheless, the optimized C_0 values showed that only a limited amount of alanine, the slowest diffusing amino acid, was released during the production. Hence, rupture of the internal droplets in the second homogenization stage was negligible for the experimental conditions used. Therefore, the initial release of the more rapidly diffusing solutes was also diffusion-controlled. Whereas the preparation happened during a short time, the elevated temperature largely speeded up the solution-diffusion migration process.

Table 4.5 : Optimized t_a and C_0 values (with their 95% confidence interval) of entrapped compounds in double emulsions as a function of the average concentration in the aqueous phase (C) and the aqueous phase pH, during storage at 37 °C.

Entrapped compound	pH	t_a (d)	C_0 (mM)
DL-alanine	7	7.5±2.30	0.50±0.17
L-alanine-L-leucine		1.80±0.07	0.37±0.08
L-leucine	1	2.67±0.11	0.26±0.16
	2	1.55±0.10	0.71±0.14
	3	1.47±0.10	1.56±0.15
	4	1.86±0.28	1.13±0.26
	7	0.98±0.08	1.14±0.06
	10	1.20±0.10	0.75±0.17
L-leucine-L-leucine	2	1.16±0.10	1.41±0.12
	3	0.84±0.02	2.38±0.08
	4	0.95±0.10	4.29±0.16
	7	0.66±0.03	3.35±0.09

4.3 Influence of Solute Concentration

In order to examine the effect of solute concentration on double emulsion stability and release kinetics, varying concentrations of L-leucine (i.e. 15, 30, 60 and 120 mM) were encapsulated in the internal water phase.

4.3.1 Particle size

From Figure 4.13, the volume weighed average droplet size of the double emulsions prepared with different solute concentrations exhibited similar diameters immediately upon preparation (about 50 μm). A significant increase was found during storage due to aggregation as explained in section 4.2.1.1 (p<0.01). The varying concentrations of L-leucine did not cause a significant difference in the increase of volume weighed droplet size during 16 days (p>0.05).

From the lineer regressions, the daily change in average droplet size increase of the double emulsions during storage (corresponded to the slope) was irrespective of concentration of the enclosed compound. The daily change in average droplet size of the samples highest for 20 mM L-leucine (1.03±0.98) μm/day. Moreover, the coefficients of determination (R^2) corresponding to the linear regressions varied between 0.67 and 0.94.

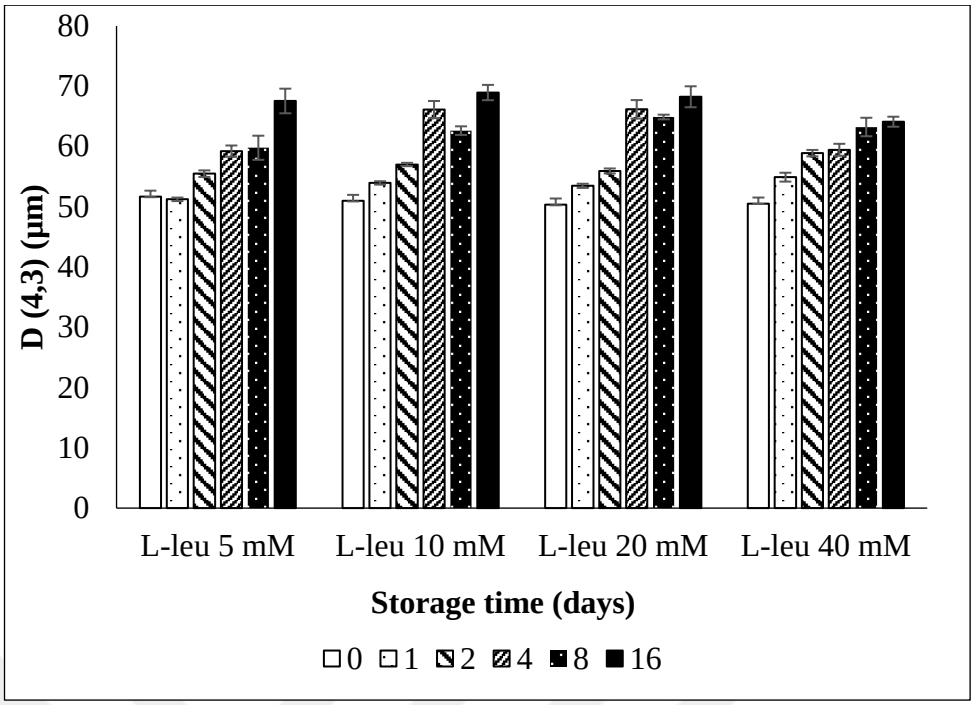

Figure 4.13 : Average droplet size of double emulsions with varying L-leucine concentrations within 16 days of storage at 37 ºC.

4.3.2 Entrapped water volume

From the analytical photocentrifugation results, the entrapped water volume fraction was found to be 70-90 % for all double emulsions (Figure 4.14). A decrease in the entrapped water volume was observed for the sample contained 40 mM L-leucine. This decrease might be due to the rapid exchange of the L-leucine as a result of the high concentration entrapped in the internal phase. Overall, the osmotic effect of the entrapped amino acid is compensated by a higher KCl concentration in the external water phase. However, when L-leucine diffuses more rapidly outward as compared to the inward diffusion of KCl, the osmotic pressure of the external water becomes larger than of the internal water, and hence will induce lower yield values due to osmotic dehydration of the internal water compartment. On the other hand, the entrapped water volume of the sample contained containing 10 mM of L-leucine increased during 16 days of storage. This increase might be as L-leucine and KCl was not equilized. However, this increase was only 0.77% daily change.

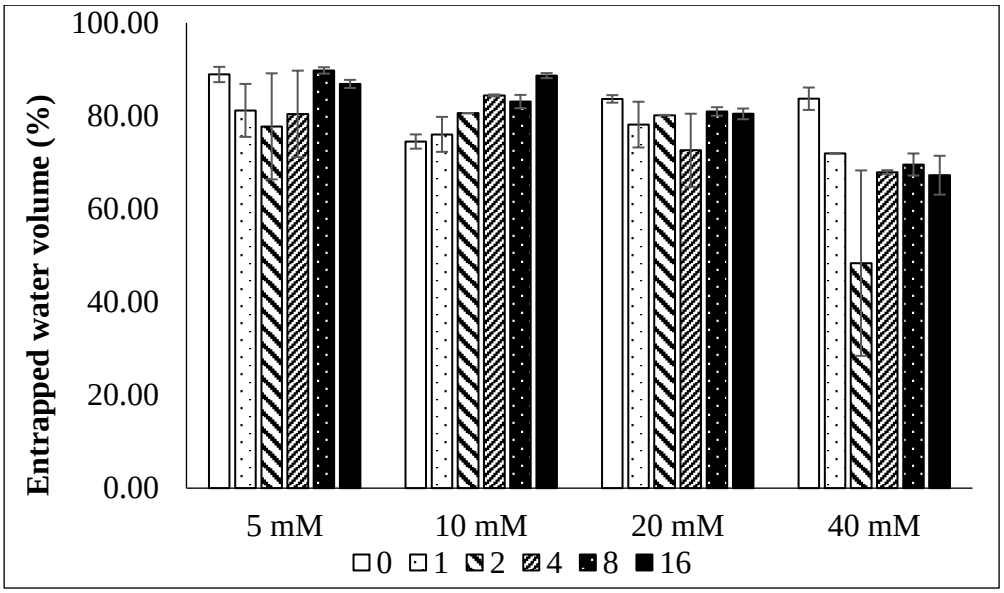

Figure 4.14 : Yield of entrapped water volume of double emulsions with varying L-leucine concentrations within 16 days of storage at 37 ºC.

4.3.3 Amino acid release

The release of L-leucine in double emulsions with varying concentrations were examined at both 4 and 37 ºC. Figure 4.15 indicates that the released L-leucine concentration approached an equilibrium after 2 weeks of storage at 4 ºC. Also, Table 4.6 shows the estimated values of t_a and C_0 of double emulsions containing different concentrations of L-leucine in the internal water phase during storage at 37 ºC.

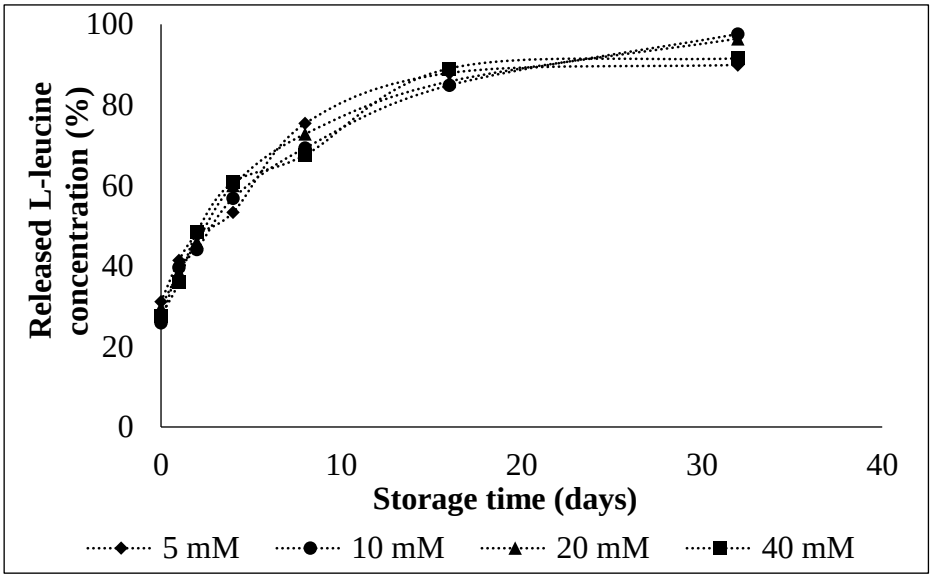

Figure 4.15 : Relative released L-leucine concentrations (i.e. actual concentration relative to the expected concentration upon homogeneous distribution over the combined aqueous phases) in the external phase during storage at 4 °C of double emulsions containing 5 mM (diamonds), 10 mM (circle), 20 mM (triangle) or 40 mM (squares) leucine at pH 7.

The released L-leucine concentration just after preparation was about 10-15% irrespective of the entrapped L-leucine concentration. This shows that the release kinetics were not affected by the entrapped solute concentration at 4 °C. However, the average residence time was significantly lower in the double emulsion that contained 120 mM L-leucine in the internal aqueous phase in comparison with the other emulsions at 37 ºC. The faster release of L-leucine as compared to KCl resulted in expelled internal water phase to equalize the osmotic pressure as it was mentioned in section 4.3.2. When the kinetic constant (t_a) data were compared at 4 and 37 °C (Table 4.6), the release at 4 °C was roughly 6-10 times slower as compared to 37 ºC. The faster release of L-leucine at higher temperature was a consequence of the increased solute solubility and higher diffusion coefficient of the dissolved L-leucine in the oil phase since the molecules gain a higher kinetic energy at a higher temperature. Hence, they permeate faster to the outer phase.

Table 4.6 : Optimized t_a and C_0 values (with their 95% confidence interval) of entrapped L-leucine in double emulsions as a function of the average concentration in the aqueous phase (C) during storage at 4 or 37 ºC.

	t_a (days)		
	4°C	**37°C**	C_0 **(mM)**
5 mM	6.86 ± 1.08^a	1.28 ± 0.22^b	0.78 ± 0.29
10 mM	8.63 ± 1.23^a	1.34 ± 0.11^b	1.55 ± 0.29
20 mM	7.46 ± 0.69^a	1.22 ± 0.18^b	2.54 ± 0.92
40 mM	6.62 ± 1.27^a	0.79 ± 0.14^a	5.19 ± 2.02

4.4 pH Effect on the Release of Amino Acids

4.4.1 Particle size

The influence of the pH of the aqueous phases on the particle size of the double emulsions was evaluated. The volume weighted average droplet size of all double emulsions was about 50 µm just upon preparation and increased up to about 60-65 µm at 37 ºC within 16 days (Figure 4.16). The significant increase in particle size during 16 days was confirmed for all double emulsions (p<0.05) with regression analysis. Considering the linear regression of the volume-weighed average droplet diameters versus storage time, there was no significant differences in the droplet size increase of the double emulsions over time (slope), depending on the pH of the aqueous phases: all values ranged from about 0.47±0.34 (for pH 4.0) to 0.75±0.27

μm/day (for pH 7.0). Furthermore, the coefficients of determination (R^2) corresponding to the linear regressions varied between 0.79 and 0.97.

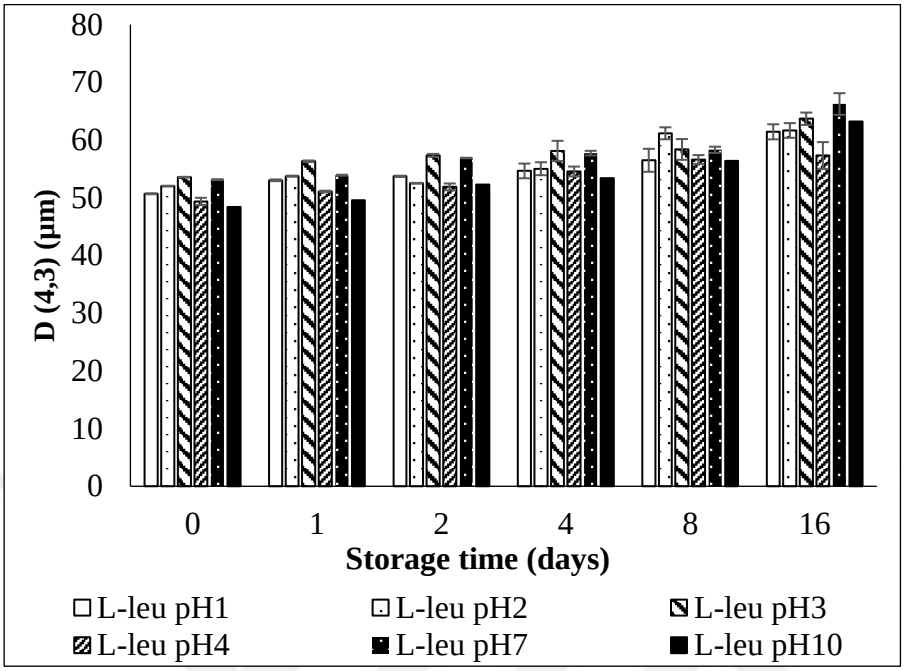

Figure 4.16 : Volume weighed average droplet size of double emulsions at different pH values 16 days of storage at 37 ºC.

4.4.2 Entrapped water volume

The yield of entrapped water demonstrated a fluctuation around 100% for double emulsions except from the samples at pH 1 and pH 3 (Figure 4.17). Coalescence of the inner water droplets and the O/W2 interface does not seem possible since there was no decrease in the entrapped water volume. The increase in the yield of enclosed water volume fraction might be a result of an osmotic imbalance, causing swelling of inner water droplets, due to the pH adjustment. Since the 15 mM of amino acid was present in the inner water phase, they could have led to a slight buffer effect. Therefore, more HCl was needed to adjust the pH than for the external water phases which contained only Tween 80, KCl and sodium azide. Another reason might be experimental error as an increased enclosed water volume fraction was not observed for the sample at pH 2. However, this reason seems unlikely since similar results were found during storage at the same pH value.

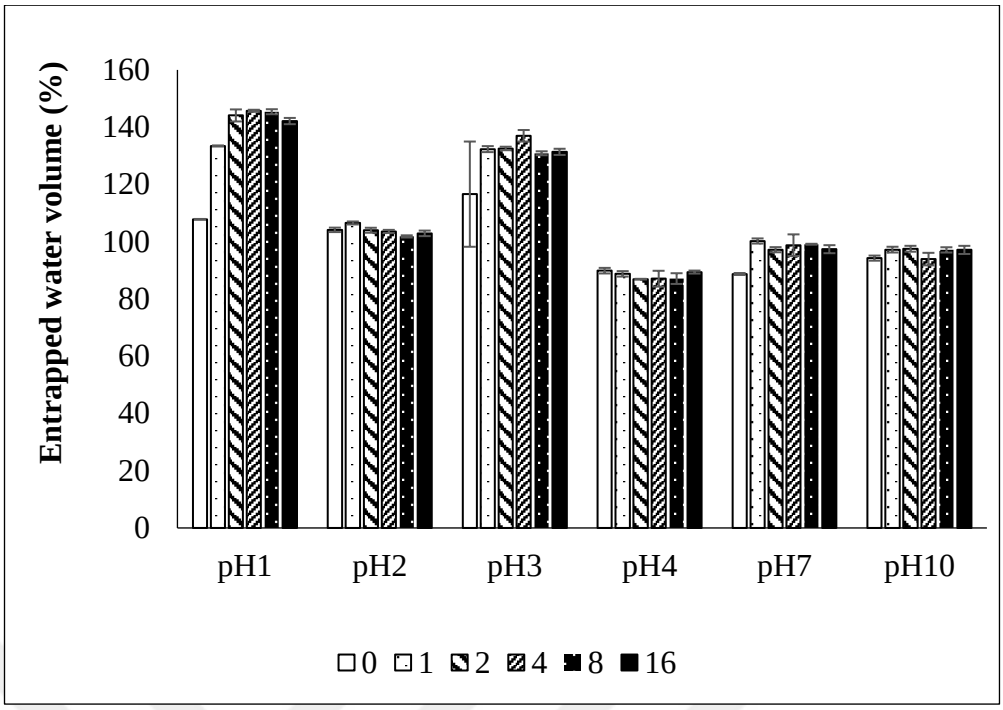

Figure 4.17 : Yield of entrapped water volume of double emulsions at different pH values 16 days of storage at 37 ºC.

4.4.3 Amino acid release

The solubility and partitioning of hydrophilic molecules with ionisable functional groups, such as carboxyl and amino groups, largely depend on the environmental pH (McClements, 2015). In the current study, the pH effect on the release kinetics was investigated. The release of double emulsions containing L-leucine at different pH values was significantly different; the only exception was the double emulsion at pH 3 which was not significantly different from the one at pH 4 (p=0.29). Figure 4.18 indicates the pH effect on the release of L-leucine during 32 days of storage at 37 ºC. It was observed that the release rate of L-leucine was lowest at pH 1, while it was highest at pH 7. Additionally, a remarkable release was observed during production for L-leucine as can be seen from the optimized C_0 values that was 1.14 mM at pH 7, whereas it was only 0.26 mM at pH 1 (Table 4.5). Hence, the initial released L-leucine concentration increased with the increased pH of the aqueous phases.

Also, from Table 4.5, the t_a of L-leucine increased as the pH of the aqueous phases decreased, which indicates a slower decrease for the positively charged species formed at acidic pH. It is clearly seen that the released L-leucine concentration increased as the pH increased. The net charge of amino acids and peptides is pH dependent. From Table 3.1, the isoelectric point of the compounds can be observed. From the pKa values of the amino acids and di-peptides used (Table 3.1), at least 99% of L-leucine

was in the zwitterionic state at pH 7. At lower pH, the amino acids and di-peptides became positively charged. The Henderson-Hasselbalch equation revealed that 95, 67, 17 and 2 % of cationic L-leucine was present at pH 1, 2, 3, and 4, respectively. Hereby, the cationic species are less permeable than the zwitterionic form due to their lower hydrophobicity. Hence, we observed that the release of neutral and weakly charged (non-polar) solutes was promoted by their higher solubility in the intermediate phase, in comparison with (polar) charged amino acids and di-peptides.

Based on the pK_a values from Table 3.1, it follows that 72% of the leucine was in anionic state at pH 10, whereas more than 99% of leucine as well as other amino acids was in zwitterionic state at pH 7. Considering the significant difference between pH 7 and 10, the anionic fraction displayed a less pronounced tendency to release compared to the zwitterionic species (Table 4.5). However, for the case of DL-alanine, there was no significant difference between the t_a values of the double emulsions at pH 7 (t_a=7.5±2.3) and 10 (t_a=7.0±0.2) stored at 37ºC.

