

**Aus dem Institut für Humanernährung und Lebensmittelkunde  
der Christian-Albrechts-Universität zu Kiel**

**Preparation of solid lipid nanoparticles from cocoa butter:  
Chacaraterization and applications in food sector**

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## **Abstract**

The fortification of food products with functional compounds such as antioxidants, vitamins and omega-3-fatty acids is a constant focus of the food research. This is employed as a preventive action in maintaining the health against nutrient deficiency in the human diet. For this purpose, ongoing efforts are exerted in order to improve the techniques for efficiently introducing functional compounds into food systems. In the recent years, the innovative applications of nanotechnological approaches started to grow in the field of food science. However, the need of suitable materials and preparation methods for a practical application in the food sector is a big challenge. The present work introduces a new approach in this area through a developed SLNs system based on the use of the cocoa butter and a food grade surfactant blend. The system was fabricated by the hot homogenization, which is a commonly used technique in the food industry. No solvents were utilized in this approach for preparation purposes. A two level factorial design was applied in order to optimize the preparation variables.

The obtained system was examined for its suitability to encapsulate bioactive compounds, where (-)-epigallocatechin-3-gallate (EGCG) and cholecalciferol were used as examples for hydrophilic and lipophilic compounds, respectively. In conclusion, the presented work describes an innovative approach, which will show potential applications in the food sector.

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## **Kurzdarstellung**

Die Anreicherung von Lebensmittelprodukten mit funktionellen Additiven wie Antioxidantien, Vitaminen und Omega-3-Fettsäuren ist ein ständiger Fokus der Lebensmittelforschung. Dies wird als eine vorbeugende Maßnahme verwendet, um die Gesundheit gegen Nährstoffmangel in der menschlichen Diät aufrechtzuhalten. Hierfür werden laufende Bemühungen ausgeübt, um die Lieferungstechniken funktioneller Substanzen in Lebensmittelsysteme zu verbessern. In den letzten Jahren begannen die innovativen Anwendungen der nanotechnologischen Ansätze im Bereich der Lebensmittel- und Ernährungswissenschaften zu wachsen. Jedoch ist der Bedarf nach geeigneten Materialien und Zubereitungsverfahren für die praktische Anwendung im Lebensmittelsektor eine große Herausforderung. Die vorliegende Arbeit stellt einen neuen Ansatz in diesem Bereich vor, wobei ein SLNs System entwickelt wurde, das auf die Verwendung von Kakaobutter und lebensmitteltauglicher Tensidmischung basiert. Die SLNs wurden durch die Hochdruckhomogenisierung hergestellt, die eine der oft verwendeten Techniken in der Lebensmittelindustrie ist. Keine Lösungsmittel wurden hierbei für Zubereitungszwecke eingesetzt. Um die Herstellungsparameter zu optimieren wurde ein zweistufiger faktorieller Versuchsplan angewendet.

Das hergestellte System wurde hinsichtlich der Eignung für die Verkapselung bioaktiver Substanzen geprüft. (-) Epigallocatechin-3-gallat (EGCG) und Cholecalciferol wurden als Modell für hydrophile bzw. lipophile Substanzen ausgesucht. Abschließend stellt diese Arbeit einen innovativen Ansatz dar, der potentielle Anwendungen im Lebensmittelsektor aufweisen wird.

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## Table of contents

List of figures .....	VIII
List of tables .....	XI
List of abbreviations .....	XII
<b>1. Motivation and hypotheses .....</b>	<b>1</b>
<b>2. General introduction .....</b>	<b>4</b>
2.1 Bioactive nanocarrier .....	4
2.2. Solid lipid nanoparticles .....	6
2.2.1. Preparation technique of SLNs .....	8
2.2.2. SLNs composition .....	10
2.2.2.1. Cocoa butter .....	10
2.2.2.2. Surfactant blend .....	13
2.2.3. SLNs for the delivery of bioactive compounds .....	17
2.2.3.1. Encapsulation of bioactive compounds .....	17
2.2.3.2. Release of encapsulated compounds from SLNs .....	20
<b>3. Methods .....</b>	<b>21</b>
3.1 Particle size and size distribution .....	21
3.2. Zeta potential .....	22
3.3. Total content (TC) and entrapment efficiency (EE) .....	23
3.3.1. EE for EGCG .....	24
3.3.2. EE for cholecalciferol .....	25
3.4. <i>In-vitro</i> release tests .....	25
3.5. Thermal analysis .....	26
3.6. Morphology measurements .....	27
3.7. Storage stability .....	27
3.8. References .....	29
<b>4. Manuscript 1: The experimental design for the preparation of SLNs .....</b>	<b>38</b>
4.1 Abstract .....	38
4.2. Introduction .....	38
4.3. Materials and methods .....	40

---

4.3.1. Materials.....	40
4.3.2. Preparation of SLNs.....	40
4.3.3. The effect of individual formulation variables.....	41
4.3.4. The experimental design.....	41
4.3.5. Particle size, PDI and zeta potential.....	41
4.3.6. Thermal measurements.....	42
4.3.7. Physical stability of SLNs.....	42
4.3.8. Data analysis.....	42
4.4. Results and discussion.....	42
4.4.1. Surfactant blend.....	42
4.4.2. One factor-at-a-time (OFAT) experiments.....	44
4.4.2.1. Effect of the homogenization pressure.....	44
4.4.2.2. Effect of the number of homogenization cycle.....	45
4.4.2.3. Effect of the cooling conditions.....	47
4.4.3. Full factorial design experiments.....	47
4.4.3.1 Effects of independent variables on particle size.....	51
4.4.3.2 Effects of independent variables on polydispersity index (PDI).....	52
4.4.3.3 Effects of independent variables on zeta potential.....	52
4.4.4. Crystal modification.....	54
4.4.5. SLNs stability.....	56
4.5. Conclusion.....	57
4.6. References.....	58
<b>5. Manuscript 2: Encapsulaion of EGCG in SLNs.....</b>	<b>61</b>
5.1 Abstract.....	61
5.2. Introduction.....	61
5.3. Materials and methods.....	63
5.3.1. Materials.....	63
5.3.2. Preparation of EGCG-SLNs.....	63
5.3.3. Characterization of EGCG-SLNs.....	64
5.3.3.1. Particle size and zeta potential.....	64
5.3.3.2. Total EGCG content (TC).....	64
5.3.3.3. EGCG entrapment efficiency (EE).....	65
5.3.4. <i>In-vitro</i> release experiments.....	66
5.3.5. Thermal measurements.....	66
5.3.6. Morphology measurements.....	67
5.3.7. EGCG stability study.....	67

---

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5.4.	Results and discussion .....	68
5.4.1.	Particle size measurements .....	68
5.4.2.	Entrapment efficiency .....	69
5.4.3.	<i>In-vitro</i> release profiles of EGCG-SLNs .....	71
5.4.4.	Crystal modification of SLNs .....	72
5.4.5.	SLNs morphology .....	74
5.4.6.	Stability profile of EGCG in SLNs .....	75
5.5.	Conclusion .....	77
5.6.	References .....	78
<b>6.</b>	<b>Manuscript 3: Encapsulation of cholecalciferol in SLNs .....</b>	<b>82</b>
6.1	Abstract .....	82
6.2.	Introduction .....	82
6.3.	Materials and methods .....	83
6.3.1.	Materials .....	83
6.3.2.	Preparation of cholecalciferol-loaded SLNs .....	84
6.3.3.	Characterization of cholecalciferol-loaded SLNs .....	84
6.3.3.1	Particle size and zeta potential measurements .....	84
6.3.3.2	Total cholecalciferol content .....	84
6.3.3.3	Cholecalciferol entrapment efficiency .....	85
6.3.4.	Accelerated stability test .....	85
6.3.5.	Thermal measurements of cholecalciferol-loaded SLNs .....	86
6.3.6.	Statistical analysis .....	86
6.4.	Results and discussion .....	86
6.4.1.	Particle size and zeta potential .....	86
6.4.2.	Cholecalciferol content and entrapment efficiency .....	87
6.4.3.	Stability index of unloaded and cholecalciferol-loaded SLNs .....	88
6.4.4.	Crystal modification of cholecalciferol-loaded SLNs .....	89
6.4.4.1	Effect of cholecalciferol loading concentration .....	89
6.4.4.2	Effect of storage time .....	90
6.5.	Conclusion .....	92
6.6.	References .....	93
<b>7.</b>	<b>General discussion .....</b>	<b>95</b>
7.1	Influence of preparation variables on SLNs characteristics .....	96
7.1.1	Cocoa butter and surfactant blend .....	96
7.1.2	Homogenization pressure and number of homogenization cycles .....	96