It should be also noted that a lower equlibrium concentration was observed for the double emulsions with lower pH values (Figure 4.18). Upon pH adjustment to acidic values, the solutes in the internal water phase become highly protonated, and hence cationic. As the released species from the internal to the external water phase were thought to be rather zwitterionic, they will become protonated in the external phase, which will cause an increased pH in the external water phase. Conversely, the release of the zwitterionic species will induce a partial depronotation of the retained cationic solute. This effect will lower the pH in the internal water phase, which facilitates a further protonation (Figure 4.18). This effect will ultimately stop the further efflux of (cationic) entrapped solute, despite of a concentration gradient between the internal and external aqueous phases. Hence, the positively charged solutes will be partly retained inside the internal water droplets.

From Table 4.5, the release profiles at different pH values were significantly different for the double emulsions containing L-leucine-L-leucine, except from the double emulsion at pH 7 which was not different from pH 2 (p=0.11) and pH 3 (p=0.31). When the t_a values of the double emulsions containing L-leucine-L-leucine were considered, the highest residence time was found for the double emulsion at pH 2 and the lowest at pH 7 (Table 4.5).

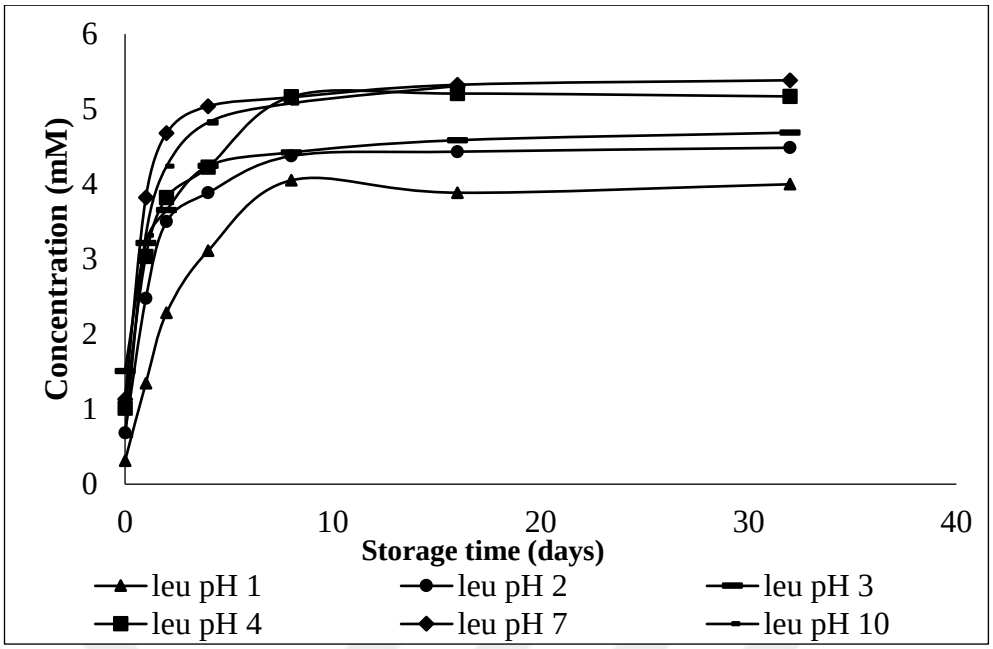

Figure 4.18 : Released L-leucine concentration in the external phase of double emulsions containing 5 mM L-leucine at pH 1 (triangle), pH 2 (circle), pH 3 (dash), pH 4 (square), pH 7 (diamond) and pH 10 (short dash) as a function of storage time at 37 °C.

Our findings are in agreement with previous studies in literature regarding the release of compounds as a function of pH. Owusu *et al.* (1992) reported that the rate of tryptophan release was higher at pH values near the isoelectric point whereas ionized forms released slower due to their lower solubility in oil. Giroux *et al.* (2019) studied the release of peptides in the gastrointestinal environment and found that it is controlled by the peptide hydrophobicity: peptides with a higher hydrophobicity index showed a higher release rate. Moreover, they found higher release rates at intestinal conditions (i.e. at pH 7) than at gastric pH (at pH 3) since neutral and weakly charged (non-polar) peptides are more soluble in oil as compared to charged peptides. In another research that the release of curcumin was examined at different pH values ranged from 3 to 7.4, the release rates became faster as the pH increased as a result of protonation of carboxylic groups at low pH (Sufi-Maragheh *et al.*, 2019).

4.5 Effect of Oil Phase Composition

4.5.1 Particle size

Figure 4.19 demonstrates the volume weighted average oil droplet size of the double emulsions stabilized with PC-depleted lecithin and PGPR mixture, prepared with MCT or LCT oil. The only significant increase during storage was found in the samples

stabilized with 1%PC-4%PGPR that containing MCT or LCT oil as well as the sample with 3%PC that containing MCT oil (p<0.05).

Regarding the double emulsions including a similar emulsifier content, the average droplet size was significantly smaller in the samples that contained MCT oil in comparison with LCT oil both just after preparation and during 8 days of storage (p<0.05). The larger droplet size of the double emulsions that contained LCT oil as compared to the ones with MCT oil is supported by the result of Balcaen *et al.* (2020). They reported that the oil droplet size of the double emulsions was larger when LCT oil was used in comparison with MCT oil. It was also stated that the viscosity of the LCT containing samples was higher than that of the MCT containing samples. Therefore, the larger droplets of the LCT containing double emulsions might be related to a stronger aggregation tendency in this emulsion.

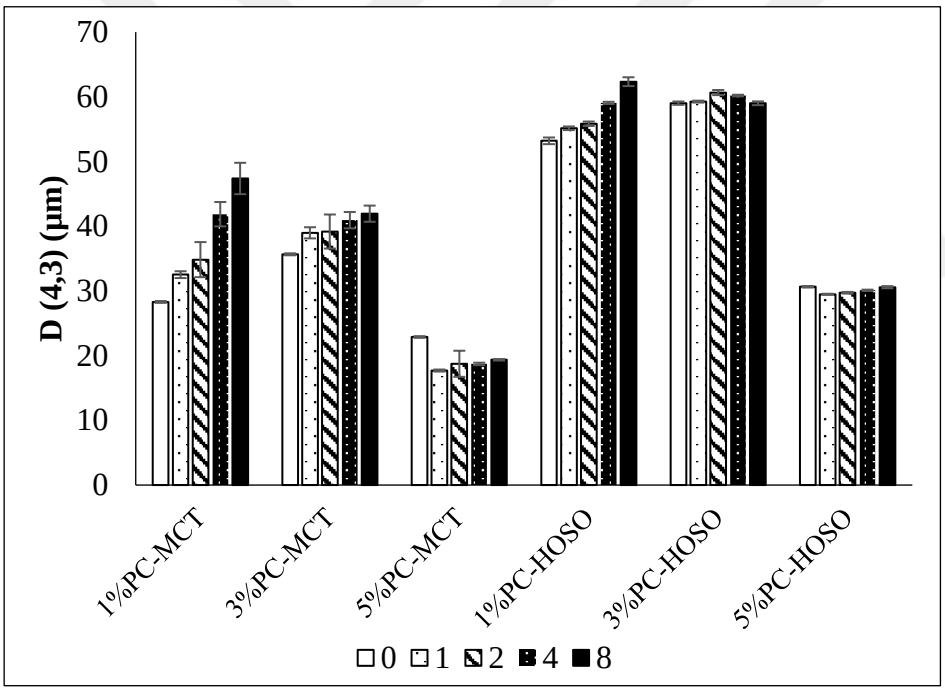

Figure 4.19 : Volume weighed average droplet size of double emulsions containing MCT or LCT oil, stabilized with PC-depleted lecithin-PGPR mixture during 8 days of storage at 37 °C.

4.5.2 Entrapped water volume

Figure 4.20 shows the influence of the oil phase on the entrapped water volume fraction during 16 days. A detrimental effect in the enclosed water volume fraction was observed for the double emulsions stabilized with 1%PGPR-4% PC depleted lecithin and 5% PC depleted lecithin that contained MCT oil. Therefore, there was no enclosed water in these samples. However, the enclosed water volume fraction of the

1%PGPR-4% PC depleted lecithin and 5% PC depleted lecithin stabilized double emulsion containing LCT oil was 40% and 11% just after preparation, respectively, as can be seen in Figure 4.27 in section 4.7.2.

The other samples, on the other hand, showed enclosed water volume around 85-100% except from the sample with 3% PC depleted lecithin that contained MCT oil which exhibited a decreasing trend during 16 days of storage.

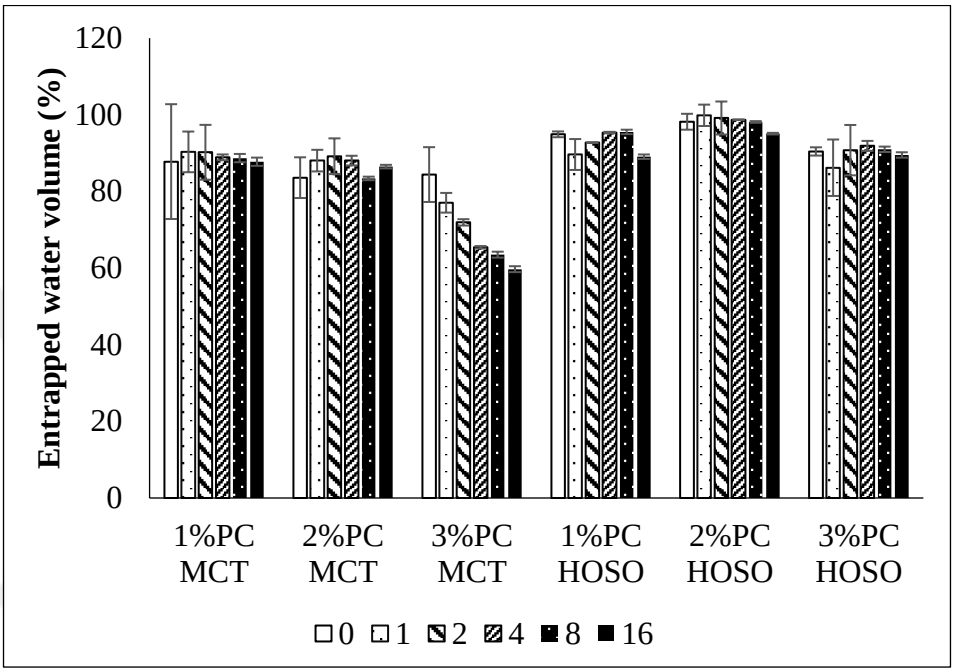

Figure 4.20 : Entrapped water volume of double emulsions contained MCT or LCT oil, stabilized with PC-depleted lecithin-PGPR mixture during 8 days of storage at 37 ^{o}C.

4.5.3 Amino acid release

From Figure 4.21, it is noted that the L-leucine concentration in the external aqueous phase directly after preparation was almost 5 mM in double emulsions prepared with MCT oil, with the exception of the emulsion with 5% PGPR. This indicates the nearly complete release of the entrapped L-leucine during emulsion preparation. On the other hand, the release of L-leucine was much slower in double emulsions prepared with HOSO. As the entrapped water volume of the double emulsions stabilised by 5% PGPR, and containing HOSO or MCT oil was found to be 91.8% and 92.9% just after preparation, respectively, it is clear that L-leucine release was not by breakdown of the water droplets. The faster release of L-leucine was likely due to the fact that MCT oil is more hydrophilic than LCT, as reflected by the solubility of water in the oils, which is more than two times higher in MCT as compared to LCT (Land *et al.*, 2005). Hence,

faster transport of L-leucine through the oil phase occurred due to the higher solubility of the solute. Moreover, MCT oil also has a lower viscosity, which gives rise to a higher diffusivity of dissolved solutes, which may also speed up molecular transport. Lutz *et al.* (2009) observed that the oil type was a significant factor in the release process, which was related to the hydrophobicity.

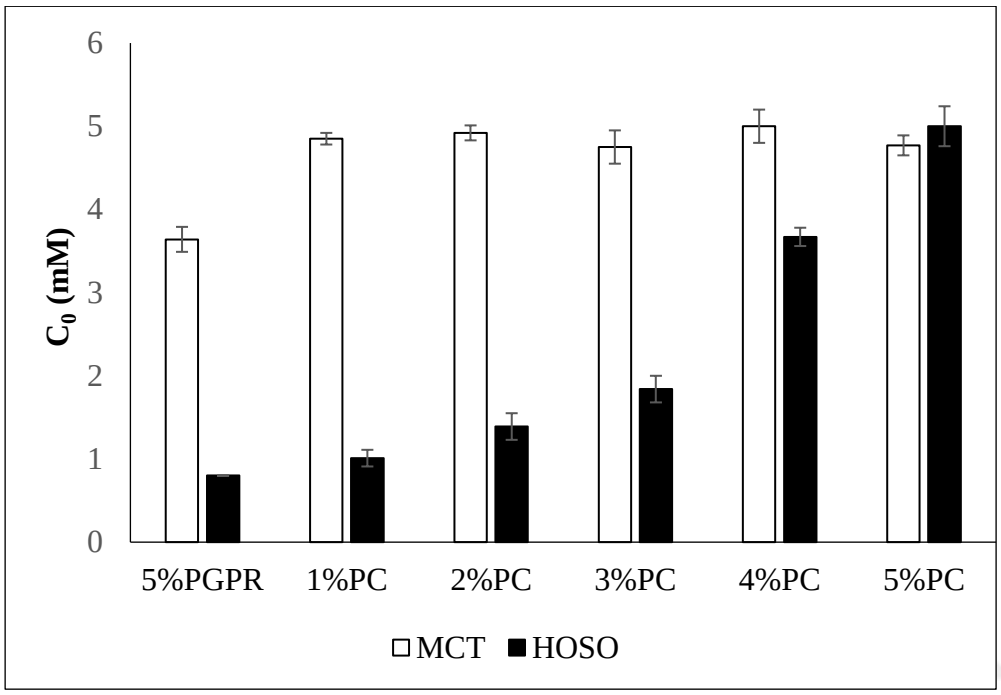

Figure 4.21 : Optimized C_0 values of L-leucine in double emulsions containing 5% of a mixture of PC-depleted lecithin and PGPR in either MCT oil or High Oleic Sunflower Oil (HOSO) at 37 ºC.

4.6 Effect of Hydrophobic Emulsifier Concentration

4.6.1 Particle size

From the particle size analysis, the volume weighted average droplet size of the PGPR-stabilised double emulsions showed a decreasing trend as the PGPR concentration increased within the studied interval ranging from 1 to 5%, directly after preparation (R^2=0.98), as indicated by the black arrow in Figure 4.22. When the average droplet size evolution during storage was considered, the double emulsion prepared with 5% PGPR was not significantly different from all other emulsions at a confidence value of 95%. Hence, it can be concluded that reducing the PGPR content from 5 to 1% had no significant effect on the size characteristics when the double emulsions were prepared by rotor-stator mixing, which is known as a less powerful emulsification technique as compared to high pressure homogenisation or microfluidisation.

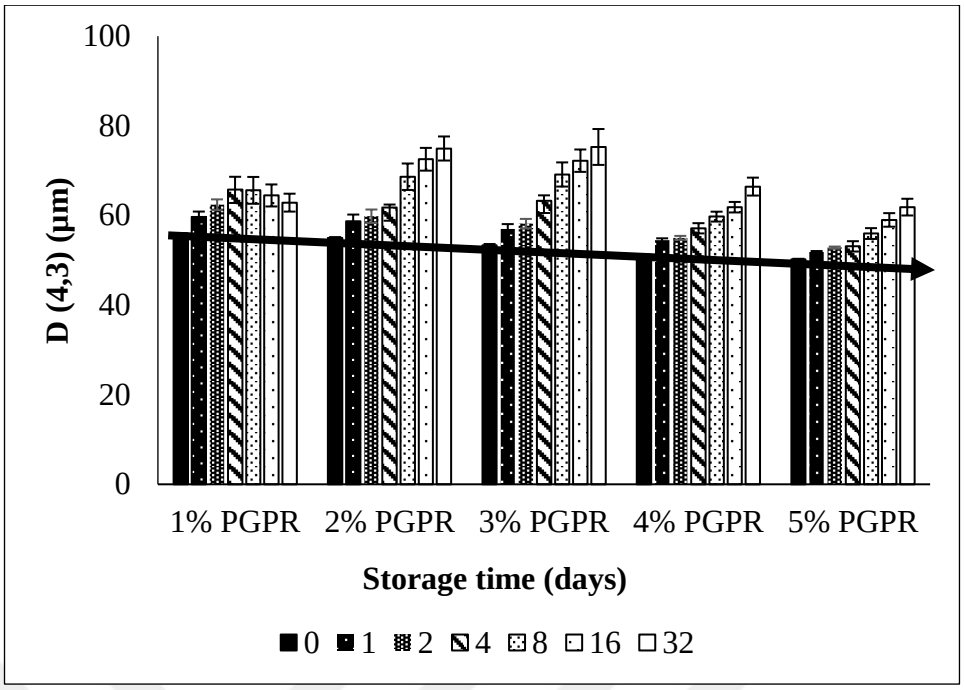

Figure 4.22 : Volume-weighted average droplet diameter (D43) of L-leucine containing double emulsions prepared with different concentrations of PGPR during storage for 32 days at 37 ^{o}C.

Table 4.7 shows the daily change of the droplet size (expressed in µm/day) of the L-leucine containing double emulsions. The droplet size increased during 32 days storage for all emulsions, except from the one with 1% PGPR whose droplet size showed an increasing trend until day 4 and slightly decreased afterwards. Besides, all double emulsions containing only PGPR showed a statistically similar droplet size increase, except for 5% PGPR which was only similar with 1% and 4% PGPR.

Table 4.7 : Average rate of change of the volume-weighted average droplet size during 32 days of storage at 37 °C (expressed in µm/day) of L-leucine containing double emulsions whose primary emulsion was stabilised by a mixture of PGPR.

PGPR (%)	Coefficient of determination (R^2)	Rate of change of D43 (µm/day)
1	0.57	0.86 ± 1.07^{ab}
2	0.98	1.60 ± 0.49^{b}
3	0.96	1.80 ± 0.72^{b}
4	0.96	0.85 ± 0.50^{ab}
5	0.99	0.70 ± 0.20^{a}

4.6.2 Entrapped water volume

Upon 2 hours centrifugation, creaming was visible from the transmission profiles of the double emulsions containing 1 to 5% PGPR (Figure 4.23). The cream layer thickness was found by considering the difference between its top and bottom positions. According to the results shown in Figure 4.23, a similar cream layer thickness was observed for the double emulsions with 1 and 5% PGPR just after preparation. Whereas the cream layer thickness of the double emulsions with 5% PGPR remained constant during 32 days of storage, the 1% PGPR stabilised double emulsion had a thinner cream layer upon storage, which indicated the gradual loss of entrapped water droplets to the outer aqueous phase.

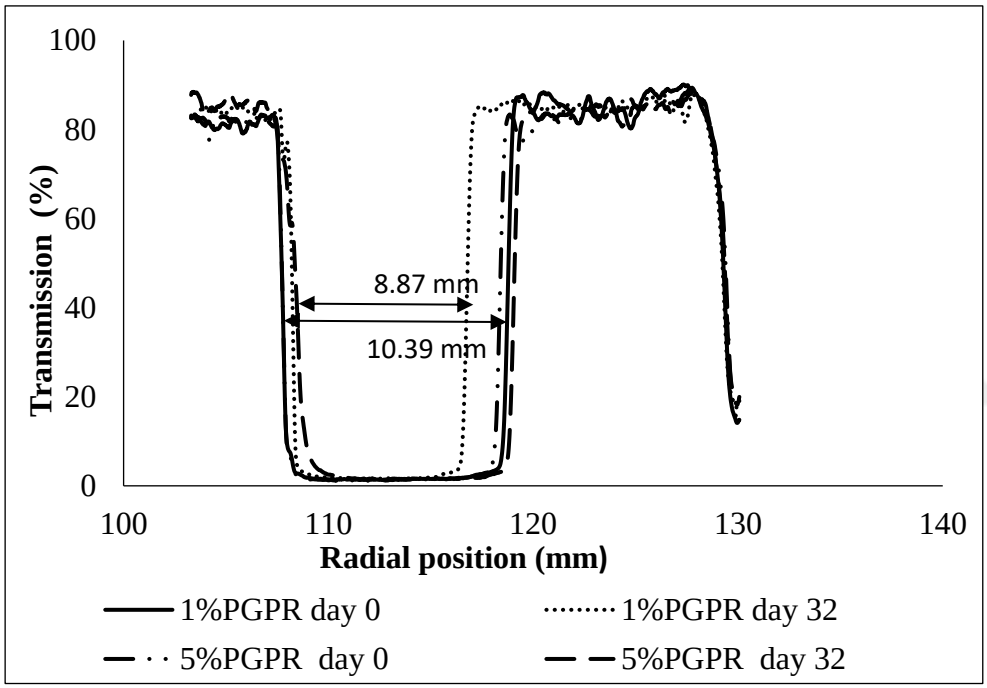

Figure 4.23 : Transmission profiles obtained upon 2 hours of centrifugation at 3000 rpm of 25:25:50 (m/m/m) W1/O/W2 double emulsions, stabilized with either 1 or 5% PGPR in the oil phase just after preparation and after 32 days of storage at 37 ^{o}C.

The yield of entrapped water of the PGPR stabilised double emulsions is presented in Figure 4.24. The enclosed water volume fraction of all double emulsions stabilized with only PGPR fluctuated around 100%, except from the double emulsion containing only 1% PGPR. From the post-hoc analysis, no significant difference was found between all double emulsions containing PGPR, except from the one with 1% PGPR which was significantly different from the other samples (p<0.01). The results show that a significant effect of storage time was found only for the double emulsion containing 1% PGPR (p<0.05). The yield of the latter emulsion decreased to 50% after

32 days of storage as a result of the transfer of the internal water droplets to the external water phase. Hence, 1% PGPR was too low to sufficiently stabilise the entrapped water droplets in the W/O emulsion. Bahtz *et al.* (2016) reported that the water migration rate increased up to a critical concentration of 1% PGPR. The double emulsion stabilized with 1% PGPR had a lower yield as the lack of PGPR at the interface may reduce the stability of the inner water droplets against coalescence with the outer water phase (Schuch *et al.*, 2015). Within the formulation conditions used in this study, 2% of PGPR in the oil phase was sufficient to obtain stable emulsions in terms of entrapped water.

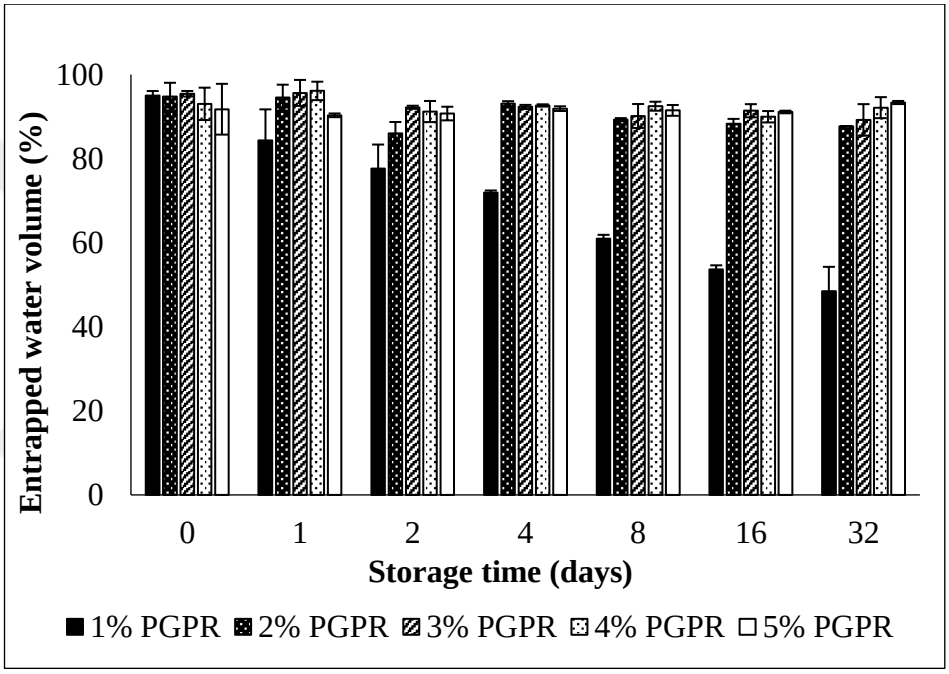

Figure 4.24 : Yield of entrapped water in double emulsions containing L-leucine prepared with varying concentrations of PGPR upon storage at 37 °C.

4.6.3 Amino acid release

From Table 4.8, the original amino acid concentration C_0 of the double emulsions which contained only PGPR was similar, i.e. within the range from 0.63-0.87 mM. Linear regression analysis indicated that the PGPR concentration effect on C_0 was not significant both at 4 ^oC (p=0.12) and at 37 ^oC (p=0.87). The estimated equilibrium concentration C_{eq} of all the PGPR containing double emulsions approximated 5 mM. It can be clearly seen that a lower concentration of PGPR decreased the release kinetics of L-leucine both at 4 and 37 ^oC: the t_a roughly doubled when the PGPR concentration of the double emulsions decreased from 5 to 1%. Moreover, the effect of the PGPR concentration on the average residence time was more clear at 4 ^oC, because of the

slower release at lower temperature. The slow release kinetics at 1% PGPR are remarkable, considering the lack of stability of the internal water droplets. As leucine release by coalescence of internal water droplets with the external aqueous phase took place in this emulsion (only), it follows that leucine release by molecular transport through the oil layer must be significantly lower at this low PGPR concentration as compared to the higher concentrations.

Garti and Lutz (2004) reported that an excess of hydrophobic surfactant aggregates in the oil phase forming reverse micelles which negatively impact the encapsulation efficieny due to the transport of the entrapped compounds. Tamnak *et al.* (2016) also reported that excessive micelles of the hydrophobic emulsifier facilitate the transport of solutes across the film of the hydrophilic surfactant.

Table 4.8 : Optimized t_a and C_0 values (with their 95% confidence interval) of L-leucine release in double emulsions containin varying PGPR concentrations at 4 and 37 ºC.

PGPR (%)	t_a (d)		C_0 (mM)	
	4ºC	**37 ºC**	**4 ºC**	**37 ºC**
1	8.63 ± 0.52^{e}	1.04 ± 0.10^{c}	0.87 ± 0.07^{b}	0.77 ± 0.13^{a}
2	7.00 ± 0.48^{d}	1.24 ± 0.06^{c}	0.73 ± 0.09^{ab}	0.78 ± 0.07^{a}
3	5.60 ± 0.49^{c}	0.77 ± 0.04^{b}	0.86 ± 0.10^{b}	0.72 ± 0.08^{a}
4	4.09 ± 0.37^{a}	0.70 ± 0.08^{ab}	0.69 ± 0.11^{ab}	0.74 ± 0.15^{a}
5	4.05 ± 0.29^{a}	0.56 ± 0.15^{a}	0.63 ± 0.09^{a}	0.80 ± 0.24^{ab}

In our previous study, the main release mechanism of encapsulated amino acids was found to be direct diffusion (Kocaman *et al.*, 2020). In this section, it was indicated that the release rate of L-leucine is also affected by the type and concentration of the hydrophobic emulsifier. Besides, the release was limited by the use of a low PGPR concentration. At this point, the CMC of the emulsifier plays a critical role regarding the migration of the entrapped compounds. The CMC of PGPR was determined by some researchers before: it was reported as 1.8% by Bahtz *et al.* (2016), whereas Nollet *et al.* (2018) mentioned that it is around 1%. Nollet *et al.* (2018) identified 3 domains regarding the PGPR concentration effect on the release of entrapped compounds: decrease in encapsulation efficiency when the PGPR concentration was lower than the CMC (0.36%), no encapsulated compound exchange when the free PGPR

concentration was close to its CMC (1%), and water transfer without entrapped compound release in the presence of higher free PGPR concentrations (≥2.5%).

In the current research, we hypothesize that above the CMC of PGPR (>1%) reverse micelles were formed which speeded up the migration of L-leucine from the internal to the external water phase without net water flux. When the PGPR concentration was close to the CMC (1%), the release of L-leucine was slower due to the smaller amount of excess micelles. On the other hand, the use of higher concentrations of PGPR enabled the entrapped water volume fraction to remain constant whereas a PGPR concentration below the CMC caused water flux from the internal to the external phase.

4.7 Partial Replacement of Hydrophobic Emulsifier with Lecithin

4.7.1 Particle size

From Figure 4.25, it was observed that when only lecithin was used as a hydrophobic emulsifier, the volume-weighted average double emulsion droplet size was much smaller compared to the PGPR-containing double emulsions: it remained about 8 µm during 8 days of storage at 20 ^{o}C, whereas the volume-weighted average oil droplet diameter of the samples containing 5% PGPR and 5% of a PGPR-native lecithin (1/1) mixture was about 53 µm just after preparation. Whereas the average droplet size of the sample that contained 5% of a PGPR-native lecithin (1/1) mixture was constant during storage, the droplet size of the double emulsions with 5% PGPR increased from 53 to about 70 µm during 8 days of storage. Hence, it is clear that the lipophilic emulsifier, which is meant to stabilise the internal W1/O interface, also affected the external O/W2 interface. From the microscopic observations (Figure 4.25), it is clear that 5% native lecithin did not enable the production of double emulsions using the formulation conditions, whereas 5% PGPR and 5% of the PGPR-native lecithin (1/1) mixture did.

Altuntas *et al.* (2017) also described that the complete replacement of PGPR by lecithin as the hydrophobic emulsifier could not be accomplished. According to Schmidts *et al.* (2010), native lecithin cannot help to produce stable W/O emulsions, but rather gives rise to O/W type emulsions. On the other hand, these authors reported that even if lecithin could not help to produce W/O type emulsions on itself, it increased the functionality of a second lipophilic surfactant, such as PGPR.

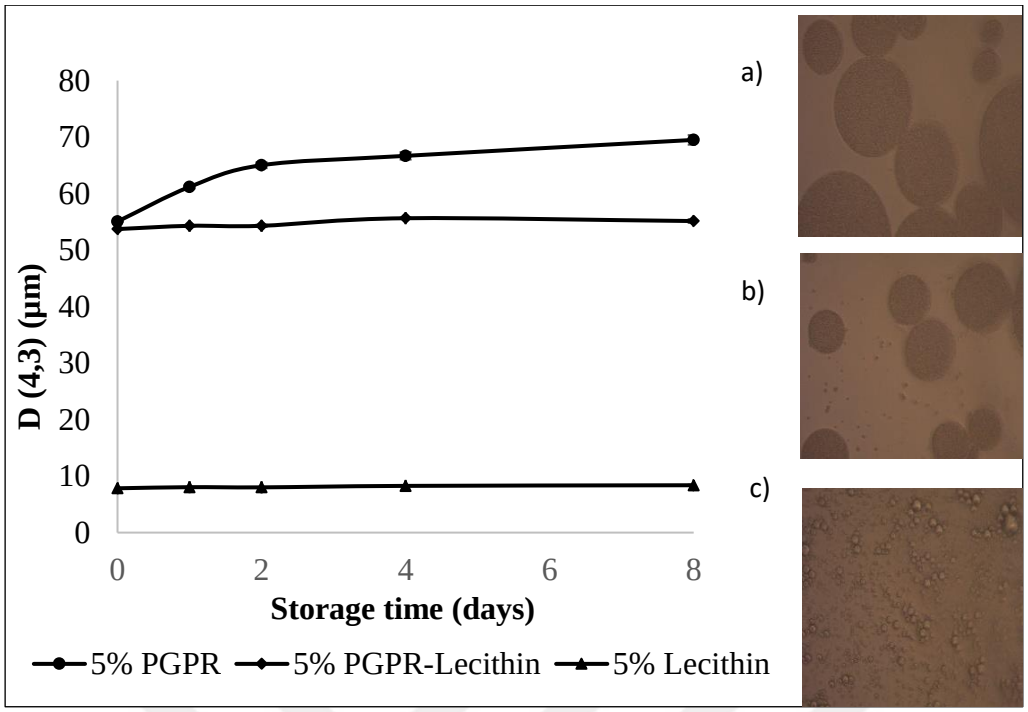

Figure 4.25 : Average droplet diameter of L-leucine containing double emulsions within 8 days of storage at 20 °C, as well as microscopic images of 10 times diluted double emulsions containing either 5% PGPR (circles; a), 5% of a PGPR-native lecithin (1/1) mixture (diamonds; b) or 5% native lecithin (triangles; c) (Error bars are smaller than markers).

In the PGPR-stabilised double emulsions, a droplet size increase was observed whereas the use of 5% of a PGPR-native lecithin (1/1) mixture resulted in a constant droplet size during storage. This shows that partial replacement of PGPR may be beneficial, e.g. for the stability of the W1/O/W2 droplet size. As especially PC-depleted lecithin has been suggested as a possible alternative for PGPR in the preparation of W/O emulsions (Altuntas *et al.,* 2017; Knoth *et al.,* 2005a; Mazo Rivas *et al.,* 2016; Scherze *et al.,* 2006), all further experiments were performed with PC-depleted lecithin.

Figure 4.26 indicates that the PGPR and PC-depleted lecithin concentration influenced the droplet size of the double emulsions. The lowest droplet size was around 30 µm after preparation and during storage in double emulsions containing 5% PC-depleted lecithin, whereas the droplet size of the emulsion stabilised by a mixture of 4% PC-depleted lecithin and 1% PGPR was about 40 µm. It can also be seen from Figure 4.26 that the droplet size of the double emulsions that contained a higher concentration of PGPR slightly increased during 1 month of storage.

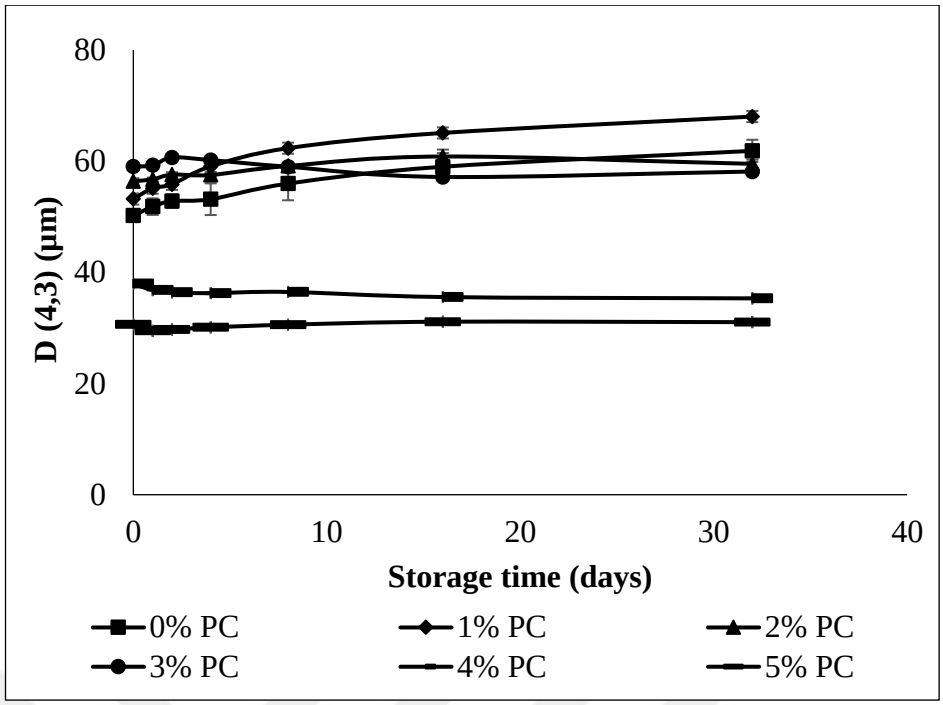

Figure 4.26 : Average droplet size of L-leucine containing double emulsions prepared with 5% of a mixture of PC-depleted lecithin (PC) and PGPR within 32 days of storage at 37 ºC (Some error bars are smaller than marker size).

On the other hand, the samples containing PC-depleted lecithin showed the most stable droplet size except for the mixture of 1% PC-depleted lecithin and 4% PGPR, which behaved similar to the double emulsions containing PGPR only (Table 4.9). Moreover, the average droplet size of the double emulsions containing 3% PC-depleted lecithin and 2% PGPR, and 4% PC-depleted lecithin and 1% PGPR slightly decreased throughout time.

Table 4.9 : Average rate of change of the volume-weighted average droplet size during 32 days of storage at 37 °C (expressed in µm/day) of L-leucine containing double emulsions whose primary emulsion was stabilised by a mixture of PGPR and PC-depleted lecithin.

PGPR (%)	PC-depleted Lecithin (%)	Coefficient of determination (R^2)	Rate of change of D43 (µm/day)
4	1	0.98	1.09 ± 0.37^{c}
3	2	0.98	0.44 ± 0.11^{b}
2	3	0.54	-0.20 ± 0.37^{a}
1	4	0.75	-0.15 ± 0.20^{a}
0	5	0.59	0.01 ± 0.19^{a}

4.7.2 Entrapped water volume

From Figure 4.27, the yield of entrapped water was about 100% for all emulsions stabilised by 5% of a mixture of PC-depleted lecithin and PGPR except those with 4% PC-depleted lecithin-1% PGPR (whose yield decreased from 36% to 15% during 32 days of storage) and 5% PC-depleted lecithin. The entrapped water volume of the latter was only 11% just after preparation, and decreased to 0% after 1 day, which means that all the internal water was transferred to the external water phase. Also, the only significant effect of storage time was found for the double emulsions containing 4% PC-depleted lecithin and 5% PC-depleted lecithin ($p<0.01$).

From the post-hoc analysis, no significant difference was found between double emulsions stabilised by 5% of a mixture of PC-depleted lecithin and PGPR except from the one with 4% and 5% PC-depleted lecithin, which were different from all other emulsions as well as from each other ($p<0.01$). When the same PGPR concentration was compared in the absence or presence of PC-depleted lecithin, the entrapped water volume of the double emulsions was not significantly different for the double emulsions containing 2%, 3% or 4% PGPR. Considering the double emulsions with 1% PGPR in the oil phase, addition of PC-depleted lecithin could not help to overcome the observed instability at too low (i.e. less than 2% in this case) PGPR concentration. In fact, lecithin addition had a negative impact on the enclosed water volume fraction (Figure 4.27): the entrapped water volume decreased from 95% to 48% during 32 days of storage for the 1% PGPR-stabilised emulsion without lecithin (in section 4.6.2), whereas the water yield decreased from 37% to 16% in the PC-depleted lecithin containing emulsion with the same PGPR content. Therefore, competition between both surfactants seemed to lead to less PGPR at the interface upon lecithin addition.

These results indicate that PGPR is superior to PC-depleted lecithin in order to reach (and maintain) a high yield of entrapped water. In fact, also when replacing PGPR by PC-depleted lecithin, at least 2% of PGPR was needed to obtain a stable yield of entrapped water. Hence, PC-depleted lecithin did not help to partially replace PGPR in our application. For completeness, it should be mentioned that Balcaen *et al.* (2020) described the succesful preparation of PC-depleted lecithin stabilised double

emulsions. However, a different formulation was used as compared to our study. Thus, glucose was used instead of KCl as osmotic agent. In addition, MCT oil was used instead of a typical long chain triglyceride oil, such as HOSO.