---

---

7.1.3	Cooling rate .....	97
7.2	EE for encapsulated compounds .....	98
7.2.1	EGCG .....	98
7.2.2	Cholecalciferol .....	98
7.3	Stability of SLNs .....	99
7.3.1	Physical stability .....	99
7.3.2	Retention of encapsulated compounds in the system .....	100
7.3.3	Stability during the <i>in-vitro</i> tests .....	101
7.4	Outlook and final remarks .....	102
<b>8</b>	<b>References .....</b>	<b>104</b>
<b>9</b>	<b>Summary .....</b>	<b>119</b>
<b>10</b>	<b>Zusammenfassung .....</b>	<b>120</b>

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## List of figures

<b>Figure 2-1:</b> Cumulative number of the documents containing the keywords food and nanoparticle in their abstracts and/or claims.....	4
<b>Figure 2-2:</b> Schematic representation of the preparation of SLNs using the hot–high pressure homogenization.....	9
<b>Figure 2-3:</b> The chemical structure of SSL.....	16
<b>Figure 2-4:</b> The chemical structure of (-)-epigallocatechin-3-gallate (EGCG).....	18
<b>Figure 2-5:</b> The chemical structure of cholecalciferol (vitamin D3).....	19
<b>Figure 4-1:</b> Average particle diameter of SLNs as a function of homogenization pressure done for three homogenization cycles. Concentrations of cocoa butter and surfactant blend used in these experiments were constant at 5% and 1.5%, respectively, n=3 ± STD. Values with different letters indicate differences significant at p<0.05.....	44
<b>Figure 4-2:</b> PDI and zeta potential of SLNs as a function of homogenization pressure done for three homogenization cycles. Concentrations of cocoa butter and surfactant blend used in these experiments were constant at 5% and 1.5%, respectively, n=3 ± STD. Values with different letters indicate differences significant at p<0.05.....	45
<b>Figure 4-3:</b> Average particle diameter of SLNs as a function of homogenization cycle number at 600 bar. Concentrations of cocoa butter and surfactant blend used in these experiments were constant at 5% and 1.5%, respectively, n=3 ± STD.....	45
<b>Figure 4-4:</b> PDI and zeta potential of SLNs as a function of the number of homogenization cycle at 600 bar. Concentrations of cocoa butter and surfactant blend used in these experiments were constant at 5% and 1.5%, respectively, n=3 ± STD.....	46
<b>Figure 4-5:</b> Surface plot of the standard error for Design as a function of composition variables.....	50
<b>Figure 4-6:</b> Surface plot of the standard error for Design as a function of process variables	51
<b>Figure 4-7:</b> Surface plots of the effects of the variables on the different responses ( <b>A:</b> particle size; <b>B:</b> PDI; <b>C:</b> Zeta potential).....	54
<b>Figure 4-8:</b> DSC heating and cooling curves of bulk cocoa butter, n=3 (± STD: 0.16 for the melting peak; 0.087 for the recrystallization peak).....	54

---

<b>Figure 4-9:</b> DSC heating and cooling curves of SLNs prepared at 1000 bar and 5 homogenization cycles using 5% and 1.5% of cocoa butter and surfactant blend and cooled at 1°C, n=3 (± STD: 0.075 for the first melting peak; 0.040 for the second melting peak; 0.025 for the recrystallization peak).....	55
<b>Figure 4-10:</b> Changes in the average particle size of SLN during a storage time of three months at 2°C considering the initial size ( <b>A</b> :112.7 nm, <b>B</b> :132.6 nm, <b>C</b> : 150.1 nm, <b>D</b> : 175.0 nm, <b>E</b> : 195 nm), n=3±STD .....	56
<b>Figure 5-1:</b> Average particle diameter of EGCG-loaded SLNs as a function of the EGCG concentration in %w/w* related to the concentration of cocoa butter All SLNs were prepared at 1000 bar for 5 homogenization cycles using 5% and 1.5% of cocoa butter and surfactant blend, respectively, n=3±STD .....	68
<b>Figure 5-2:</b> PDI and zeta potential of EGCG-loaded SLNs as a function of the EGCG concentration in %w/w* related to the concentration of cocoa butter. All SLNs were prepared at 1000 bar for 5 homogenization cycles using 5% and 1.5% of cocoa butter and surfactant blend, respectively, n=3±STD .....	69
<b>Figure 5-3:</b> Entrapment efficiency and total EGCG content in EGCG-loaded SLNs as a function of the EGCG concentration in %w/w* related to the concentration of cocoa butter, n=3±STD .....	70
<b>Figure 5-4:</b> EGCG release (%) from loaded SLNs (1% w/w EGCG) under gastric conditions (pH=1.2, containing pepsin) and under intestinal conditions (pH=6.8, containing pancreatin) at 37°C, n=3±STD .....	71
<b>Figure 5-5:</b> TEM images of SLNs: Blank ( <b>A</b> ), loaded with 1% w/w EGCG ( <b>B</b> ), loaded with 1.5% w/w EGCG( <b>C</b> ) .....	75
<b>Figure 5-6:</b> EGCG stability in three different formulations (aqueous EGCG, aqueous EGCG-SSL and EGCG-SLNs containing 1% w/w EGCG) during 45 days at 2 °C in dark, n=3±STD ..	76
<b>Figure 6-1:</b> Entrapment efficiency and total cholecalciferol content of cholecalciferol-loaded SLNs as a function of the cholecalciferol concentration in %w/w* related to the concentration of cocoa butter used for the preparation of 500g of SLN formulation, n=3±STD .....	88
<b>Figure 6-2:</b> Change of the stability index of blank SLNs and SLNs loaded with 0.4% w/W of cholecalciferol as a function of the centrifugation time, n=3±STD .....	88
<b>Figure 6-3:</b> DSC heating curves of SLNs as a function of the loading concentration of cholecalciferol .....	89

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**Figure 6-4:** DSC heating curves of SLNs loaded with 0.4%w/w of cholecalciferol as a function of the storage time.....91

**Figure 6-5:** Entrapment efficiency and total content of encapsulated cholecalciferol (a) as well as particles size and PDI of SLNs loaded with 0.4%w/w of cholecalciferol as a function of the storage time.....91

**Figure 7-1:** Schematic representation of the overall approach of the present thesis.....95

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## List of tables

<b>Table 2-1:</b> Triacylglycerol content of cocoa butter.....	11
<b>Table 2-2:</b> The classification and melting temperature of cocoa butter polymorphs.....	12
<b>Table 4-1:</b> Average particle diameter of SLNs as a function of the typ of surfactant. SLNs were prepared at 600 bar for three homogenization cycles. Concentrations of cocoa butter and surfactant blend used in these experiments were constant at 5% and 1.5%, respectively. 43	
<b>Table 4-2:</b> Effect of different cooling temperatures and methods on particles properties of SLNs prepared at 600 bar for 5 homogenization cycles using 5% and 1.5% of cocoa butter and surfactant blend, respectively, n=3± STD.....	47
<b>Table 4-3:</b> The full factorial design with the resulting properties of obtained SLNs, n=3 .....	49
<b>Table 4-4:</b> ANOVA for designed experiments showing statistical results for particle size.....	49
<b>Table 5-1:</b> Melting and recrystallization temperatures of EGCG-SLNs with two different concentrations of EGCG.....	73
<b>Table 6-1:</b> Average size, PDI and zeta potential of SLNs, unloaded and loaded with different concentrations of cholecalciferol, as a function of the cholecalciferol concentration in %w/w* related to the concentration of cocoa butter. All SLNs were prepared at 1000 bar for 5 homogenization cycles using 5% and 1.5% of cocoa butter and surfactant blend, respectively, n=3±STD.....	87
<b>Table 6-2:</b> Melting enthalpy of 0.4%w/w cholecalciferol loaded SLNs as a function of the loading concentration of cholecalciferol, n=3±STD.....	90