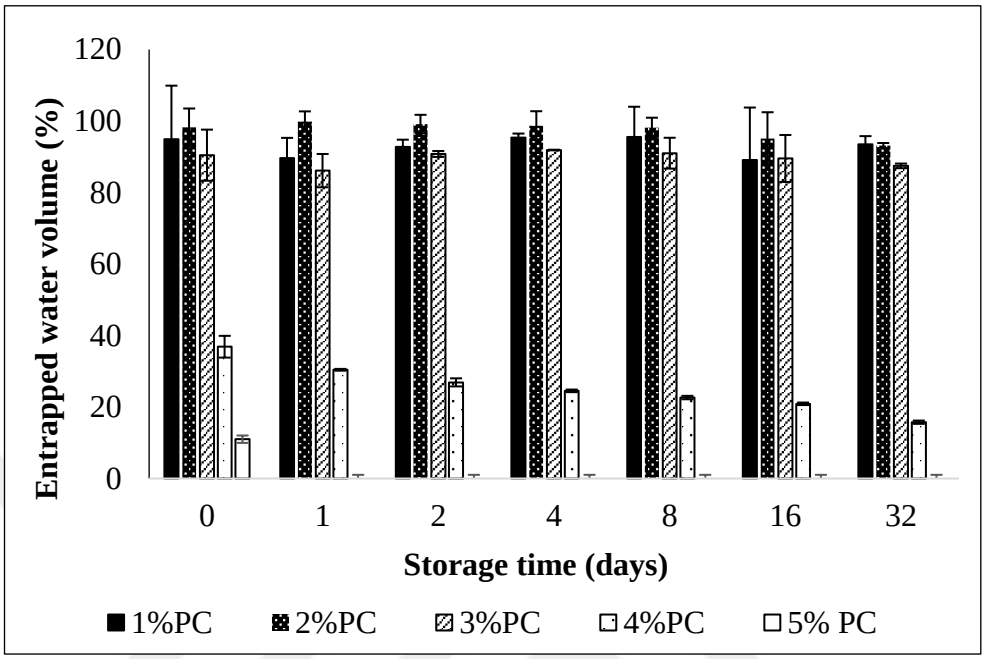

Figure 4.27 : Yield of entrapped water (%) in double emulsions containing L-leucine prepared with 5% of a mixture of PC depleted lecithin (PC) and PGPR in the oil phase, within 32 days at 37°C.

4.7.3 Amino acid release

The release of L-leucine was examined in double emulsions that were prepared with varying concentrations of the hydrophobic emulsifiers PGPR, native lecithin and PC depleted lecithin. The initial leucine concentration in the external aqueous phase (C_0) was 1.44±0.24 mM just after preparation for the double emulsion that contained 5% of a PGPR-native lecithin (1:1) mixture whereas the C_0 value was nearly halved in the 5% PGPR-stabilised double emulsion. Moreover, the average residence time (t_a) at 4°C decreased from 4.05±0.29 days to 1.63±0.31 days upon partial replacement of PGPR by native lecithin. Hence, the addition of native lecithin caused faster release of L-leucine from the internal to the external water phase, both during preparation and during storage. For completeness, it can be mentioned that the estimated equilibrium concentration (C_{eq}) was close to the expected value (5 mM) for both emulsions.

From Table 4.10, the C_0 of the double emulsions that contained a PC-depleted lecithin-PGPR mixture was higher as compared to double emulsions that contained only PGPR. Moreover, it is clearly seen that the L-leucine concentration in the external aqueous

phase directly after preparation increased as the PC-depleted lecithin concentration increased in the double emulsion. On the other hand, the average residence time t_a was much lower in PC-depleted lecithin-containing double emulsions as compared to the emulsions with only PGPR. In fact, the emulsion prepared with 5% depleted lecithin (and no PGPR) was even not a double emulsion. The storage temperature also affected the release rate of L-leucine. On average, the release was about 7 times faster upon storage at 37 ^oC as compared to 4 ^oC.

Table 4.10 : Optimized t_a and C_0 values (with their 95% confidence interval) of L-leucine release in double emulsions containing PC depleted lecithin and/or PGPR at 4 and 37 ^oC.

PGPR (%)	PC-depleted lecithin (%)	t_a (d)		C_0 (mM)	
		4 ^oC	37 ^oC	4 ^oC	37 ^oC
0	5	0	0	5	5
1	4	0.04 ± 0.01^{a}	0.03 ± 0.02^{a}	5.28 ± 0.02^{d}	3.67 ± 0.04^{e}
2	3	0.37 ± 1.03^{b}	0.61 ± 0.09^{bc}	3.37 ± 0.10^{c}	1.84 ± 0.16^{d}
3	2	3.29 ± 0.73^{c}	0.49 ± 0.09^{b}	1.32 ± 0.24^{b}	1.39 ± 0.16^{c}
4	1	3.43 ± 0.54^{c}	0.56 ± 0.06^{bc}	1.60 ± 0.15^{b}	1.01 ± 0.11^{b}
5	0	4.05 ± 0.29^{c}	0.56 ± 0.15^{bc}	0.63 ± 0.09^{a}	0.80 ± 0.24^{ab}

4.8 Effect of Hydrophilic Emulsifier Concentration

4.8.1 Particle size

The influence of Tween 80 concentration on droplet size distribution is presented in Figure 4.28. The oil droplet size distributions were fairly similar for the double emulsions that contained 0.5 and 1% Tween 80 with the main volume of droplets between 15 and 130 μm immediately after preparation. The emulsion with 2% Tween 80 had slightly smaller particle size distribution as compared to the other emulsions. For all double emulsions, a rather monomodal size distribution was observed.

The average droplet size of the double emulsions stabilized with 0.5, 1 and 2% Tween 80, exhibited an increase for all double emulsions within 16 days (p<0.05) (Figure 4.29). The emulsions contained 2% Tween 80 had a smaller increase in average droplet size than the other emulsions. It is possible that for the samples containing less than 2% Tween 80, the interface between oil and outer aqueous phase was not covered by

emulsifer properly. This effect could cause the instability in the system as a result of insufficient repulsion and therefore more interaction between droplets.

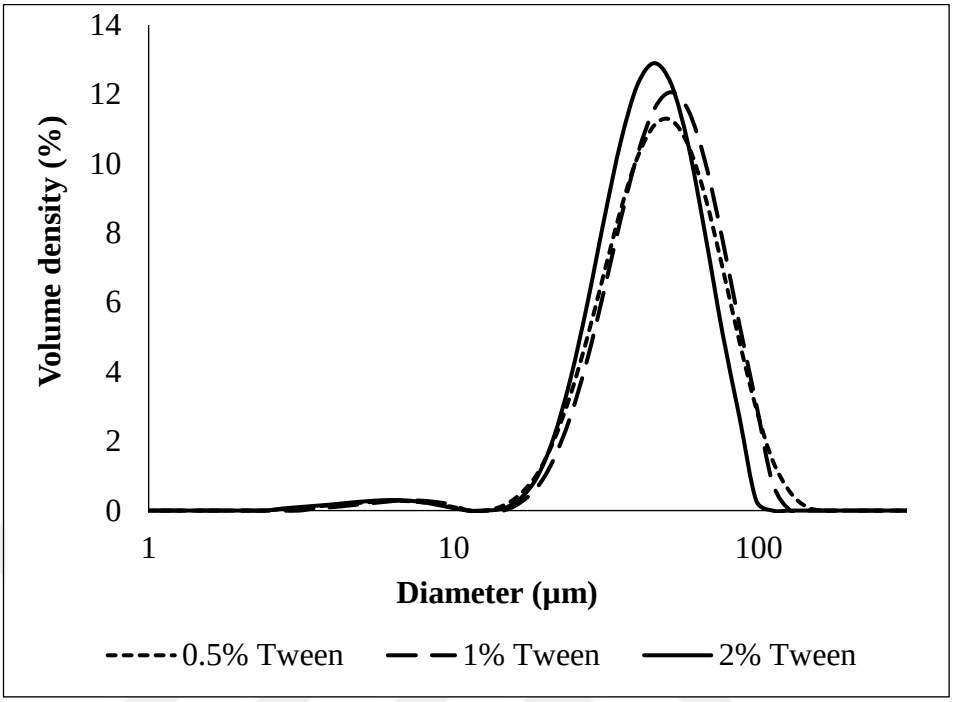

Figure 4.28 : Droplet size distribution of 15 mM L-leucine containing double emulsions with 0.5, 1 and 2% Tween 80 directly after preparation by Ultra-turrax treatment at 17500 rpm.

It was also stated by Ilić *et al.* (2017) that the use of Tween 80 concentration below 3% caused extensive coalescence while above this concentration only partial coalence was observed within the droplets. It should be also noted that the concentration of hydrophilic emulsifier can affect the activity of the hydrophobic emulsifier. It was reported that the use of low concentration of Tween 80 caused the PGPR to take place in O/W2 phase which resulted in a detrimental effect wherease high Tween 80 concentrations hindered its effect in only W1/O interface (El Kadri *et al.*, 2015).

Looking at the Tween 80 concentration effect, the daily change of the volume weighted mean diameter values of the double emulsions stabilized with 0.5, 1 and 2% Tween 80 was found to be 5.84±0.83, 4.23±0.55 and 3.22±0.40 µm/day, respectively, during 16 days of storage at 4 ºC. A significant difference was found between the possible combinations considering the increase in average droplet size (p<0.05). Also, the coefficient of determination (R^2) was above 0.96 for all double emulsions when a polynomial curve fit was applied to the increase of average rate of change of the volume-weighted average droplet size.

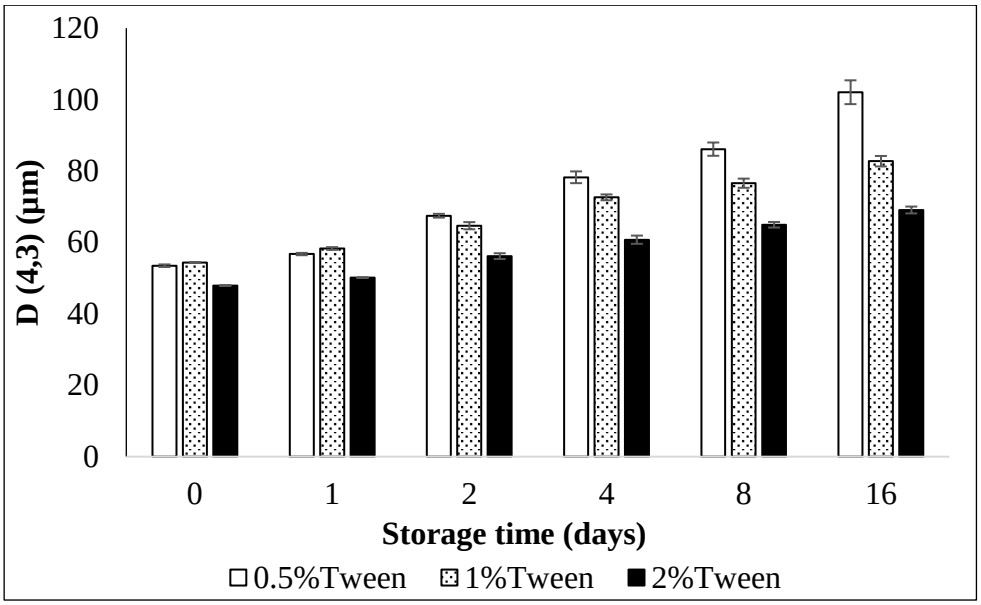

Figure 4.29 : Average droplet size of 15 mM L-leucine containing double emulsions stabilized with 0.5, 1 and 2% Tween 80 as a function of storage at 4 ºC after preparation by Ultra-turrax treatment at 17500 rpm.

4.8.2 Entrapped water volume

Concerning the effect of the Tween 80 concentration on the entrapped water volume fraction, no significant difference was observed between the samples. The encapsulated water volume fraction was about 90% during 8 days of storage (Figure 4.30). It can be concluded that water transport is not influenced by the hydrophilic surfactant concentration studied in the current study.

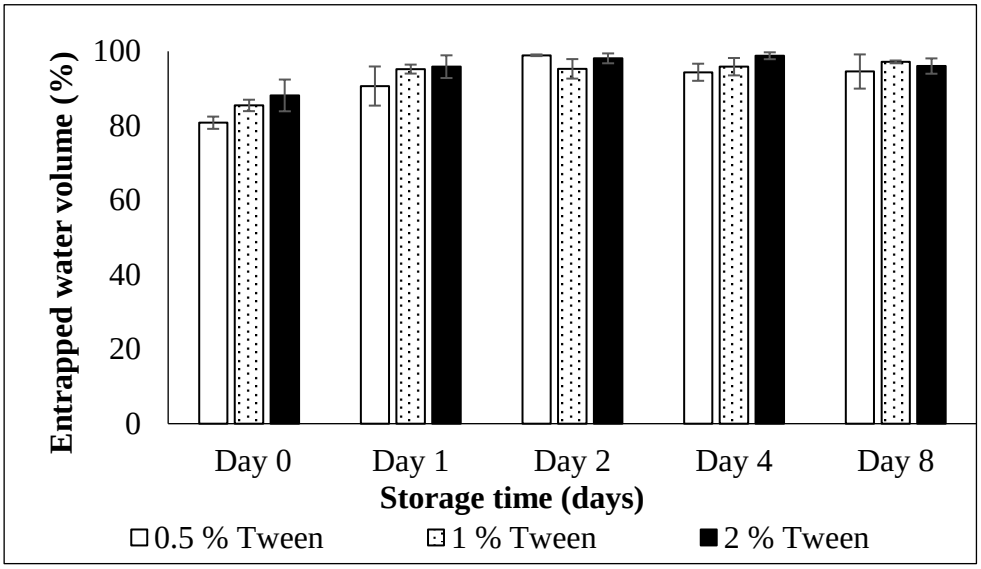

Figure 4.30 : Entrapped water volume fraction of double emulsions containing 15 mM L-leucine prepared with varying Tween 80 concentrations in the external water phase during storage at 4 °C.

4.8.3 Amino acid release

The thin film layer in the oil and outer water phase interface can be modified by the used Tween 80 concentration which also effects the water flux and release of solutes Tamnak *et al.* (2016). It was also stated that the use of too high emulsifier concentration may cause reduced elasticity and interfacial stability of the emulsions. In this context, Tween 80 concentration should be used in combination with hydrophobic emulsifier as the optimum concentration to enable stability by occuring the steric repulsion of oil droplets (Opawale and Burgess 1998).

From Table 4.11, it can be seen that the hydrophilic emulsifier concentration did not lead to a clear difference on the release of L-leucine. The initial release was nearly 20% in double emulsions with varying Tween 80 concentration. Additionally, no relation between the droplet size and the release was observed in the reseach with different Tween 80 concentrations: although the higher Tween 80 concentrations enabled smaller droplets, the release of L-leucine was not different for the different Tween 80 concentrations. The increase in droplet size at lower Tween 80 concentrations was probably due to flocculation, which did not change the identity of the droplets. Hence, the target compound migrated by passing through a similar distance for all double emulsions with different Tween 80 concentrations.

Table 4.11 : Optimized t_a and C_0 values (with their 95% confidence interval) of L-leucine release in double emulsions with varying Tween 80 concentrations during storage at 4 ^{o}C.

AA	Tween 80 (%)	t_a (days)	C_0 (mM)
L-leu (15 mM)	2%	4.07±0.27^{a}	1.00±0.10^{a}
	1%	3.96±0.25^{a}	0.92±0.07^{a}
	0.5%	4.41±0.35^{a}	0.85±0.10^{a}

4.9 Effect of Homogenization Intensity and Xanthan gum

4.9.1 Particle size

Considering the effect of emulsification method on the double emulsion stability, multimodal and monomodal particle size characteristics were observed for microfluidized double emulsions and those prepared with Ultra-turrax, respectively. As can be seen from Figure 4.31 a, several peaks have been observed after microfluidization at 140 bar for the double emulsions with and without xanthan gum.

Although very small droplets were produced during the microfluidization, a uniform droplet size distribution could not be achieved. This can be explained by the insufficient homogenization time and/or intensity. Also, as the sample unit of the microfluidizer was small for the sample volume to be emulsified, homogenization was carried out by dividing the sample into several batches, which might have resulted in a non-uniform oil droplet size distribution. Yildirim *et al.* (2017) also found that the use of xanthan gum did not enable the fabrication of the evenly dispersed droplets even at higher shear rates as a result of highly viscous structure formed by xanthan gum. Hence, larger oil droplet size was found in case the xanthan was used in the external water phase. On the other hand, the double emulsions prepared with Ultra-turrax were characterised by a more uniform particle size distribution in comparison with the microfluidization method.

Considering the xanthan gum effect, the peak of the size distribution shifted to the left after the inclusion of xanthan gum which demonstrates the decrease in droplet size (Figure 4.31 b). Moreover, the influence of the homogenization level on the size distribution can be also seen from Figure 4.31 b where the peak of the sample emulsified at 24000 rpm was found to the left of the sample produced at 17500 rpm, irrespective of the inclusion of xanthan gum. In other words, a more intense Ultra-turrax treatment allowed to produce a smaller droplet size as a result of increased shear stress. The micrographes inserted into Figure 4.31 prove the latter claim looking at the smaller droplet size of the emulsion homogenized at 24000 rpm.

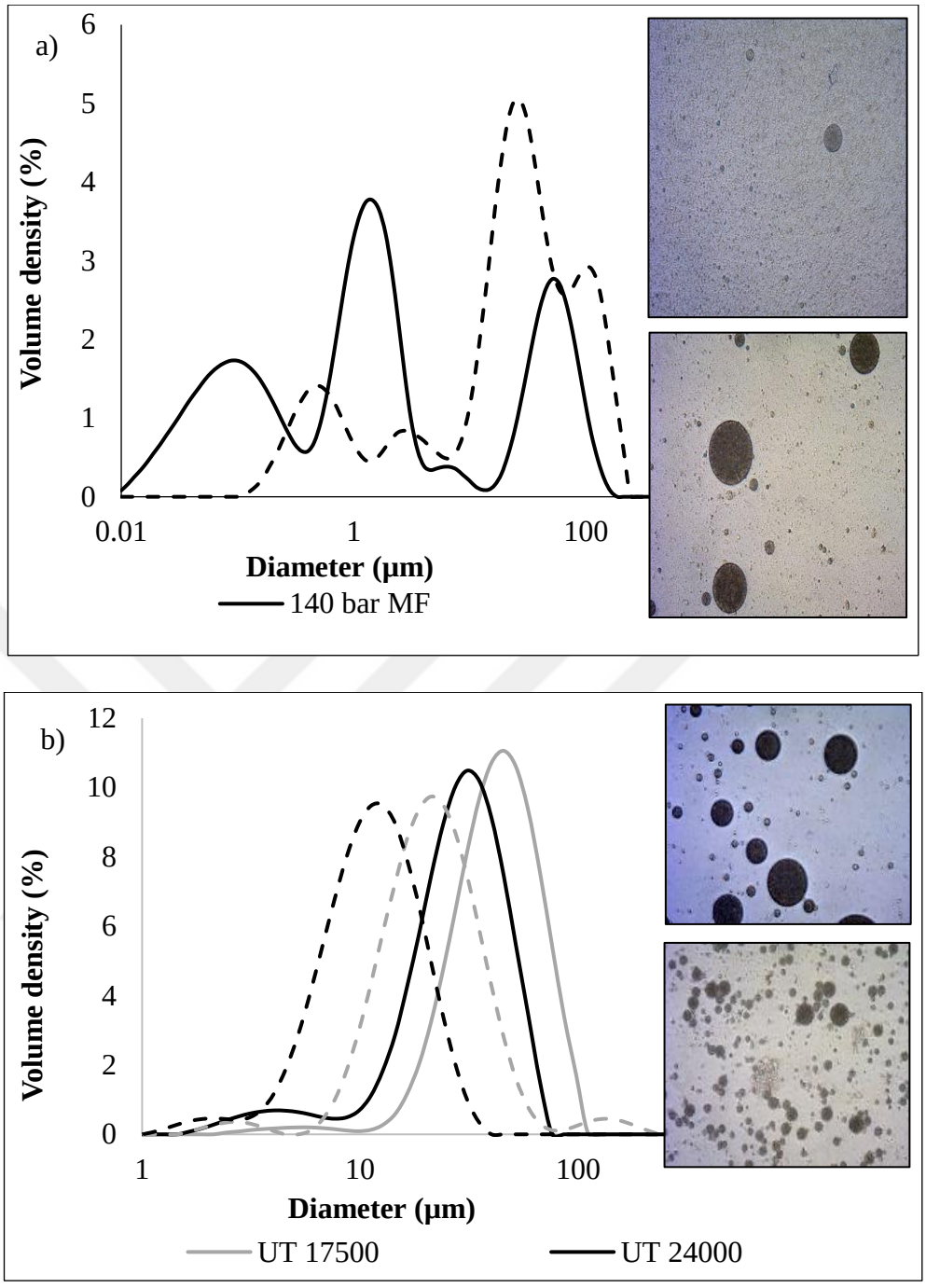

Figure 4.31 : Droplet size distribution of 120 mM L-phenylalanine containing double emulsions homogenized using microfluidization (a) or Ultra-turrax treatment (b) with (dashed line) or without (full line) xanthan gum directly after preparation: the insets present the micrographs of the ten times diluted samples homogenized with 140 bar microfluidization (a) and 24000 rpm Ultra-turrax treatment (b) without (top) and with xanthan gum (bottom).