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## List of abbreviations

DLS:	Dynamic light scattering
DMG:	Mono- and diglycerides of fatty acids
DSC:	Differential scanning calorimetry
EE:	Entrapment efficiency
EGCG:	-(-)epigallocatechin-3-gallate
FF-TEM:	Freeze fracture-Transmission electron microscopy
HLB:	Hydrophile-lipophile-balance
HPH:	High pressure homogenization
HPLC:	High performance liquid chromatography
PDI:	Polydispersity index
SEC:	Size exclusion chromatography
SGF:	Simulated gastric fluid
SIF:	Simulated intestinal fluid
SLNs:	Solid lipid nanoparticles
SSL:	Sodium stearyl-2-lactylate
TC:	Total drug content

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## 1. Motivation and hypotheses

Colloidal delivery systems with sizes in the nanometer range have been well investigated and attracted a great deal of attention by the pharmaceutical researchers and in the associated industry. These innovative systems offer promising properties and advanced functionalities over the traditional approaches (Weiss et al. 2008). This includes the improved stability and bioavailability as well as the controlled solubility and release of encapsulated bioactives (Aditya and Ko 2015; Neves et al. 2015). In recent years, nanotechnology has found fascinating applications in the food sector as a result of the hard efforts by food scientists (Aditya and Ko 2015) to meet the stiff challenge that, today, consumers often consider food materials more than a source of nutrients, but rather expect them to contribute to the health and wellness (Sozer and Kokini 2009; Weiss et al. 2008). In this area, nanotechnology is seen as one of the most promising technologies for the functional food industry, whereby two main groups of nanodelivery systems can be distinguished: *liquid systems* including nanoemulsions, nanoliposomes and nanopolymer, and *solid systems* including solid lipid nanoparticles, polymeric nanoparticles, nanocrystals and complexes (Sabliov et al. 2015).

Solid lipid nanoparticles combine the advantages and avoid the drawbacks of emulsions, polymeric nanoparticles and liposomes (Mukherjee et al. 2009; Naseri et al. 2015) and offer excellent biocompatibility and biodegradability. Moreover, due to their enormously enhanced surface area, they exhibit a faster dissolution rate and an improved bioavailability in the human body than microsized systems (Mitra et al. 2013).

As lipid based delivery systems for food applications, solid lipid nanoparticles could be fabricated using various high melting lipids with GRAS status (Aditya and Ko 2015) including, among others, triglycerides, fatty acids, waxes, steroids, partial glycerides and hard fats. What they all have in common that they are solid at room temperature as well as at body temperature (Das and Chaudhury 2011). Although no commercialized products containing bioactive-loaded solid lipid nanoparticles are available yet, some works have been already done in this direction (Eltayeb et al. 2013; Strasdat and Bunjes 2013). Examples of lipid components used already in the literature to prepare solid lipid nanoparticles for different uses are stearic acid, tristearin, tripalmitin, and cocoa butter (Pardeshi et al. 2012). Cocoa butter with its melting point close to body temperature is a vegetable fat consisting of a complex mixture of triacylglycerols (saturated, monounsaturated and polyunsaturated), which, themselves, show several different polymorphic varieties. This gives the cocoa butter a complex structural behavior (Loisel et al. 1998). Traditionally, cocoa butter is the key component in the manufacturing of chocolate. However, cocoa butter was recently used for innovative applications; for instance, as a shell material in the production of microcapsules by

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using electrospray technique in order to encapsulate aqueous based food ingredients (e.g. flavors and aromas) (Bocanegra et al. 2005; Grumezescu 2016). Furthermore, cocoa butter has also been used in the literature, alone and in combination with other lipids, to prepare solid lipid nanoparticles for pharmaceutical uses (Kim et al. 2005) and also for food applications (Qian et al. 2013). However, no study, to date, has been conducted to investigate the effects of the different preparation variables on the properties and stability of solid lipid nanoparticles made of cocoa butter for applications in the food industry.

The aim of the present work is the development of solid lipid nanoparticles using cocoa butter as particle core and to investigate the system suitability for encapsulating bioactive compounds in order to be introduced into food products with the physio-chemical characterization of obtained nanodispersion to be conducted after preparation and during the storage time.

The implementation of this approach involves the verification of following hypotheses:

- a. Cocoa butter has a melting point, which is very close to the body temperature. Nevertheless, it exhibits suitable structural characteristics with regard to its interesting polymorphism as a result of the complex triglycerides composition. The crystal modification of cocoa butter could be desirably affected by different parameters, such as shear amount, thermal and crystallization conditions. This suggests a potential use for the preparation of solid lipid nanoparticles.
- b. The optimal preparation variables could be defined using the design of experiments (DOE). This enables the investigation of the effect of each variable, as well as the interaction effects between the different variables on the particle size properties of the obtained solid lipid nanoparticles.
- c. The combined use of sodium steryl-2-lactylate (SSL) and mono- and diglycerides of fatty acids (MDG) as a surfactant blend enables the stabilizing of the nanodispersion against coalescence. The ability of SSL to stabilize colloidal systems is caused, in particular, by the pickering mechanism and MDG affect the polymorphism of the cocoa butter core. Thus, the combination of the two mechanisms may enhance the stability of SLNs.
- d. Cooling rate has a strong influence on the lipid crystallization in general. It is hypothesized that the cooling of the developed SLNs directly after the preparation process is a crucial step, which will exert several effects. Applying lower cooling

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temperature will positively influence the particle characteristics of the SLNs (particle size, size distribution, zeta potential and crystal modification).

- e. The core of the solid lipid nanoparticles is capable to encapsulate both hydrophilic and lipophilic bioactives. A successful encapsulation depends on the crystal structure of the particle lipid core obtained. The encapsulation of the polyphenol "*epigallocatechin-3-gallate*" in the fabricated solid lipid nanoparticles by this study will enhance the stability of epigallocatechin-3-gallate against degradation, which is caused by the environmental factors or induced under gastrointestinal conditions. A higher entrapment efficiency as well as a longer storage stability will be achieved for the lipid soluble vitamin "*cholecalciferol*".

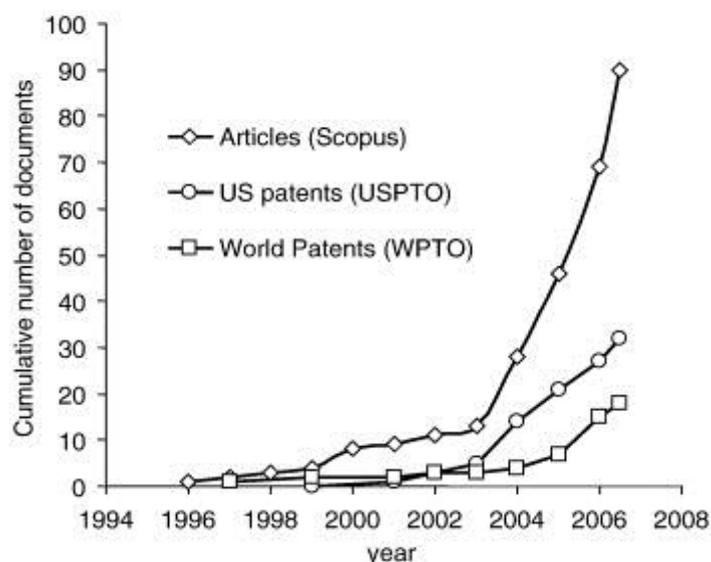
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## 2 General introduction

### 2.1 Bioactive nanocarrier

Nanocarrier refer to systems being developed to deliver specific substances such as vitamins, antioxidants, omega-3 fatty acids, nucleic acids, proteins, minerals and flavors in a nanoparticulated form to enable an improved absorbance in the human body. This offers several benefits to the human health since nanoencapsulated substances show quite different properties compared to that of conventional bulk equivalents, including bioabsorbance and metabolism (Grumezescu 2016).

The numerous nanotechnology initiatives of the late nineties, in combination with the continuous advances in nutraceutical industry in order to create food-grade additives, have supported the growth of food nanotechnology. In order to follow the growth of nanotechnology in the field of food science figure (2-1) shows the development of the term “nanoparticles” as a cumulative number of the publications, which contain the keywords food and nanoparticle in their abstracts and/or claims (Acosta 2009).