The particle size results indicated that the homogenization type and intensity, as well as the presence of xanthan gum, all influenced the particle size distribution. Bou *et al.* (2014) also reported that the homogenization factors and emulsion composition influence the size distribution of the emulsions. The average droplet size of the double emulsions prepared using varying homogenization intensities with and without xanthan gum was demonstrated in Figure 4.32. The average droplet size of the double emulsions showed a decreasing trend in the absence or presence of xanthan gum as the homogenization level increased from 17500 to 21500 rpm as indicated by the black arrows in Figure 4.32 (p<0.05). Also, the increased viscosity of the continuous phase slows down instability mechanisms such as coalescence and flocculation in emulsions. Besides, the decreased droplet size might result from the higher shear stress applied to the double emulsion. This outcome is consistent with the results of Leal-Calderon *et al.* (2012). In their study, xanthan gum addition enhanced the viscosity of the continuous phase which enabled the formation of smaller droplets and, therefore a better stability of the emulsion. It should be also noted that the average droplet size increased during 8 days of storage time for the samples without xanthan gum (p<0.05), whereas no significant change was found for the samples with xanthan gum (p>0.05).

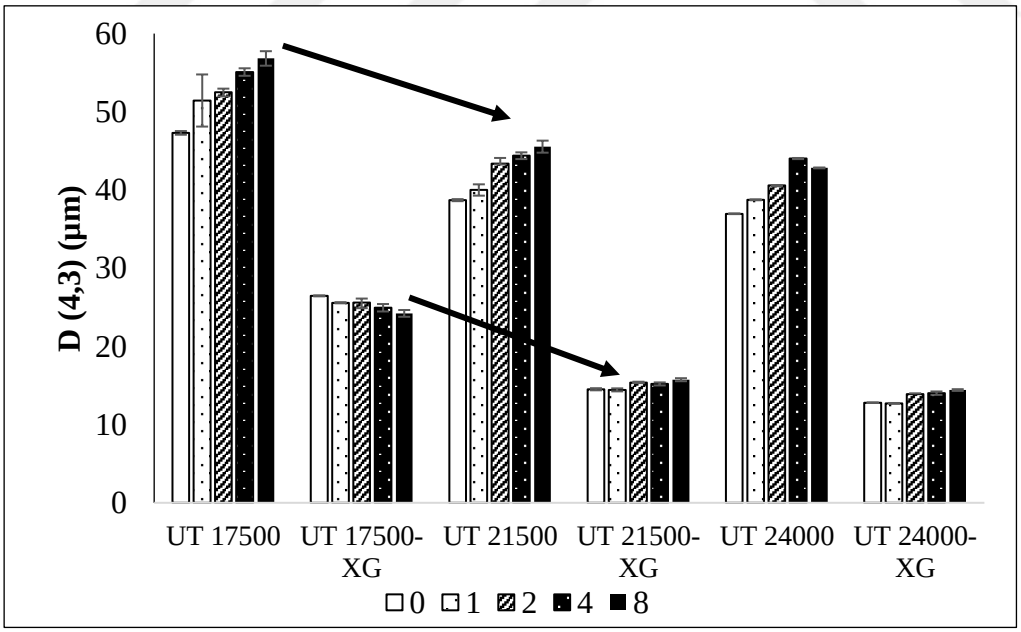

Figure 4.32 : Average droplet size of 120 mM L-phenylalanine containing double emulsions homogenized at 17500, 21500 and 24000 rpm, prepared with or without xanthan gum as a function of storage time (0, 1, 2 or 8 days) at 37 ºC.

Table 4.12 illustrates the rate of change of the average droplet size of the double emulsions during 8 days. Considering the increase of average droplet size, the addition

of xanthan gum caused a significant difference for the same level of emulsification intensity ($p<0.05$). However, no significant difference was found in the use of different homogenization levels regarding the average droplet size increase except from the sample containing xanthan gum and prepared with 17500 rpm which was significantly different from the other emulsions.

Table 4.12 : Average rate of change of the volume-weighted average droplet size of 120 mM L-phenylalanine containing double emulsions stored at 37 °C for 8 days (expressed in µm/day).

UT (rpm)	XG	Coefficient of determination (R^2)	Rate of change of D43 (µm/day)
17500	-	0.98	2.78 ± 0.30^{c}
21500	-	0.94	2.64 ± 0.41^{c}
24000	-	0.98	2.06 ± 0.54^{c}
17500	+	0.92	-0.45 ± 0.13^{a}
21500	+	0.78	0.29 ± 0.14^{b}
24000	+	0.83	0.51 ± 0.17^{b}

4.9.2 Entrapped water volume

The transmission profiles represent the variation of the droplet concentration. High transmission indicates a low droplet concentration, whereas a low transmission shows a high droplet concentration. From Figure 4.33 a, b and c, the sample prepared at 17500 rpm displayed a higher transmission as compared to the ones prepared at 21500 and 24000 rpm after centrifugation started. The pronounced changes in the transmission profiles for the sample prepared at 17500 rpm indicated the rapid movement of the oil droplets during centrifugation. In the creaming mechanism, bigger droplets move faster to the top of the sample than smaller ones. As the droplet size of the double emulsion prepared at 17500 rpm was larger, the movement of the droplets was faster during analytical centrifugation. A similar observation can be seen by comparing the (initial) evolution of the transmission profiles of the emulsions obtained using the Ultra-turrax and microfluidization methods: as a further consequence of the high intensity applied upon microfluidization at 140 bar, a lower transmission can be seen for the microfluidized sample, indicating a smaller droplet size (Figure 4.33 d). On the other hand, the transmission profiles of the sample stabilized with xanthan gum and prepared at 17500 rpm demonstrated that an complete separation was not observed during 2 hours of centrifugation (Figure 4.33 e).

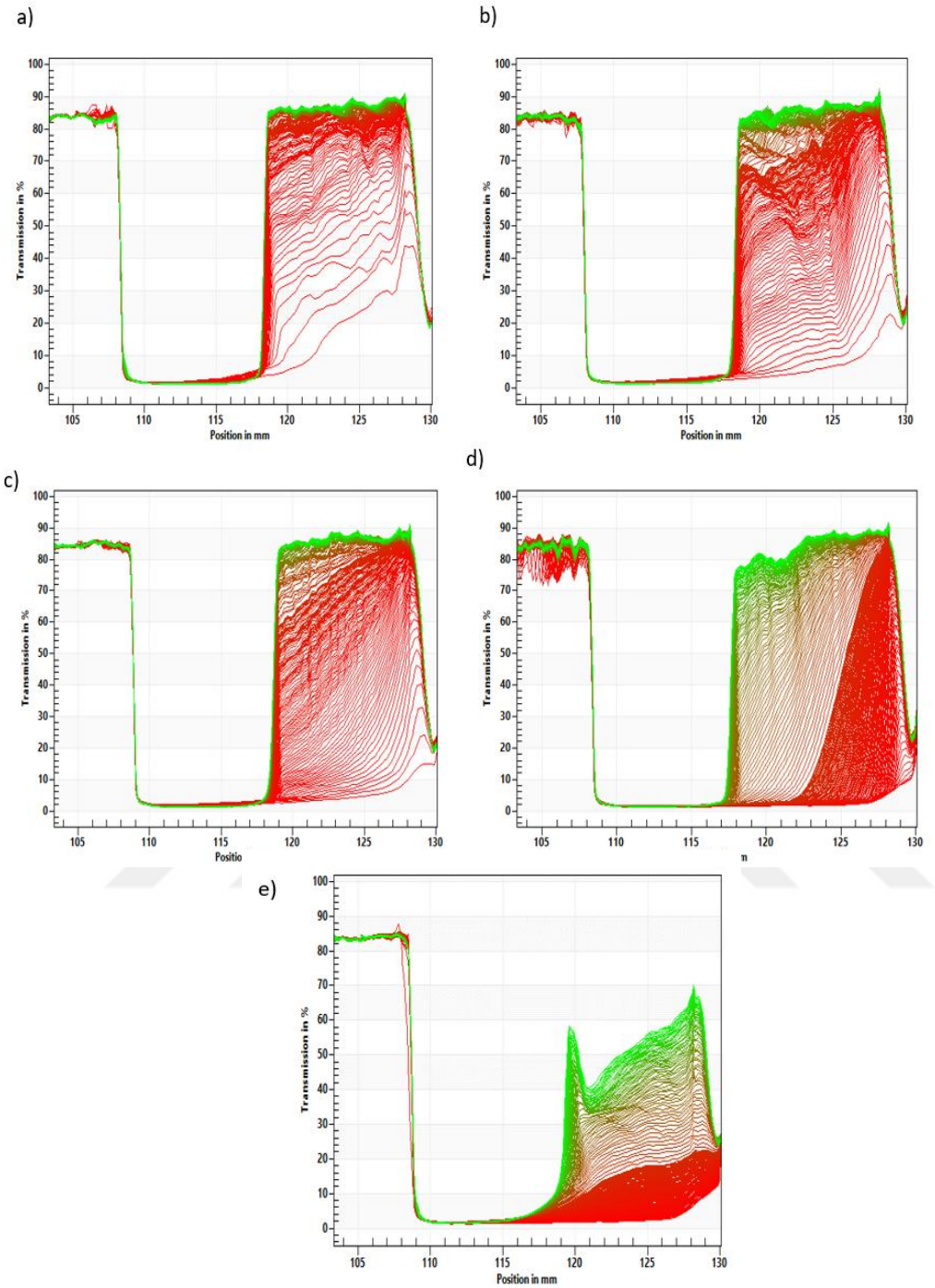

Figure 4.33 : Transmission profiles of the double emulsions containing 120 mM of L-phenylalanine in the absence (a-d) and presence of xanthan gum (e), homogenized with 17500 rpm Ultra-turrax (a, e), 21500 rpm Ultra-turrax (b), 24000 rpm Ultra-turrax (c), 140 bar microfluidizer (d) after 2 hours of analytical centrifugation at 3000 rpm.

The lumisizer tubes containing the samples just after analytical centrifugation are shown in Figure 4.34. The serum phase of the samples that containing xanthan gum was not entirely clear after 2 hours of centrifugation. This can be explained from the fact that the oil droplets can be hardly closely packed as a result of the increased viscosity of the continuous phase and the reduced oil droplet size.

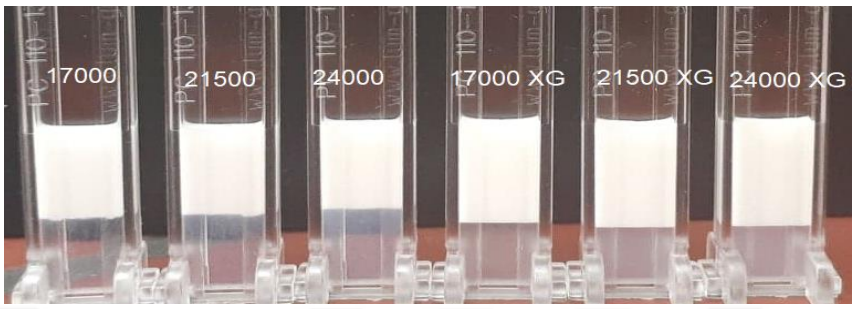

Figure 4.34 : Phase separation right after centrifugation at 3000 rpm for 2h of double emulsions containing 120 mM L-phenyalanine, with the opaque cream layer at the top and the more transparent serum layer (external water) at the bottom.

The entrapped water volume fraction of the samples homogenized with different levels of Ultra-turrax treatment without xanthan gum is shown in Figure 4.35. The yield of entrapped water values were about 80% immediately after preparation and were reduced to 60% after 8 days of storage. The reducement in the yield of entrapped water can be as a result of faster diffusion of amino acid as compared to the KCl which was used to maintain osmotic pressure as it was discussed in section 4.3.2.

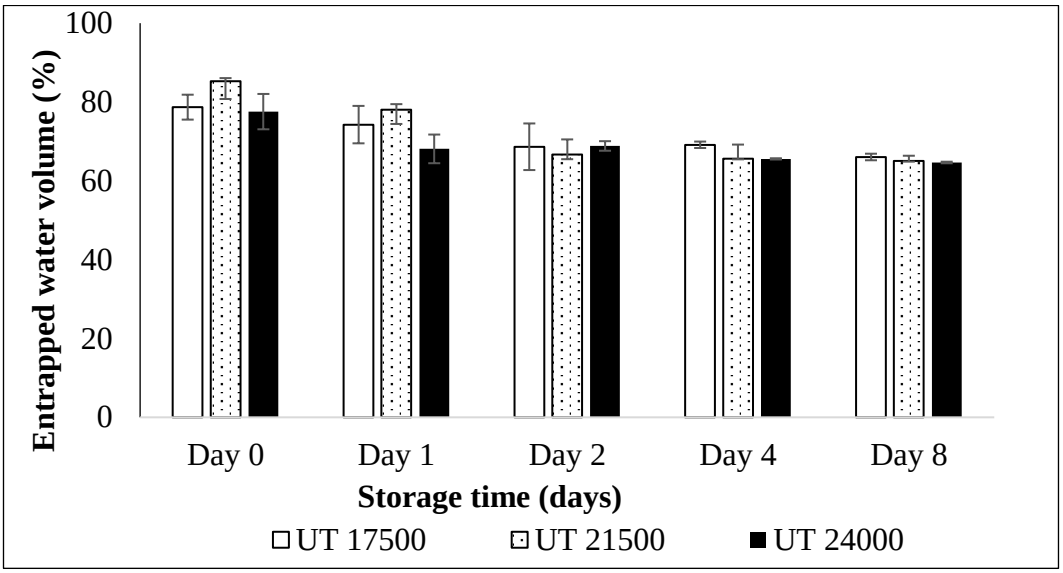

Figure 4.35 : Entrapped water volume fraction (%) of double emulsions containing 120 mM L-phenylalanine prepared with varying Ultra-turrax homogenization levels during storage at 37 °C.

4.9.3 Amino acid release

The released L-phenlyalanine concentration during 8 days of storage is demonstrated in Figure 4.36. In general, the release of the double emulsions homogenized using an identical intensity without xanthan gum was slower as compared to the samples with xanthan gum. However, the effect of homogenization intensity could not be clearly observed probably due to the high hydrophobicity of the L-phenylalanine. Therefore, L-leucine release was also examined to observe more reliable release kinetics.

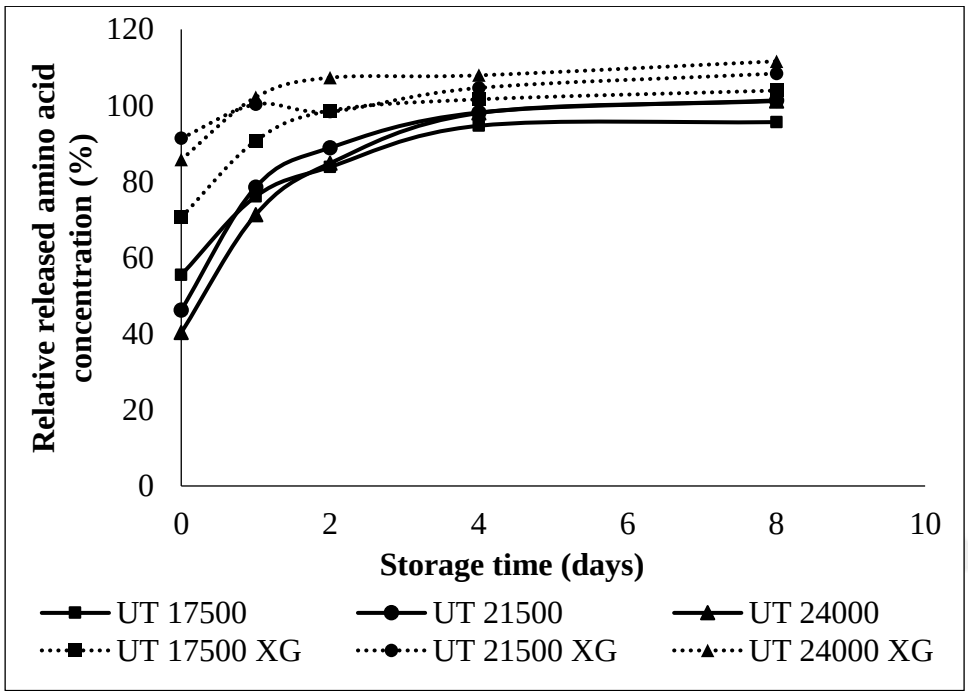

Figure 4.36 : Released L-phenylalanine concentrations in the external phase of double emulsions with or without xanthan gum, prepared with different homogenization levels of Ultra-turrax during storage at 4 °C.

As can be seen from Figure 4.37, the released L-leucine concentration was inversely proportional to the homogenization speed. The released L-phenylalanine concentrations of the double emulsions homogenized with 17500, 21500 and 24000 rpm were 17.73%, 22.02% and 25.51% just after preparation, respectively. This indicates that the encapsulation efficiency of the L-phenylalanine was larger when the homogenization level was lower. The difference in encapsulation efficiency which changed with emulsification level became more pronounced during storage. This outcome is supported by Schuch *et al.* (2014) as they reported that there is a correlation between the encapsulation efficiency and the droplet size of the double emulsions. It was argued that the presence of larger droplets enables a better encapsulation efficiency.

The faster release of L-leucine in double emulsions with xanthan gum can be also explained due to the smaller oil droplet size of these samples resulting from xanthan gum addition. As can be clearly observed from Figure 4.37, it took about 2 days to reach the equilibrium concentration for the samples with xanthan gum, whereas no equilibrium was observed within the time frame of 16 days for the samples without xanthan gum. Opperman *et al.* (2016) investigated the impact of xanthan gum on the droplet size and water yield of double emulsions. They concluded that the addition of xanthan gum induced an increase in the viscosity of the aqueous phase which caused a more effective break up of oil droplets. This facilitated a smaller oil droplet size and reduced the yield of the internal water droplets. A decrease in the oil droplet size causes a larger interfacial area between the oil droplets and the external water phase. In other words, the possibility of interaction and solute transport increase for smaller droplets as compared to larger ones (Opperman *et al.*, 2016).