**Figure 2-1:** Cumulative number of the documents containing the keywords food and nanoparticle in their abstracts and/or claims

As seen in figure (2-1), the term nanoparticles became relevant in the food science at first after 2000 (Acosta 2009). In September 2003, the United States Department of Agriculture (USDA) has published the roadmap of nanotechnology in agricultural and food industries (Momin et al. 2012). Since then, many researchers and several organizations are working hard on the development of appropriate nanotechniques and materials to employ this

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innovative field in the food sector taking the drug delivery system as inspiring model for this purpose (Acosta 2009). This is due to the similar principles used in the fabrication of nanostructured materials with encapsulated drug molecules for pharmaceutical applications as well as those with bioactive molecules for functional food applications (Acosta 2009; Sanguansri and Augustin 2006). Unlike the case with pharmaceutical products, only food-grade ingredients, which are generally regarded as safe GRAS, are acceptable to be used and incorporated when nanotechniques are applied to the food industry (Sanguansri and Augustin 2006).

While the term nanotechnology refers, generally, to particles of sizes between 1 and 100 nm, food nanotechnology embraces the production, processing and application of particles of sizes between 10 and 1000 nm (Grumezescu 2016; Li et al. 2010). Due to their very small size, encapsulation of bioactives in nanocarrier systems allows the improving of stability during processing and storage without remarkable impact on the food texture, taste and color (Ezhilarasi et al. 2013). In general, two different approaches could be used for nanomanufacturing, which are: “*top down*” based on mechanical or chemical techniques and “*bottom up*” based on self-assembly concept (Huang 2012; Ravichandran 2010). The recent advances in the nanoencapsulation and the design of novel food delivery systems were based on the use of natural food components (Livney 2011) including lipids, proteins and polysaccharides (Sahu 2014). Lipids gain a special attention of food researcher and processor due to their desired structural, sensory and rheological properties like texture and mouthfeel, which make them suitable for delivering bioactives in food systems (Aditya and Ko 2015). The encapsulation of bioactive fat-like molecules in lipid-based nanodelivery systems enables the integration of such bioactives in food and beverages (Chaudhry and Castle 2011). The use of nanoencapsulation would protect the sensitive bioactives from environmental factors and against unwanted changes during food processing and storage (Sanguansri und Augustin 2006). The suitability and potentiality of the nanotechnological approaches in the food processing sector are revealed and examined by researchers and food processors (Huang et al. 2010). To date, there is still a gap between the commercial development and the societal acceptance regarding the application of nanotechnological principles in the food sector (Sahu 2014). The increasing research in this area, in order to explore and evaluate the different potential benefits and risks associated with such innovative applications, decreases this gap gradually (Frewer et al. 2014). Nanotechnology is expected to shape the future of the food processing sector (Huang 2012) by making extraordinary changes and developments in this field (Sahu 2014). This includes the creation of food products with novel structures showing improved sensory properties and quality (Chaudhry et al. 2008; Huang 2012). An example, low-fat nanostructured products like mayonnaise or ice cream would be as creamy as their full-fat alternatives, which places a

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healthier choice on the food market (Chaudhry et al. 2008). In addition, nanotechnology allows the development of new devices, processes and analytical tools which adds significant value to food processing and safety (Huang 2012). However, only extensive research could bring these expectations into reality (Sahu 2014).

## **2.2 Solid lipid nanoparticles**

Liposomes were for a long time the only nanocarrier system to be considered. The first report indicating the potential advantages of liposomes as drug delivery vehicles was at the FIP 1979 congress (Egbaria und Weiner 1990). 1986 liposomes found their uses for cosmetic applications. Major problems correlated with the liposomal applications were low physical stability and low drug activity because of the lacking of specific targeting (Wissing et al. 2004). The progressive development has led throughout 1990 to the appearance of another innovative carrier system on the pharmaceutical market; called: the polymeric nanoparticles (Patidar et al. 2010; Wissing et al. 2004). These are good biocompatible, physically stable and efficiently protect the active substances. In addition, polymeric nanoparticles offer site-specific targeting and good control over release kinetics of encapsulated compound (Silva et al. 2011; Wissing et al. 2004). Though, disadvantages associated with polymeric nanoparticles are the cytotoxicity aspect because of the use of nonbiodegradable polymers sometimes and the difficulties combined with the large scale production (Patidar et al. 2010). This has restricted the use of polymeric nanoparticles in the pharmaceuticals (Müller et al. 2000; Wissing et al. 2004). Hard efforts by several research groups, in order to find a suitable alternative to the polymeric nanoparticles (Üner and Yener 2007), have resulted in the development of solid lipid nanoparticles in the middle of the 1990s (Nerella et al. 2014; Patidar et al. 2010; Wissing et al. 2004). Nanosized particles show a greater surface area per mass unit and are, therefore, expected to be more biologically active compared to their equivalents of larger size, which is of great importance for functional food applications (Sozer 2009).

An important advantage of solid lipid nanoparticles (SLNs) is that they are prepared from biocompatible and biodegradable lipids exhibiting an excellent tolerability. Moreover, the possible manufacturing without using solvents would avoid the problems associated with acute and chronic toxicity (Bleve et al. 2011; Garud et al. 2012; Nerella et al. 2014; Pardeike et al. 2009). Hence, the SLN are often defined as nanosafe carrier (Bleve et al. 2011). Furthermore, they combine the advantages of older colloidal nanocarriers including emulsions, liposomes and polymeric nanoparticles and simultaneously overcome most of the potential drawbacks (Patidar et al. 2010; Ramteke et al. 2012). The encapsulation of both

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lipophilic and hydrophilic compounds in solid lipid nanoparticles under optimized condition is possible (Attama et al. 2012; Patidar et al. 2010; Tiwari et al. 2015).

The use of SLNs as drug delivery system was first mentioned in the year 1991 (Aditya and Ko 2015; Teja et al. 2014) as alternative to polymeric nanoparticles (Silva et al. 2011). Based on the successful uses of SLNs in pharmaceutical and cosmeceutical applications, the realization of similar concepts for food applications seems to be necessary. This would enable the food processors to successfully introduce the bioactives into food products and, thus, enhance the nutritional and health value as well as the quality of foods (Weiss et al. 2008).

SLNs are described as suspensions of lipid nanocrystals in aqueous medium (Jenning et al. 2002). Three main components are generally needed for the preparation of SLNs, which are lipids, surfactants and water (Mukherjee et al. 2009; Pardeike et al. 2009). As colloidal O/W system SLNs differ from submicron emulsion of liquid oil droplets that the liquid oil is replaced by crystallized lipids, i.e. lipid matrix being solid at room as well as body temperature (Jenning et al. 2002; Lucks and Müller 1999). This offers better controlled release and improved stability of encapsulated active substances (Helgason et al. 2008; Weiss et al. 2008) as the mobility of encapsulated substances in crystallized lipid matrix is more restricted as that in liquid lipid matrix. Moreover the existing of encapsulated active substances within individual lipid particles prevents the accumulation of bioactive molecules at the surface of particles. Thus, the active substances are effectively protected and the rate of chemical degradation might be significantly retarded (Helgason et al. 2009). Furthermore, it has been shown that the digestion of lipid particles in the human body is dependent on the physical nature of the lipid as well as the stabilizers used in the preparation. When solid lipid matrix is used the digestion takes a longer time, which results in a more sustained release of encapsulated substances (Helgason et al. 2009; Pardeike et al. 2009). The resulting products of lipid particles digestion are fatty acids and glycerol, which are natural compounds present in the human body (Pardeike et al. 2009). Moreover, the utilization of SLNs in the food industry is increasingly gaining the interest of manufactures due to the following attributed specific advantages (Aditya and Ko 2015; Weiss et al. 2008):

- ✓ Easy and large-scale production which is of particular industrial importance.
- ✓ The renouncement of the use of organic solvents promotes their application in food products.
- ✓ Suitability for several sterilization techniques used for food products.
- ✓ The ability to be introduced to liquid and solid foods in form of nanodispersions and/or powder after lyophilization.

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- ✓ The availability of food-grade raw materials, i.e. lipids, surfactants and co-surfactants, in large-scale.
  - ✓ The best suitability for the oral delivery which is the optimal route of administration for nutraceuticals.

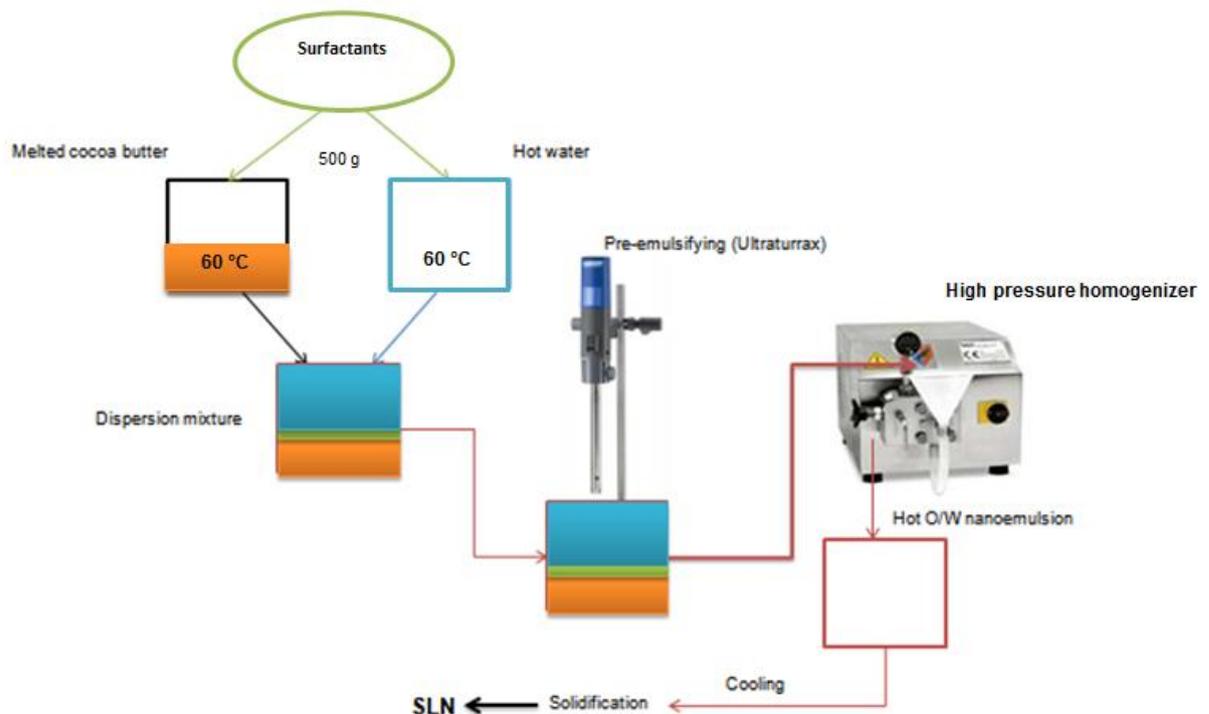
General disadvantages attributed with SLNs are, on the one hand, the relatively low incorporation rate for some drugs and active substances and, on the other hand, the bioactive expulsion related with  $\alpha$ -to- $\beta$  polymorphic transition of the core matrix leading to the change of the roughly spherical particles to disc-like shaped particles. This results in significantly increased oil-water surface area (Qian et al. 2012). A further problem is attributed to the high water content of solid lipid nanodispersions (Garud et al. 2012; Ramteke et al. 2012) as the nanoparticles tend to adsorb water and swell. These drawbacks decrease the system stability and increase the gelation problem (Patidar et al. 2010; Qian et al. 2012). Disadvantages mentioned above could be controlled by optimizing the different preparation variables and storage conditions. The lyophilization of liquid nanodispersion can solve the problem with gelation tendency (Garud et al. 2012).

### **2.2.1 Preparation technique of SLNs**

Different techniques could be applied for the production of the SLNs such as ultrasonication, high pressure homogenization (cold and hot), supercritical fluid method, precipitation techniques etc. (Müller et al. 2000; Naseri et al. 2015; Ramteke et al. 2012; Sagis 2015). The production of SLNs in the present work was carried out by applying high pressure homogenization method which is currently one of the most preferred techniques (Mehnert and Mäder 2001; Sagis 2015).

In comparison to the other methods, high pressure homogenization technique is distinguished that it is easy to scale up and requires a short time to be carried out (Pardeike et al. 2009; Sagis 2015) and, thus, is considered as the most feasible industrial technique (Sagis 2015). In contrast to high shear mixing or ultrasonication method the high pressure homogenization method functions more effectively in the production of submicron sized dispersions of solid lipids showing sizes below 500 nm and low content of microparticles (Mehnert and Mäder 2001). Two different approaches are to be considered when talking about the high pressure homogenization technique; hot and cold (Al-Haj and Rasedee 2009; Mehnert and Mäder 2001). In this work, the hot approach illustrated in figure (2-2) was applied to prepare the SLNs from cocoa butter.

The hot approach starts with the melting of lipid at approximately 5-10 °C above the melting point. The bioactives to be encapsulated are dissolved or solubilized in the melted lipid. The aqueous phase containing the surfactant or surfactant blend having the same temperature of the lipid melt is added and the resulting mixture is processed to a pre-emulsion using a high-shear mixing device, which is then subjected to the high pressure homogenizer, where pressures between 100 and 2000 bar could be applied. During the homogenization the fluid pre-emulsion is pushed up through a narrow gap under high pressures and accelerates on a very short distance to very high velocity. The very high shear stress and cavitation forces formed during the process achieve the disruption of the emulsion droplets down to the submicron range (Mehnert and Mäder 2001; Mukherjee et al. 2009; Sagis 2015). Each passage through the high pressure homogenizer is called cycle and the required number of homogenization cycles is dependent on the raw materials employed and the different preparation parameters. It has been shown that emulsion temperature increases with increasing the number of homogenization cycles under high pressures (Kumar and Karimulla 2012; Pardeike et al. 2009). The hot fluid product resulted from the homogenizer is more likely a nanoemulsion as the lipid particles therein are still in liquid state. The solid lipid nanodispersion is only obtained when the hot nanoemulsion is cooled down to temperatures much below the crystallization temperature of the lipid as bulk material in order to allow the formed particles to crystallize (Bunjes et al. 2003; Mehnert and Mäder 2001).



**Figure 2-2:** Schematic representation of the preparation of SLNs using the hot–high pressure homogenization

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## 2.2.2 SLNs composition

### 2.2.2.1 Cocoa butter

There is a wide choice of lipids and lipid components with GRAS status, which could be employed for the fabrication of SLNs (Aditya and Ko 2015). The physical and chemical characteristics like melting point, crystallization velocity, viscosity, polarity composition, chain length etc. are essential features when selecting the lipid for this purpose. Most important is the polymorphic state of the lipid. This will identify the type of the crystals obtained upon cooling and, thus, the functionality, as well as the stability of SLNs (Aditya and Ko 2015; Mehnert and Mäder 2001; Qian et al. 2012). Lipid polymorphism is defined as the ability of the lipid to form different types of crystalline structures under different conditions. The crystal (polymorph) is built up from molecules arranged in a fixed pattern, which is called a lattice. Different molecular packings result in different crystal shapes. Crystals are also considered as solids of the same composition, which arranges in a regular three dimensional pattern (Lasic and Martin 1995). The smallest structure of the crystal cell is called sub-cell, the structure of which identifies the polymorphic form existing. In general, there are three specific sub-cell types of lipids correlating to the crystal forms:  $\alpha$ ,  $\beta'$  and  $\beta$ . The metastable  $\alpha$ -form exhibits a hexagonal chain packing, while the  $\beta'$ -form possesses an orthorhombic packing and the  $\beta$ -form has a triclinic parallel one (Mastai 2015, Weiss 2008). Lipid polymorphism is commonly examined using x-ray technique, differential scanning calorimetry and nuclear magnetic resonance (Lasic and Martin 1995).

An appropriate degree of microstructural disorganization of the lipid matrix is necessary (Mukherjee et al. 2009; Gomes et al. 2013). This is needed in order to enable the generation of particles with more disordered crystalline structure, which involves enough voids to ensure a higher incorporation rate and a less expulsion of encapsulated bioactive compounds during storage (Gomes et al. 2013; Weiss et al. 2008). The least ordered  $\alpha$ -subcell of spherical crystals meets all these requirements and is, hence, the favorable form for the SLNs. In contrast, the utilization of pure homogeneous lipids with a sharp melting point induces the transition of  $\alpha$ - to the most ordered  $\beta$ -subcell with plated-like crystals, which tends to expel the encapsulated compounds. Furthermore, this transition causes the instability of the nanodispersion because the  $\beta$ -subcell is corresponding to higher surface-to-volume ratios. The new surfaces resulted by this polymorphic transition would require a higher amount of surfactants to be fully covered. If this is not available in the system an aggregation occurs (Gomes et al. 2013; Qian et al. 2013; Weiss et al. 2008).