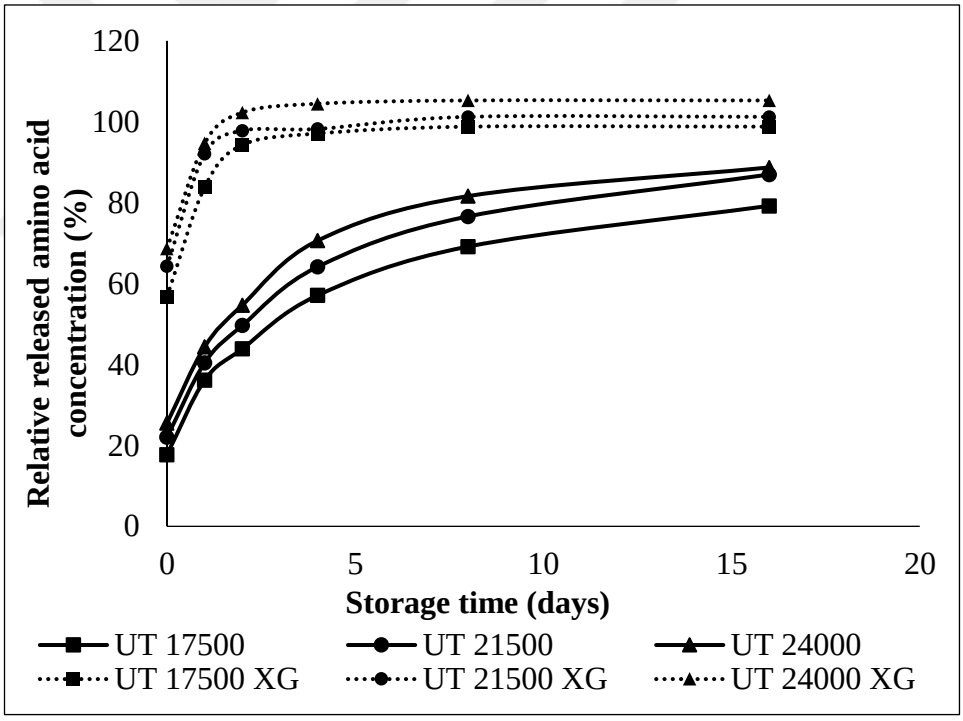

Figure 4.37 : Released L-leucine concentration in the external phase of double emulsions with or without xanthan gum, prepared with different homogenization levels of Ultra-turrax during storage at 4 °C.

Table 4.13 indicates the estimated t_a and C_0 values of double emulsions prepared by microfluidization and Ultra-turrax at varying intensities. Considering the C_0 values of the double emulsions without xanthan gum, almost all L-phenylalanine was already released during the microfluidization process. For the samples prepared using

microfluidization that contained xanthan gum, about 80% of L-phenylalanine was already released to the external water phase directly after preparation.

The double emulsions prepared with Ultra-turrax treatment, on the other hand, displayed a slower release compared to the ones prepared with microfluidization. An inverse relation was observed between the Ultra-turrax homogenization speed and the t_a value in double emulsions that contained L-leucine and L-phenylalanine. In other words, increasing the homogenization intensity induced a faster release of the amino acids. The t_a of the encapsulated L-leucine was about 2 times higher in the double emulsion without xanthan gum and prepared at 17500 rpm than in the emulsion homogenized at 24000 rpm. It is assumed that the entrapped compound in the larger droplets has to travel over a longer route, whereas the migration takes a shorter distance and hence time in smaller droplets. Regarding the xanthan gum effect, the t_a of L-leucine was 4 times higher in the double emulsion emulsified at 17500 rpm without xanthan gum as compared to the one that contained xanthan gum. It can be concluded that upon inclusion of xanthan gum in the samples prepared with Ultra-turrax treatment, the entrapped amino acids showed a more pronounced tendency to be released.

Table 4.13 : Optimized t_a and C_0 values (with their 95% confidence interval) of L-phenylalanine and L-leucine release in double emulsions in the absence or presence of xanthan gum, prepared with different homogenization levels of Ultra-turrax during storage at 4 ^{o}C.

Technique/ Intensity	**AA**	**t_a (days)**	**C_0 (mM)**	**t_a (days)**	**C_0 (mM)**
		Without XG		**With XG**	
MF 105 bar	L-phe (120 mM)	0	38.2 ± 0.7^{e}	0	32.5 ± 0.9^{f}
MF140 bar		0	42.4 ± 0.4^{f}	0	31.2 ± 1.0^{e}
UT 17500		1.52 ± 0.18^{b}	22.2 ± 0.6^{d}	0.98 ± 0.06^{c}	28.2 ± 0.3^{d}
UT 21500		1.47 ± 0.06^{b}	16.1 ± 0.4^{b}	0.75 ± 0.08^{b}	34.3 ± 0.7^{g}
UT 24000		1.19 ± 0.10^{a}	18.6 ± 0.6^{c}	0	36.6 ± 1.1^{h}
UT 17500	L-leu (15 mM)	4.07 ± 0.27^{d}	1.0 ± 0.1^{a}	0.93 ± 0.06^{c}	2.8 ± 0.0^{a}
UT 21500		2.86 ± 0.32^{cd}	0.9 ± 0.1^{a}	0.66 ± 0.10^{b}	3.2 ± 0.1^{b}
UT 24000		2.17 ± 0.37^{c}	0.9 ± 0.2^{a}	0.03 ± 0.01^{a}	3.4 ± 0.0^{c}

4.10 Release of Amino Acids in a Simulated Gastrointestinal System

The release of L-leucine and glycine was observed in a simulated gastrointestinal system. The digestion of the samples was not examined in the mouth since the double emulsions do not involve components that can be digested by the effect of the saliva secreted in the mouth. It was expected that the digeston of the samples mainly occured in the gastric and intestinal environments.

The absorbance spectra of the samples after 2 hours of gastric digestion is shown in Figure 4.38. The blank solution, which indicates the external water phase without amino acid (green), showed the slightest absorbence compared to the other samples. The absorbance values obtained for both glycine and L-leucine solutions at 340 nm were higher than the peaks of double emulsions with glycine or L-leucine. This clearly indicates the retardation of the amino acid release due to the double emulsion encapsulation. The double emulsion without amino acids showed an absorbance as well, which was probably due to the compounds formed during the digestion. These compounds might have reacted with TNBS during the amino acid determination and hence increased the absorbance values. On the other hand, the difference in the absorbance of blank double emulsion and of double emulsions with amino acids indicates the amino acid effect. As L-leucine released faster than glycine because of its higher permeability, the difference in absorbance with the blank emulsion was higher for the L-leucine than for the glycine double emulsion.

The absorbance spectra of 5 mM L-leucine solution and L-leucine double emulsion after 2 hours of gastric digestion is shown in Figure 4.39. As expected, the absorbance of the L-leucine containing double emulsion increased during digestion in the gastric environment. Also, the absorbance of the L-leucine solution (5 mM) was the highest among all samples. The release of L-leucine from a double emulsion during gastric and intestinal digestion is presented in Figure 4.40. According to these results, the enclosed L-leucine gradually released in the gastric environment, which confirms the protective characteristics of the oil barrier. The concentration of the L-leucine solution was found as 5 mM during gastric digestion, whereas the initial release for L-leucine was only about 0.2 mM and increased significantly to about 1.5 mM after 2 hours of gastric digestion ($p<0.05$).

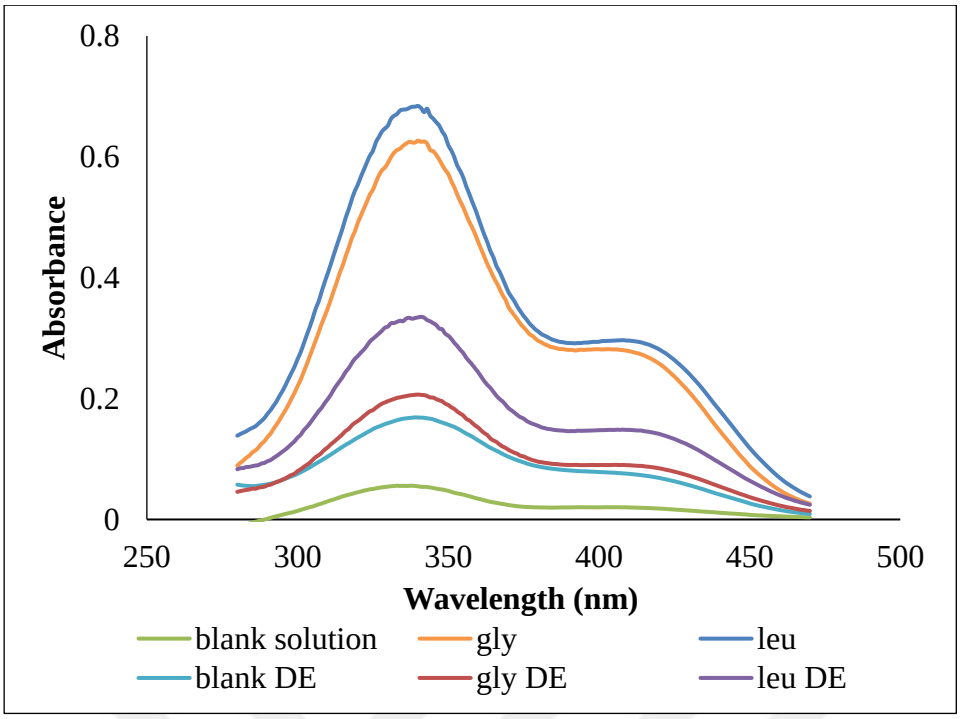

Figure 4.38 : Absorbance spectra of the solutions and double emulsions with or without 5 mM L-leucine and glycine after 2 hours gastric digestion.

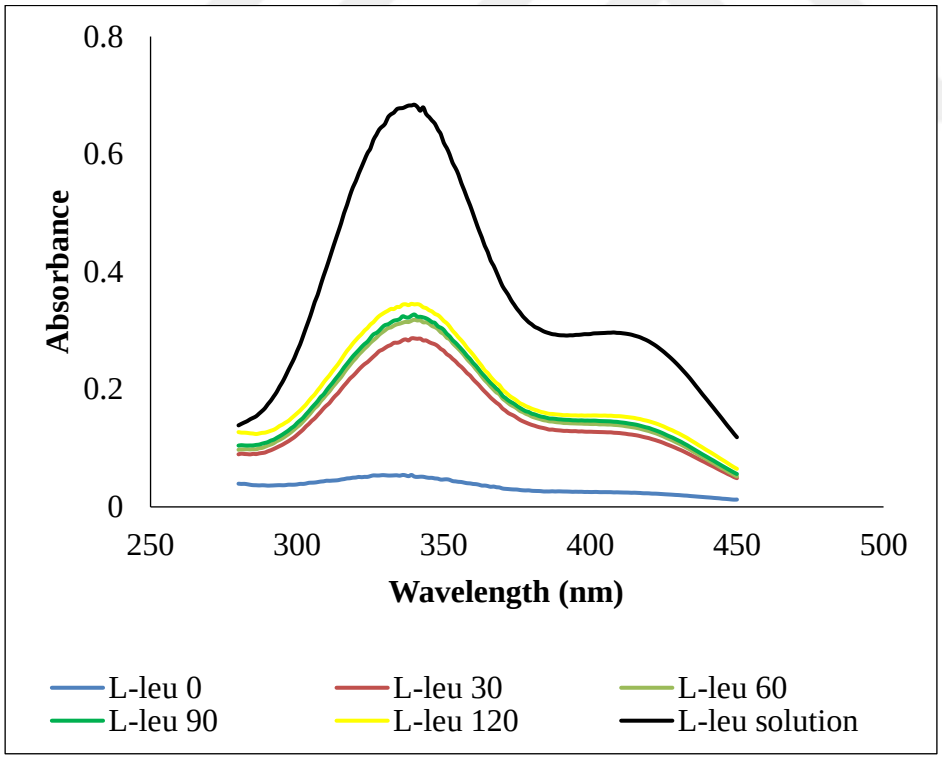

Figure 4.39 : Absorbance spectra of the samples with 5 mM L-leucine solution and released L-leucine concentration in double emulsions during 2 hours gastric digestion.

In the gastric environment, limited digestion was expected for the double emulsion as it does not contain protein based components to be digested by the effect of the pepsin included in the gastric juice. Moreover, the amino acids enclosed in the double emulsions can not be broken into smaller pieces as they are the basic units of proteins. As 75% of the double emulsions consisted of water whereas 25% of the composition was oil, the main digestion was expected in the intestinal phase.

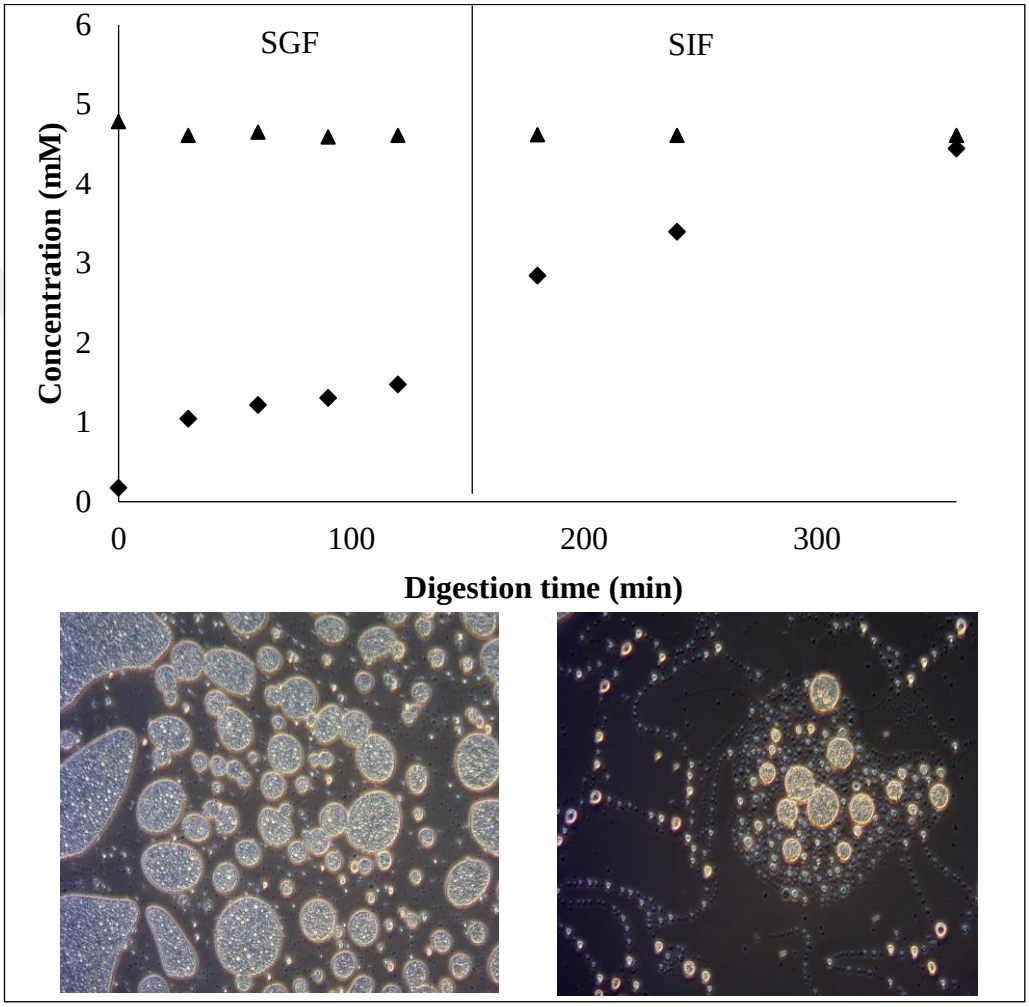

Figure 4.40 : Released L-leucine concentrations in the external phase of double emulsions during 6 hours gastrointestinal digestion. (i.e. 2 h gastric phase, followed by 4 h of intestinal phase). The insets show micrographs of the samples after 2 hours of gastric digestion followed by 4 hours of intestinal digestion (right).

After the release in the gastric environment, the released L-leucine concentration increased to about 3 mM in the intestinal fluid. Subsequently, the released L-leucine concentration reached to about 4.5 mM after 6 hours of gastrointestinal digestion. The released amino acid kinetics were fitted to the Higuchi model which is applicable for a diffusion-controlled release mechanism (Higuchi, 1961). The R^2 was found to be

0.87 considering the release during the gastrointestinal digestion. When the model was fitted to only the gastric release, the R^2 increased up to 0.96. This can be explained from the effect of the digested triglycerides during the intestinal digestion. The release of amino acids might be governed by diffusion in the gastric environment, whereas oil digestion can change this mechanism as well as the relase rate. The insets in Figure 4.40 demonstrate the rupture of oil droplets due to the effect of the digestive fluid. The micrograph on the left indicates the presence of a large number of oil droplets even after gastric digestion. However, the intestinal digestion remarkably reduced the size and number of oil droplets. The presence of empty oil droplets (i.e. without water droplets inside) with a small size also revealed the lipase enzyme effect which broke down the oil droplets.

The poorer fit when considering the complete gastrointestinal process might be due to the contribution of the intestinal fluid components to the absorbance. It should be noted that some fluctuations were observed in absorbance values of the samples digested in the intestinal environment. Giroux *et al.* (2016) also reported that monitoring peptide release from double emulsions in gastrointestinal digestion was difficult using spectrophotometry due to the complexity of the digestive fluids. The most significant effect might be due to the bile extract which had an orange color in the current research. As the quantification of amino acids was based on a colorimetric method, this effect substantially increased the absorbance values. Thus, this compound was not involved in the experiment to prevent this interference.

The release of the amino acids was thought to depend on both the physicochemical and chemical effects in the gastrointestinal system. It was found that the osmotic balance between the water compartments is crucial in terms of double emulsion stability (Mezzenga *et al.*, 2004). The physicochemical effects to which the emulsions are exposed are mainly the extreme pH conditions, which can cause an osmotic imbalance between the water phases of the double emulsions. In section 4.4 (where the pH effect was examined), it was found that a lower pH enabled a slower release as charged species showed a tendency to remain in the internal water droplets. However, in those experiments the pH of the two water phases was similar. In the case of a very low pH in the outer aqueous phase (in the gastric environment), the osmotic balance will become disturbed which will result in an instability of the double emulsions. In

order to prevent the effect of an osmotic imbalance, hydrophilic gelling agents were found to be effective by Opperman *et al.* (2016).

Another effect that facilitates the release of entrapped solutes is the hydrolysis of the triglycerides, which takes place during intestinal digestion. By the hydrolysis of the oil which forms the intermediate phase in the double emulsions, the protective barrier between both aqueous phases becomes disrupted. This accelates the release rate of entrapped amino acids as a result of thinning of the lipid barrier. This also reveals that the release of amino acids during digestion is not purely driven by diffusion. Giroux *et al.* (2016) investigated the peptide release from double emulsions in a simulated gastrointestinal environment. They reported that the release of peptide hydrolysates was mainly governed by lipolysis (i.e. degradation of the oil phase), rather than by diffusion. They also reported that the oil type of the double emulsions significantly affected the release mechanism and rate. It was found that longer chain triglycerides slowed down the release in comparison with shorter chain triglycerides. Kaimainen *et al.* (2015), on the other hand, examined the release of betalain in the intestinal environment. They reported that the release was rather due to the lipase and bile salts, whereas environmental factors such as osmolarity, temperature and pH did not significantly influence the release of betalain. They also stated that lipase bridging flocculation induced aggregation of oil droplets which resulted in inhibition in further release.

4.11 Diffusion Analysis of L-phenylalanine in Double Emulsions with High Resolution NMR

Detailed analysis of the temperature effect on encapsulation and release is complicated due to not only the sample incubation, but also the centrifugation to obtain a transparent external aqueous phase for spectrophotometric analysis should be performed at constant temperature. Especially the centrifugation step is troublesome as high speed centrifugation typically leads to a temperature increase. Moreover, centrifugation takes time, which makes it hard to observe the kinetics on a time scale less than 1 hour. In order to overcome these problems, high resolution NMR diffusometry was evaluated. As the diffusion behaviour of an amino acid in the internal and external aqueous phase is different (due to restricted and unhindered diffusion, resp.), the two fractions can be analysed without the need for a physical separation

(e.g. by centrifugation). This analysis is based on the diffusivity measurement of entrapped L-phenylalanine which was chosen due to its aromatic side chain that can be easily detected by NMR and discriminated from the contributions of oil and water. In a preliminary test, the protons involved in double bonds due to the unsaturation of HOSO overlapped with the amino acid signal. Hence, glyceryl trioctanoate oil was used as the oil phase due to the high saturation level and its liquid state down to 11 ^{o}C. In another experiment, a double emulsion with 15 mM of L-phenylalanine did not show a clear peak. Thus, 60 mM of L-phenylalanine was encapsulated in order to enable the detection of clear diffusion peaks.