For the preparation of SLNs, lipids are typically used in a concentration of 5 to 10% of the mixture weight (Kumar and Karimulla 2012; Pardeike et al. 2009), while higher lipid

concentrations resulted in larger particle size with broader particle size distribution (Mehnert and Mäder 2001; Vitorino et al. 2011).

Cocoa butter is an edible vegetable light yellow fat (Minifie 1989), known especially for its functional and technological importance in the chocolate manufacturing being the main lipid phase. It plays an essential role by providing the unique texture, mouthfeel and flavor release to chocolate as well as other confectionery products, where it is used in combination with other vegetable oils such as hydrogenated soybean oil and palm oil fractions (Garti and Widlak 2012; Toro-Vazquez et al. 2005). The functional properties of cocoa butter are related to its triacylglycerides composition and crystallization behavior (Sonwai and Mackley 2006). The triacylglycerols content of cocoa butter consists primarily (>80%) of monounsaturated triacylglycerols, which are composed of three fatty acids: palmitic, stearic and oleic acids (Fessas et al. 2005; Quast et al. 2011). In these monounsaturated triglycerides the oleic acid is esterified at the second position of the glycerol molecules leading to the building of three main monounsaturated triacylglycerols: 1,3-dipalmitoyl-2-oleoylglycerol, 1-palmitoyl-2-oleoyl-3-stearoylglycerol and 1,3-distearoyl-2-oleoylglycerol. The complex polymorphism of these triacylglycerols results in a very complex thermal and structural behavior of cocoa butter (Loisel et al. 1998; Minifie 1989). Although this composition is dependent on the origin of cocoa butter, the mass fractions of the main triacylglycerols do not differ significantly (Fessas et al. 2005). Table (2-1) shows the percentages of the triacylglycerols content of cocoa butter (Minifie 1989):

**Table 2-1:** Triacylglycerol content of cocoa butter

<b>Triacylglycerols</b>	<b>Percentage, %</b>
<b>Monounsaturated:</b>	
✓ 1,3-distearoyl-2-oleoylglycerol (SOS)	18-22
✓ 1-palmitoyl-2-oleoyl-3-stearoylglycerol (POS)	52-57
✓ 1,3-dipalmitoyl-2-oleoylglycerol POP	4-6
<b>Diunsaturated:</b>	
✓ 1,3-dioleoyl-2-stearoyl (OSO)	6-12
✓ 1,3-dioleoyl-2-palmitoyl glycerol (OPO)	7-8
<b>Trisaturated:</b>	
✓ Tristearin (SSS) and tripalmitin (PPP)	2.5-3
<b>Triunsaturated:</b>	
✓ Triolein OOO	1

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For cocoa butter six polymorphs (crystalline forms) were observed (Wille and Lutton 1966). According to the literature, several classifications of the cocoa polymorphs could be found with various associated melting point. However, these polymorphs could be generally defined as illustrated in the table (2-2) and in the order of increasing melting point and stability of the sub-cell structure (Loisel et al. 1998; Manning and Dimick 1985; Révérend et al. 2010).

**Table 2-2:** The classification and melting temperature of cocoa butter polymorphs

<b>Crystal polymorphs</b>	<b>Melting point, °C</b>
$\gamma$ (I)	0-18
$\alpha$ (III)	18-23.3
$\beta'$ (III)	23.3-25.5
$\beta'$ (IV)	25,5-27,5
$\beta$ (V)	27,5-33,8
$\beta$ (VI)	33.8-36.3

The cooling rate during the crystallization is one of the most important factors. It plays a central role in determining and controlling the crystal form that would occur. It has been observed, that the rapid cooling of the melted cocoa butter at low temperatures induces the crystallization in  $\gamma$ - and  $\alpha$ -forms. In contrast, the cooling at moderate temperatures promotes the occurrence of  $\beta'$ (III) and/or  $\beta'$ (IV) (Ribeiro et al. 2012; Wille and Lutton 1966).

The differential scanning calorimetry (DSC) is the commonly used technique for analysing the polymorphic crystals of cocoa butter (Manning and Dimick 1985). The defining of the crystalline forms using the DSC is based on the melting temperatures obtained from the recorded curves. The crystal polymorph plays a crucial role in regard to the lipid functionality in the various food products. In this spirit, the desired crystal modification (polymorph) of cocoa butter depends on the function of the final product containing it. The optimization of the different conditions and variables during the preparation procedures enables the generating of the desired polymorph. For instance, in the chocolate manufacturing the desired crystal modification of the cocoa butter is the most stable one:  $\beta$ (V), which ensures the obtaining of a good structure and popular sensory properties. In contrast, the required crystal modification of cocoa butter as particle core of the SLNs is the metastable one:  $\alpha$ (II).

(Kim et al. 2005) has prepared SLNs using the cocoa butter as core material. Curdlan was used as a surfactant and Tween 80 was used as a co-surfactant in order to prevent the system aggregation and also the gelling of the curdlan. The SLNs were produced for drug

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delivery purposes. Up to 1% of cocoa butter, the nanoparticles showed a size in the range of 140 to 200 nm with a unimodal distribution and the polydispersity index remained  $<0.4$ . When the lipid concentration exceeded 1%, the particle size significantly increased to  $>200$  nm. The production was carried out by stirring the hot material mixture (40 °C) using a magnetic bar and the obtained dispersion was allowed to cool at room temperature. Based on these results, it would be possible to prepare SLNs using the cocoa butter by using a more effective technique in order to obtain a system with improved properties, which is suitable for applications in the food sector.

### **2.2.2.2 Surfactant blend**

Surfactants are essential components for the preparation and stabilization of lipid dispersions (Mehnert 2001). The type, as well as the concentration of the surfactants employed, has a main impact on the overall quality of the colloidal systems (Bleve et al. 2011; Vitorino et al. 2011). This impact works in two ways. Firstly, surfactants reduce the interfacial tension between the immiscible phases, i.e. oil (lipid) and water, which facilitates the droplet break up. Secondly, they build a mechanically strong and elastic interfacial film at the oil droplet surface that acts to prevent the coalescence with other oil droplets (Dickinson 1992; Subramaniam 2016). Commonly used surfactants in the preparation of lipid dispersions are low-molecular weight surfactants, such as polysorbates, poloxamers and phospholipids.

Depending on their electrochemical charge in aqueous systems, surfactants are generally divided into two groups: ionic and nonionic. Ionic surfactants are either anionic or cationic. Some types of surfactants, such as lecithin, contain both anionic and cationic groups, where the surface-active properties of these surfactants are pH-dependent (Bastida-Rodríguez 2013). It has been found that ionic surfactants enable the production of SLNs with considerably smaller size compared to the size obtained when nonionic surfactants are used (Bunjes et al. 2003; Mehnert and Mäder 2001). The use of a suitable combination of surfactants instead of one surfactant had shown much better results. This includes a lower particle size and a higher storage stability of the SLNs system (Bleve et al. 2011; Mehnert and Mäder 2001; Sahin and Sumnu 2006a; Nik et al. 2012). A possible explanation is the formation of intermolecular complexes at the oil/water interface and the creation of stronger interfacial films around the oil droplets. This ensures an efficient prevention of the coalescence (Nik et al. 2012).

In addition to the control of the particle size and the stability of the nanodispersion, surfactants may play another important role in the SLNs systems. They can affect the crystallization process of the lipid particles and, therefore, the polymorph generated, as well

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as the polymorphic transition during the preparation and the storage time (Vitorino et al. 2011; Weiss et al. 2008). Furthermore, they may interact with the fat crystals and, thereby affect some of their properties. For instance, it has been demonstrated that lecithin molecules adsorbed to the fat crystals and increased, thereby, their polarity (Rousseau 2000). Further research on this area is needed in order to obtain more specific informations regarding the type of the interactions between the dispersed lipid and the surfactant molecules (Bunjes et al. 2002). In addition, it has been evident that the lipid dispersions stabilized with ionic surfactants (e.g. sodium glycocholate) were almost completely in the  $\alpha$ -modification and exhibited an extraordinarily high stability during the crystallization process. The dispersion stabilized with nonionic surfactants tended, in contrast, to transform more rapidly into the stable  $\beta$ -modification that no existence of the  $\alpha$ -modification could be observed during the crystallization process (Bunjes et al. 2002).