In the analysis, freshly prepared samples were used to avoid amino acid release before analysis. In order to compare the free and restricted diffusion profiles of L-phenylalanine, NMR was carried out on two samples. First, 60 mM L-phenyalanine was dissolved in the internal water phase including deuterium oxide (D_2O), potassium chloride and sodium azide which was expected to give rise to free diffusion. Second, a double emulsion containing 60 mM L-phenylalanine in the internal water phase was considered, which was thought to diffuse slower due to the droplet confinement.

The spectra of the L-phenyalanine aqueous solution (red line) and of the L-phenyalanine in the double emulsion (blue line) are shown in Figure 4.41. In the literature, the peaks belonging to the aromatic ring in a L-phenylalanine solution are observed at 7.1-7.4 ppm (Corsaro *et al.*, 2015). The L-phenyalanine solution exhibited several peaks apart from the aromatic structure indicating the contibution of the protons: the peaks around 3 ppm are due to the methylene group next to the aromatic ring, whereas the contribution around 4 ppm is due to the –CH- group between the amino and the carboxyl group. From the spectra, several sharp peaks are observed for the L-phenylalanine solution, whereas only a single broad peak (shown by a black arrow) was obtained for L-phenylalanine contained the double emulsion at 7.1-7.4 ppm. As the NMR peak width is inversely proportional to the mobility, this broad peak indicates that the mobility of the L-phenyalanine in the double emulsion was much lower as compared to the simple aqueous solution. Whereas D_2O was used for solution and emulsion preparation, a signal was detected at about 4.7 ppm as a result of hydrogen deuterium oxide (HDO) formation from the exchange between hydrogen and D_2O. For the L-phenyalanine containing double emulsion, a large contribution of the specific groups of the glyceryl trioctanoate oil was observed. The methyl and

methylene groups of the triglycerides were found at 0.85-0.95 and 1.2-1.4 ppm, respectively. Moreover, the typical contribution of the protons close to the ester bond is observed at about 3.67 ppm (Satyarthi *et al.*, 2009).

Figure 4.42 compares the diffusion behaviour of water and L-phenylalanine in the 60 mM solution that contained the chemicals which were used to prepare the internal water phase. Considering the decay curves of L-phenylalanine and water, clear mono-exponential profiles were obtained for both samples showing free (unhindered) diffusion.

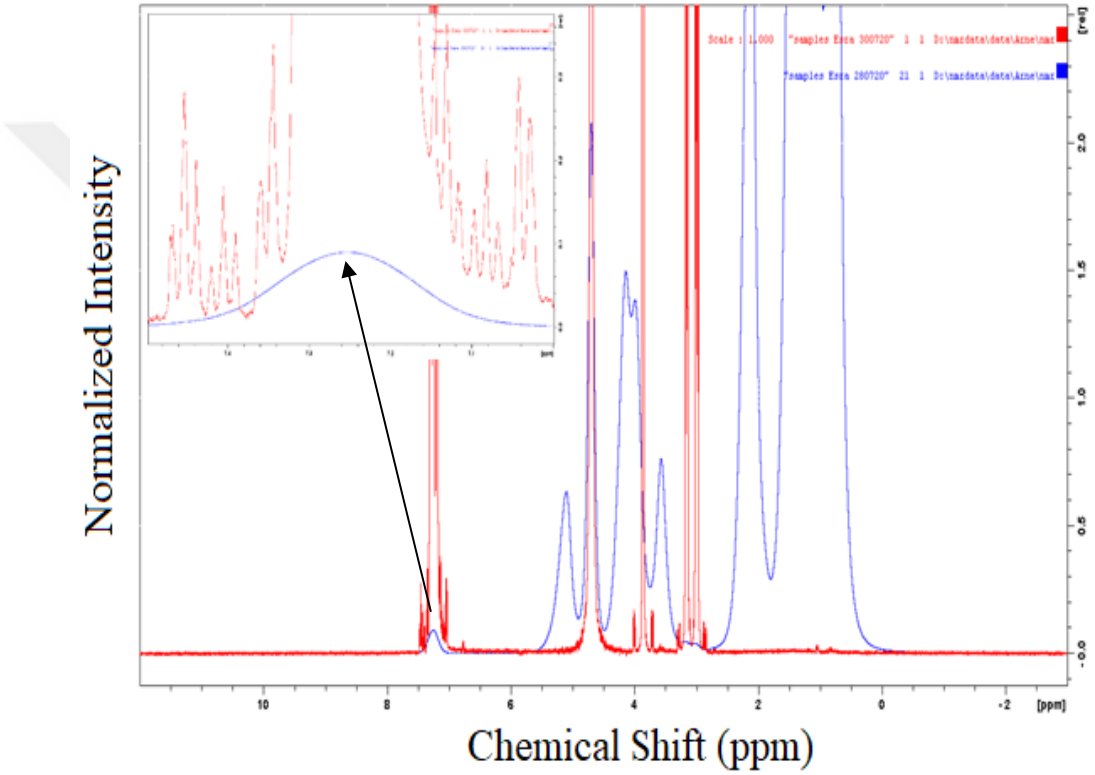

Figure 4.41 : H-NMR spectra of 60 mM L-phenylalanine in the internal water solution (red line) and of a double emulsion that contained 60 mM L-phenylalanine (blue line) in the internal water phase after 20 min of measurement at 20 °C.

It was reported that the enclosed compounds in a double emulsion exhibit a slower movement due to the obstruction of the oil droplets. However, the released molecules diffuse much faster in the external aqueous phase since they are not resticted anymore (Vermeir *et al.*, 2014). Regarding the diffusion of L-phenylalanine containing double emulsions, two fractions were detected in this study: slowly and fast diffusing components with different NMR parameters. The slowly diffusing component indicates the enclosed L-phenylalanine as the enclosed solute is restricted by the boundaries of the internal water droplets. The released L-phenyalanine, on the other

hand, does not experience the boundaries of the internal droplets anymore and hence diffuses much faster.

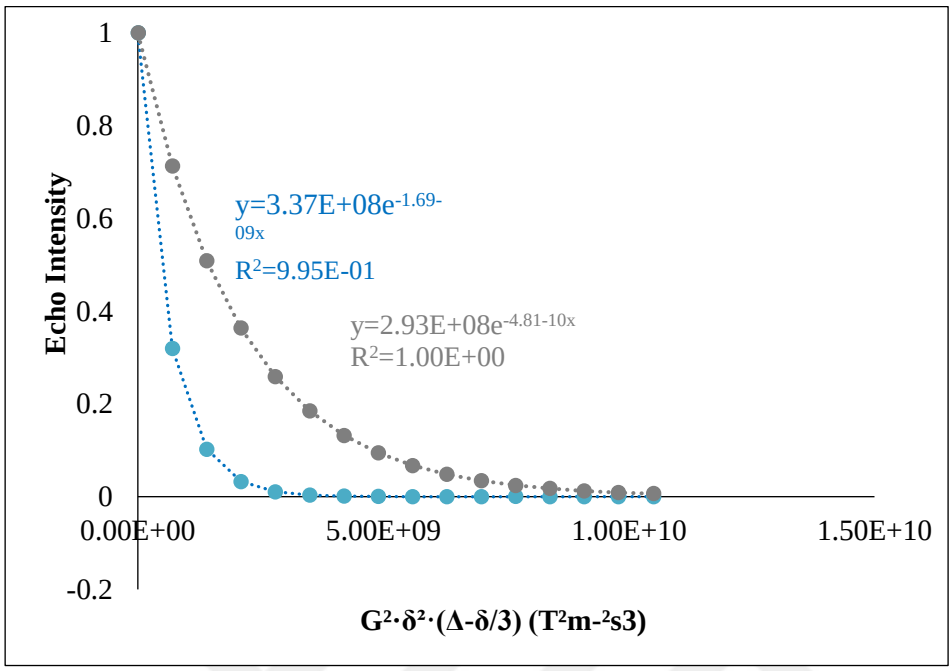

Figure 4.42 : Echo intensity decay the NMR-signal of water (blue line) and of L-phenylalanine in a 60 mM solution (grey line) prepared in the internal water phase; the measurements were performed at 20 °C.

The first and last measurement of the L-phenylalanine decay in double emulsions measured at varying temperatures is shown in Figure 4.43. The results indicate that a similar bi-exponential decay profile was found for the samples incubated at different temperatures. The decay of the encapsulated L-phenylalanine was very slow, being nearly a horizantal line. The intercept values of the L-phenylalanine decay are very similar at the start (Figure 4.43a) and at the end of the incubation period (Figure 4.43b), indicating that the same amount of phenylalanine was always detected. Considering the first and last measurement, the baseline values were significantly lower for the last measurement. Hence, the contribution of the slowly decaying fraction (which is thought to correspond to the entrapped L-phenylalanine) obviously decreased during the incubation period.

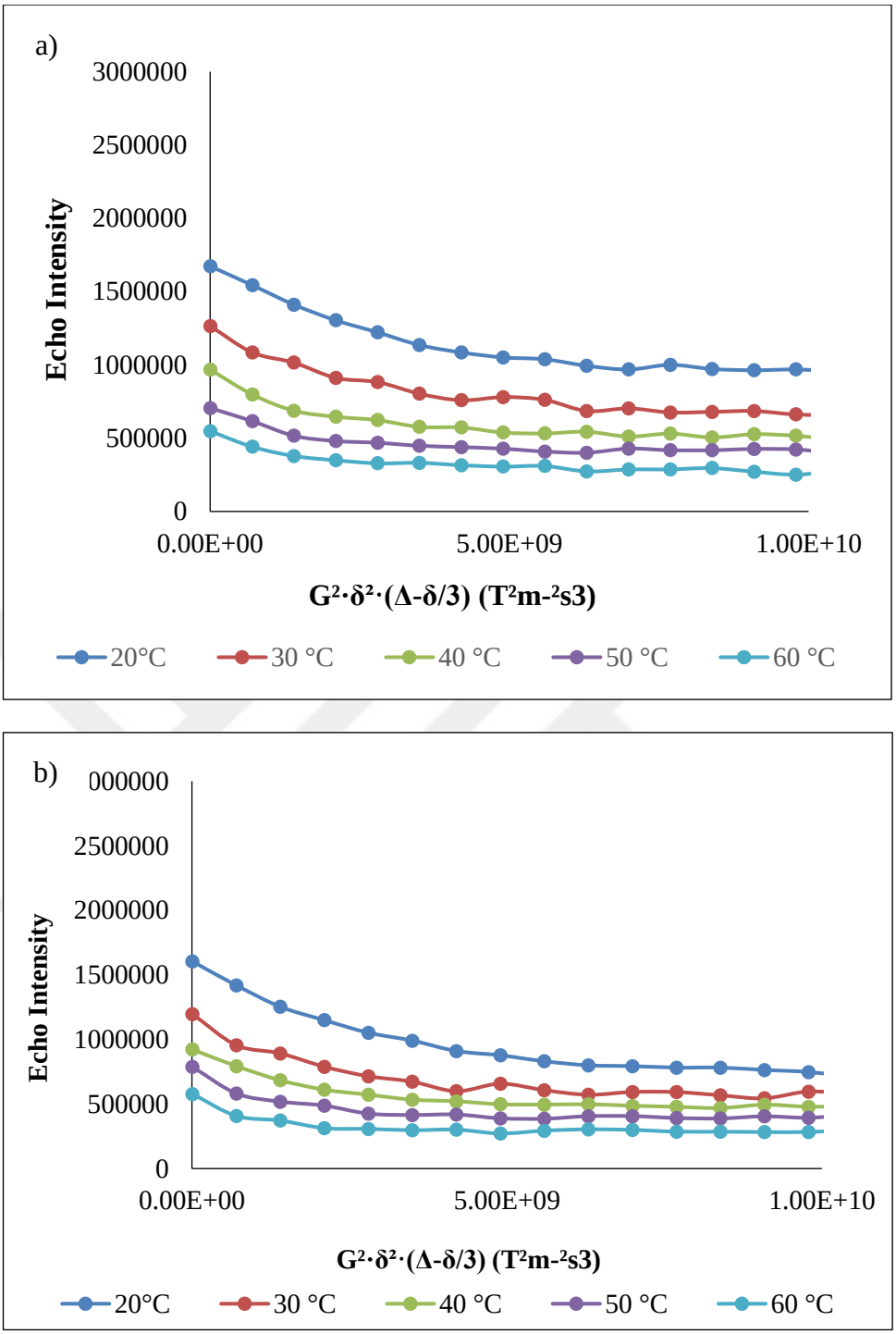

Figure 4.43 : Echo intensity of double emulsions containing 60 mM L-phenylalanine during incubation at different temperatures after the first (a) and last measurement (b).

Figure 4.44 demonstrates the water and L-phenylalanine decay in double emulsions stored at 30 (a) and 50 °C (b) as a function of incubation time. It can be clearly seen that the first and last decay profile overlap for water, which indicates that the enclosed water volume fraction did not change during the incubation at 30 or 50°C.

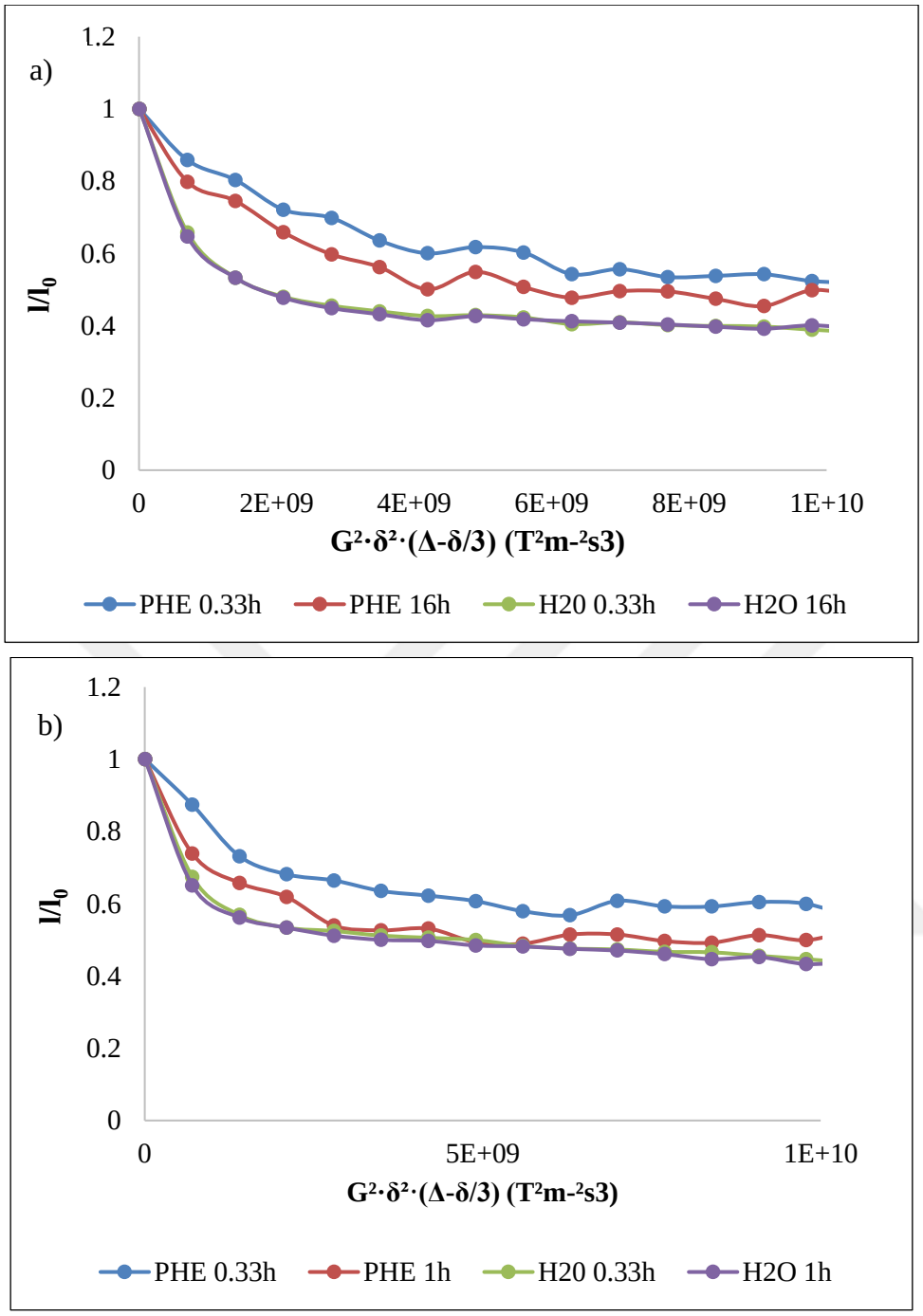

Figure 4.44 : Normalized echo intensity of water and L-phenylalanine in a double emulsion during incubation at 30 °C (a) and 50 °C (b).

The diffusion coefficient of the inner (D_{slow}) and outer water (D_{fast}) exhibited similar values for the first and last measurement both at 30 or 50°C (Table 4.14). On the other hand, the decay curves of L-phenylalanine did not overlap in the first and last measurement, both at 30 and 50°C. The lower intensity of the last measurement as compared to the first one indicates the release of L-phenylalanine during incubation.

Based on the 25/25/50 composition of the double emulsion used in this research, the enclosed water volume fraction is expected to be 1/3. However, Table 4.14 shows that the slowly diffusing component (of both water and L-phenlyalanine) was always larger. This may be due to some contribution of the triglycerides present (which are much more intense in signal) as these will also diffuse much slower than water or amino acid in water. On the other hand, the slowly diffusing fraction, corresponding to the entrapped L-phenyalanine, was around 60% in the first measurement, whereas it decreased to about 50% in the last measurement at both 30 and 50°C. Considering the diffusion coefficients, higher values for the fast diffusion coefficient were obtained at higher temperature, which is a logical consequence of the higher kinetic energy. Also, the diffusion coefficient of the released L-phenyalanine (D_{fast}) was higher at 50 °C in comparison with 30 °C.

Table 4.14 : Bi-exponential fit parameters of water and L-phenylalanine in a double emulsion during incubation at 30 °C and 50 °C.

		T = 30°C			T = 50°C		
		Slow (%)	**D_{fast} (10^{-12} m^2/s)**	**D_{slow} (10^{-12} m^2/s)**	**Slow (%)**	**D_{fast} (10^{-12} m^2/s)**	**D_{slow} (10^{-12} m^2/s)**
Phe	**First (0-20 min)**	60.5	489	15.3	58.8	652	0.4
	Last	48.4	540	1.6	50.6	884	0.0
H_2O	**First (0-20 min)**	46.6	1386	18.7	55.0	1757	21.2
	Last	45.3	1370	14.6	54.3	1915	21.5

Regarding the release behaviour, the main problem was that during (or maybe even before) the first 20 minutes of measurement, a large fraction of the enclosed amino acid has been released. Using the colorimetric method, the released amino acid content was found to be 47.4% just after preparation. It thus seems that some additional amino acids were released before the NMR analysis. This may have occurred during the storage period (at low temperature) between double emulsion preparation and NMR measurement, but also during the optimisation of the NMR parameters before the actual measurement. During the latter period, the sample is already at the desired temperature, and hence some additional release will surely happen. Therefore, only around 60% of the entrapped L-phenyalanine (slow diffusion part) of the double emulsion remained present before the NMR analysis. As such, the observation of differences in the release kinetics of the double emulsions at different temperatures became difficult.