The surfactant concentration employed determines the efficiency of the surfactant to emulsify and stabilize the SLNs dispersion. The existence of a high amount of the surfactant in the system ensures a sufficient reduction of the interfacial tension in order to facilitate the particle partition during the homogenization procedure. This results in smaller particles. The preparation method used also plays an important role because the different preparation methods are associated with different dispersion degrees (Mehnert and Mäder 2001). The smaller the particles obtained, the higher the connected increase in the surface area. In the case of the high pressure homogenization, the rapidly occurred increase of the surface area requires the utilizing of an excessive amount of the surfactant molecules. This is needed in order to completely cover the new surfaces and, thereby, avoid the agglomeration (Helgason et al. 2009). However, the use of high concentrations of surfactants in turn increases the risk of possible associated toxic side effects (Mehnert and Mäder 2001).

All types of surfactants consist of molecules containing the two opposing groups: hydrophilic and lipophilic. The balance of the size and strength of the hydrophilic and lipophilic groups is called Hydrophile-lipophile-balance (HLB) (Griffin 1949). This value is given on a scale of 1 to 20, where 1 indicates a very high affinity of the surfactant for the oil phase and 20 indicates a very high affinity for the aqueous phase (Hasenhuettl and Hartel 2008). Usually, the preparation of O/W emulsions requires the use of high HLB surfactants, while low HLB surfactants are useful for the preparation and stabilization of W/O emulsions (Griffin 1949; Hasenhuettl and Hartel 2008). Oils, fats, waxes etc. also exhibit an individual HLB value known as "Required HLB". This means, to prepare a good and stable emulsion it is necessary to use a surfactant, which has a HLB value equal to the "Required HLB" of the oil/fat being employed for the preparation. At this point, the blending of surfactants of low and

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high HLB to match the needed HLB value is possible and even more favorable than the use of a single surfactant with the appropriate HLB value in order to obtain better results, which has been proven over and over again (Hasenhuettl and Hartel 2008). Although, the HLB values of a number of surfactants are given, the calculation of the HLB value of a surfactant is possible using the following equation (ICI Americas Inc. Wilmington 1984):

$$\text{HLB} = 7 + \sum \text{hydrophilic group numbers} - \sum \text{lipophilic group numbers} \quad (\text{I})$$

This equation explains that HLB value is the rate of the weight percentage of hydrophilic groups to the weight percentage of lipophilic groups in the emulsifier molecule (Sahin and Sumnu 2006b).

When a blend of surfactants (A) and (B) is used, the required amount of each one to reach the "Required HLB" of the X (oil, fat etc.) is calculated from the next equations (II and III) (ICI Americas Inc. Wilmington 1984)

$$\% (A) = \frac{100(X - \text{HLB}(B))}{\text{HLB}(A) - \text{HLB}(B)} \quad (\text{II})$$

$$\% (B) = 100 - \% (A) \quad (\text{III})$$

If more than one oil/fat is used for the preparation, it is possible to calculate the "Required HLB" for the oil/fat blend (Y) consisting , for example, of 30% oil (a), 50% oil (b) and 20% oil (c) to be emulsified in the water phase as follows (ICI Americas Inc. Wilmington 1984):

$$\text{Req. HLB}(Y) = 30\% * \text{Req. HLB}(a) + 50\% * \text{Req. HLB}(b) + 20\% * \text{Req. HLB}(c) \quad (\text{IV})$$

Unlike other works in the field of SLNs, where the used surfactants are usually types of polysorbates (tweens), poloxamers, phospholipids etc., the surfactant used in the present work was a blend of Sodium stearyl-2-lactylate (SSL) and Mono- and diglycerides of fatty acids (MDG), in order to develop a new surfactant blend and examine its suitability for the preparation of an SLNs system. The required amounts of SSL (HLB=8.3) and MDG (HLB=3-4) for the preparation of the SLN dispersions in the present work, considering the "Required HLB" of cocoa butter (HLB=6), were calculated using the equations (II) and (III). The obtained results were:

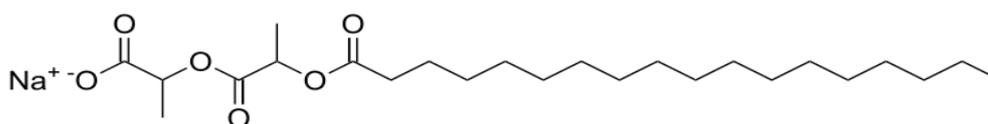
%SSL= 54%; %MDG= (100-54) =46%.

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### **Sodium stearoyl-2-lactylate (SSL; E 481)**

SSL (Figure 2-3) is an anionic low molecular surfactant, which is chemically synthesized by the esterification of stearic acid with lactic acid in the presence of sodium carbonate or sodium hydroxide (Kurukji et al. 2013; European Food Safety Authority (EFSA) 2013; Zinoviadou et al. 2011). It is a food grade surfactant and authorised as food additive in the EU (Commission Regulation (EU) No 231/2012). According to the Confederation of the Food and Drink Industries of the EU, the maximum level of SSL could be used must not exceed 5g/kg (European Food Safety Authority (EFSA) 2013). SSL is typically used as an emulsifier and/or rheology modifier in food products (Kurukji et al. 2013). Its molecular formula and weight are C<sub>24</sub>H<sub>43</sub>O<sub>6</sub>Na and 450.58 g/mol, respectively (European Food Safety Authority (EFSA) 2013). It is known for its capacity to form ordered crystalline aggregates upon dispersity in water (Kurukji et al. 2013).

The chemical structure of SSL is:



**Figure 2-3:** The chemical structure of SSL

SSL is most commonly used in the bakery industry; particularly in the production of the pan bread (Kurukji et al. 2013). Among the bilayer-forming surfactants, it has been demonstrated that SSL showed much better results of an increased foam stability compared to whey protein isolate and Tween 80 (Kurukji et al. 2013). Most recent, SSL was investigated for its potential to stabilise O/W emulsions through the pickering mechanism (Kurukji et al. 2013; Flores et al. 2007; Zinoviadou et al. 2011). These works confirmed, that SSL either alone or in combination with other surfactants could effectively stabilise O/W emulsions to coalescence. The ordered stable bilayer aggregates of SSL were adsorbed at the O/W interface. Kurukji et al. (2013) showed that the adsorption of SSL aggregates at the O/W interface was faster when the aqueous dispersion of SSL was processed with the pressure homogenizer. This is due to the significant size reduction after the homogenization.

### **Mono- and diglycerides of fatty acids (MDG; E471)**

MDG (Figure 2-4) consist of a mixture of mono- and diglyceryl esters of saturated and unsaturated long chain fatty acids. Generally Recognized by the FDA as safe (GRAS) (Food and Drug Administration (FDA), 2016). MDG have many applications as food processing

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aids. They are principally used as emulsifiers. This function also translates into stabilization and preventing of food separation and extending the shelf life of the system. The use level of MDG in foods under the FDA listing must not exceed the current good manufacturing practices. MDG are not limited by an Acceptable Daily Intake (ADI) level.

Monoglycerides exhibit a stronger surface activity than diglycerides due to their two free hydroxyl groups (Hasenhuettl and Hartel 2008). Mono- and diglycerides also increase the interfacial area of the oil or water droplets dispersed in an emulsion, which therefore require more energy to coalesce into larger droplets. Consequently, the break up of the emulsion is reduced (Clark 2013). The MDG facilitate the dispersion of oil into water or water into oil. In many applications, this creates a shelf-stable product by preventing separation of oils from other liquids in products such as salad dressings, peanut butter, and ice cream (Hasenhuettl and Hartel 2008). Furthermore, the deterioration of the physical properties of the end product is retarded.

### **2.2.3 SLNs for the delivery of bioactive compounds**

#### **2.2.3.1 Encapsulation of bioactive compounds**

The term “*bioactive compound*” refers to a compound having a therapeutic, nutritional, and/or disease-preventive effect (Livney 2011). Several bioactive compounds such as curcumin (Noack et al. 2012), ergocalciferol (Patel and San Martin-Gonzalez 2012) and  $\beta$ -Carotene (Nik et al. 2012) were encapsulated in SLNs.