From Figure 4.45, the slow diffusing fraction of L-phenylalanine was found as 57% and 56% upon 20 min incubation at 20°C and 50°C, respectively. The lower fraction of slowly diffusion components is expected at higher temperature due to the faster release. however, the differences between different time points were limited. In the ideal case, all amino acid would be encapsulated originally (and hence there would be only slow decay), whereas upon complete equilibration, 2/3 of the amino acid should be released into the external phase (and hence is expected to diffuse rapidly). For the diffusion of L-phenylalanine in the double emulsion at 20°C, the equilibrium concentration was seen after 16 hours of incubation: the relative decay decreased to 44% of its initial value.

An overview of the parameters calculated with bi-exponential fittings are demonstrated in Table 4.15. Considering the fraction of fast decaying L-phenylalanine, an increase was observed during incubation at all temperatures. As expected, a proportional relation was found between the fast diffusion coefficient and the temperature. Hence, the fast diffusion coefficient became higher when the incubation temperature increased. On the other hand, the slow diffusion coefficient was very slow (mostly around 0), with only a few exceptions. Comparing the L-phenylalanine diffusion in the internal and external aqueous phase at 20°C, the diffusion coefficient was 482.10^{-12} m^2/s for the internal aqueous phase and 341.10^{-12} m^2/s for the external water phase (Table 4.15). The lower diffusion coefficient in the external water phase may be due to the presence of xanthan gum which restricts the movement of this phase. The addition of emulsifiers and stabilizers may substantially affect the diffusion coefficient of the aqueous phases.

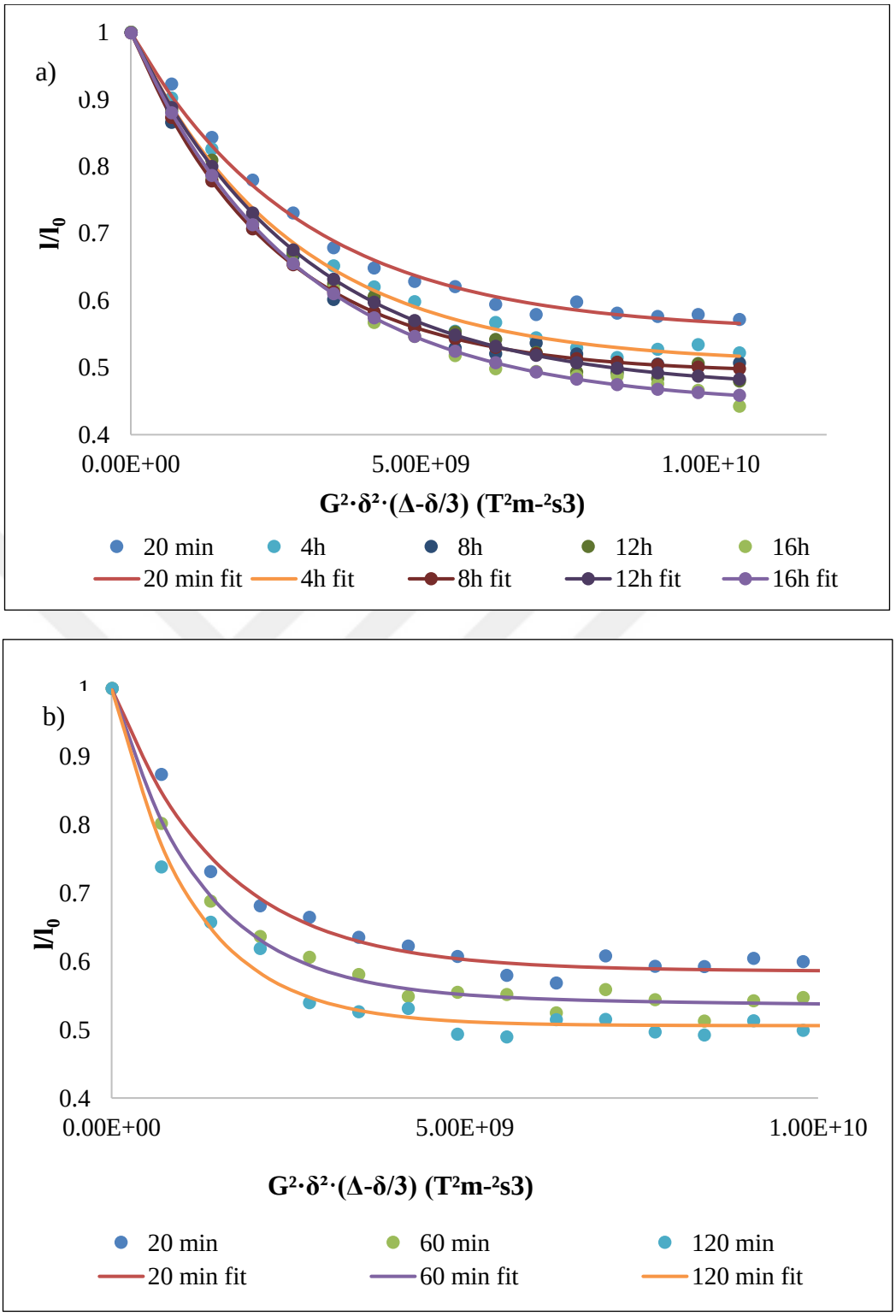

Figure 4.45 : Experimental (dotted line) and biexponential fitted (full line) normalized echo intensity of the L-phenylalanine signal of the double emulsion during incubation at 20 °C (a) and 50 °C (b).

Table 4.15, I_{fast} represents the absolute contribution of the fast decaying L-phenylalanine; this fraction is thought to be more reliable as it is not affected by some background triglyceride signal; this is shown graphically in Figure 4.46. The intensity of the fast diffusing part of the L-phenylalanine signal increased during incubation. This is related to the increased fraction of fast diffusing L-phenylalanine during the incubation which caused an increase in their signal. It should be also noted that the sum of the fast and slow fractions was 100% for most of the cases (Table 4.15). However, this value was above 100% for some cases (shown in red bold in Table 4.15). This might be due to the creaming of the oil droplets which resulted in observation of the higher slowly diffusing fraction.

Table 4.15 : Bi-exponential fit parameters of a 60 mM L-phenylalanine containing double emulsion during incubation at varying temperatures.

Temp (°C)	Time (h)	slow (%)	fast (%)	slow fraction (%)	D_{fast} (10^{-12} m^2/s)	D_{slow} (10^{-12} m^2/s)	I_o	I_{fast}
	0.33	55.33	44.67	100	340.68	0.00	1672613	747122
	4.00	50.57	49.43	91.4	359.63	0.00	1572351	777220
	8.00	49.15	50.85	88.8	409.09	0.00	1599708	813496
20 °C	12.00	46.71	53.29	84.4	335.66	0.00	1563456	833154
	16.00	44.43	55.57	80.3	345.88	0.12	1604684	891649
	0.33	**60.48**	**47.30**	100	489.11	15.29	1263796	597723
	4.00	50.57	49.43	83.6	462.26	1.88	1131726	559417
	8.00	49.45	50.55	81.8	489.03	0.00	1160805	586795
30 °C	12.00	49.24	50.76	81.4	523.88	0.00	1155362	586507
	16.00	48.35	51.65	79.9	540.29	1.64	1197278	618376
	0.33	60.57	39.43	100	782.32	15.14	966788	381203
40 °C	2.00	**54.13**	**40.24**	89.4	677.50	1.57	956927	384291
	4.00	51.64	48.36	85.3	552.68	0.00	922559	446152
	0.33	**58.82**	**42.81**	100	651.57	0.37	705107	301877
50 °C	1.00	54.74	45.26	93.1	787.66	1.78	742457	336009
	2.00	**50.60**	**45.57**	86.0	883.66	0.00	788339	359217.40
	0.33	62.85	37.15	100	1011.52	24.72	547413	203373.30
60 °C	0.67	49.43	50.57	78.6	894.27	0.00	586925	296796.80
	1.00	51.06	48.94	81.2	1134.61	2.25	578232	283008.20

Concerning the temperature effect, it can be seen that the intensity increase was higher at higher incubation temperatures. Whereas the increase in fast decaying component was sharp at 60 ^{o}C, rather horizantal lines were obtained for the lower temperatures (i.e. 20, 30 ^{o}C) (Figure 4.46). Sabatino *et al.* (2011) investigated the influence of the temperature in a range from 5 to 45 ^{o}C on the entrapped water volume fraction using low-resolution NMR pfg diffusometry. They found that the enclosed volume decreases at higher temperature as a result of the enhanced permeability of the bilayer. This indicates the faster release at higher temperatures which was also observed in section 4.2.1.3.1.

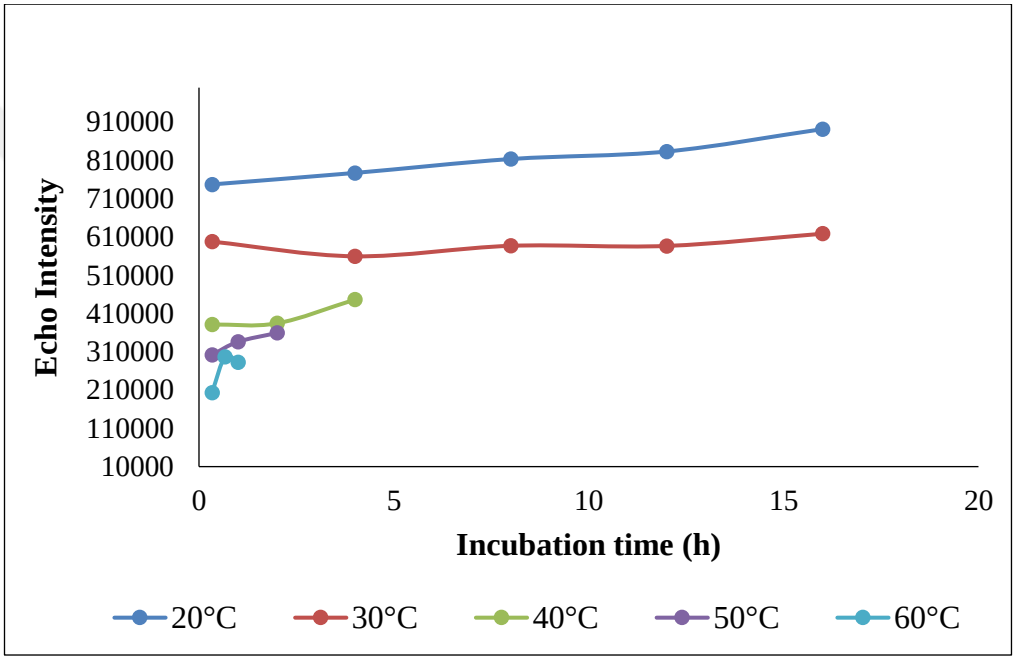

Figure 4.46 : Echo intensity of the fast decaying fraction of 60 mM L-phenylalanine containing double emulsions during incubation at different temperatures.

5. CONCLUSIONS AND FUTURE RESEARCH

5.1 Conclusions

There is an increasing research interest towards double emulsions since they have been proved to be effective for the encapsulation of bioactive compounds. Various applications of double emulsions can be used in different industries for many purposes. For example, encapsulation of both hydrophilic and hydrophobic compounds in double emulsions may provide protection against unfavourable environmental conditions. Also, the undesired flavor and taste of nutraceuticals can be masked via double emulsions. Moreover, it is possible to produce fat reduced food products by substituting some part of the fat content by water. Incompatible compounds can be also included in the same system in the separated aqueous phases of the double emulsions. This thesis contributes to the production and characterization of double emulsions considering the decisive factors for hydrophilic solute encapsulation and release. As such, this contribution is intended to provide guidance concerning the formulation of functional products to encapsulate bioactives in the pharmaceutical and food industry. From **section 4.1**, due to the high absorbance of the TNBS reagent, the original method regarding the amino acid quantification was modified. The optimum experiment conditions (i.e TNBS concentration, reaction time) were determined for amino acid and di-peptide determination.

From **section 4.2**, it was found that both hydrophobicity and temperature significantly influence the release rate of encapsulated amino acids. Based on the increasing fraction of released amino acid and the constant internal water volume fraction, direct diffusion was thought to be the primary transport mechanism of amino acids. It was also seen that amino acids were most permeable at the highest storage temperature studied (37 ^{o}C), which was in line with the increased amino acid solubility in oil. Looking at the hydrophobicity, the permeability became higher with higher hydrophobicity of the amino acids. This was also a result of the higher diffusion rate with better amino acid solubility in the oil phase. The results also suggest that amino acid and di-peptide release from the internal aqueous phase of double emulsions is controlled by the

molecular properties of the entrapped compound (with hydrophobicity overruling molecular size).

In **section 4.3**, it was found that when the concentration of the entrapped compound is too high (i.e 120 mM), the entrapped water volume as well as the release kinetics can be effected. In case solute concentration is high, the solute velocity is higher in comparison with the osmotic agent. This causes a higher osmotic pressure in the external water phase, and therefore a water flux towards external water phase to maintain osmotic balance. The decreasing trend in the entrapped water volume also speeded up the release rates of the amino acid. In order to prove the assumed more rapid amino acid exchange (as compared to KCl), both the concentration of amino acid and of KCl can be determined in the external aqueous phase during storage. The latter can be obtained by measuring the K^+ concentration, e.g. by atomic absorption spectrometry.

In **section 4.4**, a faster release of the amino acids and di-peptides was found at neutral pH as compared to gastric and basic pH conditions, which was due to the higher solubility of zwitterionic compounds in oil as compared to (more polar) charged solutes.

In **section 4.5**, the oil droplet size of the double emulsions stabilized by LCT oil was larger than those of MCT oil, which can be an effect of aggregation occurs stronger in LCT stabilized double emulsions. Comparing the encapsulation and release in MCT-oil based double emulsions to their LCT-based analogues, it was obvious that the release kinetics were much faster through the less hydrophobic MCT-oil.

In **section 4.6**, the water yield of the PGPR stabilized samples was close to 100% during 32 days of storage, irrespective of the PGPR concentration, except from 1% PGPR. Among the PGPR-stabilized double emulsions, the average residence time t_a of L-leucine was lowest in 5% PGPR-stabilised double emulsions, whereas it was highest in double emulsions containing only 1% PGPR, which indicated the faster release of L-leucine in the presence of excess reverse PGPR micelles. Hence, an excess of hydrophobic emulsifier should be avoided. In fact, PGPR concentrations which were too low to fully stabilise entrapped water droplets still gave rise to the slowest leucine release kinetics.

In **section 4.7**, the use of native or PC depleted lecithin faciliated a reduced oil droplet size of the double emulsion. This shows that the inclusion of lecithin fractions might enable stability in terms of droplet size. However, native or PC depleted lecithin cannot be an alternative to PGPR to produce stable double emulsions with high encapsulation efficiency using the formulation considered in this work: partial replacement of PGPR by PC-depleted lecithin gave rise to a faster release of the entrapped solute.

In **section 4.8**, 2% Tween 80 addition in double emulsions enabled more stable emulsion as compared to lower concentrations in terms of oil droplet size. This shows that the O/W2 interface was not filled with emulsifier sufficiently which does not help the droplets to repel each other. A modification of Tween 80 concentration did not lead to a difference in release kinetics, despite of a (limited) change in droplet size. This is probably due to flocculation which does not cause a change in the identity of the droplets.

In **section 4.9**, it was found that also other parameters, such as homogenization method and intensity and xanthan gum affected the droplet size. High homogenization level and xanthan addition decreased the droplet size remarkably. Also, an inverse relation was found between the double emulsions oil droplet size and the amino acid release kinetic constant, which means a faster release as a consequence of a decreased droplet size. This is thought to be due to the shorter distance over which the amino acid has to migrate from the internal to the external aqueous phase in smaller droplets.

In **section 4.10**, the release in the gastrointestinal environment exhibited a gradual transport to the external water phase, showing the proper protection of the enclosed amino acids. From a practical point of view, these results provide guidance in the design of colloidal systems for the encapsulation and sustained release for nutritional applications. Whereas an initial evaluation of the release in the gastrointestinal system was performed by spectrophotometry, it is advised to study the release using more sophisticated techniques (such as NMR) which might enable to understand the contribution of other compounds (i.e. bile salts, enzymes).

In section 4.11, the results suggest that the enclosed amino acid and water can be detected by H-NMR. Besides, their diffusion behaviour can be followed. These analyses proved the proportional relation between the fast diffusion coefficient and the incubation temperature. A similar outcome was reported using the spectrophotometric

TNBS method. On the other hand, a constant enclosed water volume fraction was also observed during incubation. The diffusion of L-phenylalanine in double emulsions exhibited the expected bi-exponential decay. However, the conditions used resulted in too much release before or during the first 20 minutes of measurement, so that further release could hardly be observed. For the future application of this technique, it is advised to choose a less hydrophobic compound to be enclosed to decrease the permeability. Overall, compared to the spectrophotometric TNBS method which is labor intensive and time consuming (as it requires a physial separation of the internal and external aqueous phases), the NMR technique offers a short analysis time, and is thus more user-friendly. Also, the amount of sample to use in the analysis is relatively small in NMR. Last but not least, it is much simpler to control the temperature during sample processing.

Recent studies have demonstrated that formulations and diets containing functional ingredients, such as amino acids, have beneficial effects on the human health. Hence, this thesis was proposed to gather fundamental information to assist the formulation of double emulsions that can be used in the production of functional foods or drinks. As the double emulsion systems studied in our research project had a rather large droplet size, it is logical to include them in foods with a high viscosity (to prevent creaming) and that do not have to be optically clear. Hence, the delivery system studied in this thesis might be incorporated in concentrated food emulsions, such as mayonnaise and salad dressings.

5.2 Future Research

In the current study and most of the research performed by other researchers, double emulsion preparation includes a two step production process: a previously formed primary W/O emulsion is used for the production of the W/O/W double emulsion. This makes the characterization of the primary emulsion an important issue, as it will undoubtedly affect the outcome of the second emulsification step. Hence, the characterization of the internal water droplets (especially their particle size distribution) is considered an important aspect in future research, e.g. comparing different emulsifiers and/or homogenization techniques. To that end, pfg-NMR diffusometry can be used.

In order to extend this study, additional research can be performed regarding the formulation parameters. A more profound investigation related to hydrophobic emulsifiers to substitute PGPR should be performed to avoid its inherent disadvantages, such as its off taste and its chemical connotation. Considering the previously reported negative impact of salts on the emulsification properties of lecithin, the use of glucose as an osmotic agent can be investigated to control the encapsulation and release in (PC-depleted) lecithin stabilized double emulsions. Hereby, glucose is expected to induce a better stability due to the absence of charge effects, which is especially important for ionic emulsifiers, such as (PC-depleted) lecithin. Also, the synthetic hydrophilic emulsifier Tween 80 can be replaced by natural stabilizers such as proteins (soy protein, whey protein, caseinate) and polysaccharides (pectin, starch, cellulose). Another promising strategy might be the use of an alternative oil phase such as Soft Palm Mid Fraction (soft PMF) which can be effective to retard the release of entrapped compounds as a further consequence of fat crystallization in the intermediate phase between both aqueous compartments. Besides, a temperature-triggered release might be enabled by the appropriate selection of the melting temperature of the fat phase used.

Whereas an initial evaluation of the release in the gastrointestinal system was performed by spectrophotometry, it is advised to study the release using more sophisticated techniques (such as NMR or HPLC) which might enable to understand the contribution of other compounds (i.e. bile salts, enzymes). In addition, to enable commercial application, the formulation parameters should be optimized for industrial production. Furthermore, the interaction between the delivery system and the food matrix should be profoundly examined.

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- **Kocaman, E**., Karaca, A. C., & Van der Meeren, P. (2020). Release of amino acids encapsulated in PGPR-stabilized W/O/W emulsions is affected by temperature and hydrophobicity. *Food Research International*, *137*, 109527.
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