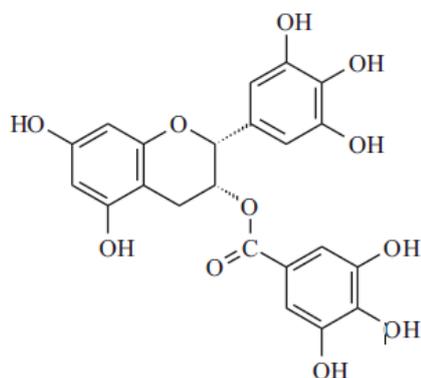
In this work, the term “*drug*” indicates in general the “*bioactive compound*”. This is because the SLNs like other nanocarrier were first developed in the field of drug delivery and, thus, the most theoretical and practical informations were collected in this area before the nanotechnology has found its applications in other fields, such as cosmetic and food industries. In the present work, (-)-epigallocatechin-3-gallate and cholecalciferol were selected as hydrophilic and lipophilic compounds, respectively, for the encapsulation in the investigated SLNs.

#### ***(-)-Epigallocatechin-3-gallate (EGCG) from green tea extract:***

EGCG (Figure 2-4) belongs to the antioxidant compounds showing several promising effects on the human health. It is one of the major tea catechins, which represents the most abundant catechin in green tea (Mähler et al. 2013). Catechins are responsible for much of the biological activity mediated by green tea. The clinical studies have confirmed the effectiveness of the green tea extract (GTE) against UV-injuries and photoaging of skin,

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oxidative DNA damage and a variety of human cancers. EGCG exhibits a great antioxidative activity. It can influence numerous processes in the brain and, thereby, shows a therapeutic efficacy in neurological disorders and aging. The daily oral administration of 180 mg/kg EGCG in the diet for 5 weeks most effectively reduced muscular dystrophy (Mähler et al. 2013). Furthermore, EGCG has been shown to have anti-inflammatory, anti-cancer (Brusselmans et al. 2003), anti-infective (Steinmann et al. 2013) and immunomodulating effects (Pae and Wu 2013). The improving of the cardiovascular health and the enhancing of weight loss were also confirmed for EGCG.



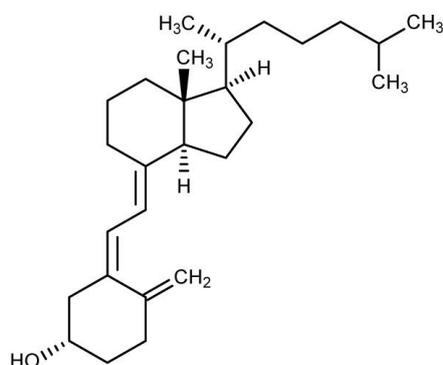
**Figure 2-4:** The chemical structure of (-)-epigallocatechin-3-gallate (EGCG)

A number of environmental factors can influence the stability of EGCG. This depends on the pH value of the surrounding medium. EGCG is rather stable at pH=2.0-5.5 and undergoes an auto-oxidation at neutral pH, yielding a yellow brown color. Many studies have confirmed, that the instability of EGCG and other catechins increases significantly in solutions having near-neutral and alkaline pH (Sang et al. 2005; Dube et al. 2010; Mochizuki et al. 2002). This indicates that EGCG exhibits a low stability in the gastrointestinal tract, which results in a very low bioavailability of EGCG after the oral administration (Zou et al. 2014). As a result of this drawback, the beneficial effects of EGCG on the human health are limited. Nanoparticulate delivery systems including the SLNs have been shown to significantly enhance the bioavailability of encapsulated bioactive compounds, like EGCG (Luo et al. 2015; Wang et al. 2013). This is performed by the protecting of EGCG against the degradation under neutral and alkaline conditions. Furthermore, SLNs have shown a sustained release of the encapsulated bioactive compounds under simulated gastrointestinal conditions (Noak et al. 2012).

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### ***Cholecalciferol***

Cholecalciferol (Figure 2-5) is one of the two major physiologically relevant forms of Vitamin D, also called vitamin D3. It exhibits a molecular formula and weight of  $C_{27}H_{44}O$  and 384.64 g/mol, respectively. The other form is called ergocalciferol (or vitamin D2). Cholecalciferol is a steroid hormone, which is synthesized from a precursor in the skin under sunlight exposure. It can also be ingested from the dietary sources (Almouazen et al. 2013; Picciano 2010). The metabolism of cholecalciferol into its active form, calcitriol, begins in the liver. There it undergoes a hydroxylation to 25-hydroxyvitamin D3 (calcidiol). This is followed by a second hydroxylation in the kidney into 1,25-dihydroxyvitamin D3 (calcitriol) (Almouazen et al. 2013; DeLuca and Zierold 1998). The classical role of calcitriol is the maintenance of the blood levels of calcium and phosphate in coordination with parathyroid hormone (Almouazen et al. 2013). This plays a dominant role in the bone metabolism. Besides the importance of calcitriol for the absorbance of calcium and the homeostasis regulation, calcitriol plays an essential role in the prevention of many chronic diseases, such as diabetes typ 2, hypertension and cardiovascular disease (Picciano 2010). 30-60% of the population in the United States and Europe suffer from vitamin D deficiency (Ginter 2009). This necessitates the development of efficient methods to incorporate vitamin D in several food products, in order to avoid the occurrence of problems like rickets in children and osteoporosis in adults.



**Figure 2-5:** The chemical structure of cholecalciferol (vitamin D3)

Cholecalciferol belongs to the lipophilic vitamins. It is very sensitive to various environmental factors such as ultraviolet radiation (light), oxygen and other environmental influences (Petritz et al. 2006). This changes the chemical structure of the cholecalciferol and lowers its health-promoting benefits. In order to improve the stability and avoid the unwanted effects during processing and storage the encapsulation of such lipophilic bioactive compounds, including both cholecalciferol and ergocalciferol, in suitable systems was and is

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still a focus of the developments in the food and nutraceutical industries (Huang et al. 2010; Luo et al. 2012).

### **2.2.3.2 Release of encapsulated compounds from SLNs**

The release profile from SLNs is a function of several factors such as the lipid matrix, the production temperature (Zur Mühlen and Mehnert 1998) and the type and amount of the surfactant used in the formulation (Üner and Yener 2007). The crystalline structure of the lipid carrier determines the mobility of the encapsulated compound and, therefore, the related release profile. Furthermore, the higher the production temperature, the higher the water solubility of the bioactive compounds during production and thus, the more pronounced is the burst release. In addition, the high amount of surfactant enables the production of smaller particles, which is combined with a larger surface area. However, it has been demonstrated that the burst release increases when the surface area of the particles increases. Moreover, surfactant molecules may interact with the outer shell of the particle core and make structural changes in its function during release. In general, for the incorporation of bioactive compounds into SLNs systems three different models are distinguished depending on the different preparation conditions (Zur Mühlen and Mehnert 1998; Zur Mühlen et al. 1998; Müller et al. 2002). These are:

#### ***The homogeneous matrix model (solid solution model):***

In this model, the encapsulated compound presents in amorphous clusters or is dispersed in the lipid matrix. Thus, the encapsulated compound interacts strongly with the lipid matrix. This model is obtained by the preparation of SLNs using the cold high pressure homogenization technique and without surfactants or solubilizing agents. Furthermore, this model is also obtained when highly lipophilic compounds were encapsulated in SLNs using the hot high pressure homogenization technique.

#### ***The drug-enriched shell model:***

The high solubility of the encapsulated compound in the aqueous phase during SLNs preparation is the main factor for obtaining this model. During the production, the bioactive compound partitions to the water phase. During the cooling of the hot SLNs formulation, the lipid precipitates when its crystallization point is reached and, hence, a bioactive compound-free lipid core is formed. During the further cooling, the bioactive compound repartitions from the water phase into the remaining liquid lipid. This leads to the forming of an outer shell,

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which contains a high concentration of the bioactive compound. This shell crystallizes surrounding the initial lipid core. Other factors such as high production temperatures and high surfactant concentrations induce the forming of the drug-enriched shell model. This model is mostly combined with a considered burst (initial) release because of the short diffusion distance.

***The drug-enriched core model:***

This is obtained with the incorporation of a bioactive compound, which is highly soluble in the lipid matrix and presents in concentrations close to its saturation solubility. The cooling of the hot SLNs formulations leads to the supersaturation of the bioactive compound in the melted lipid matrix. The bioactive compound precipitates prior to the lipid recrystallization. During the further cooling, the lipid crystallizes forming a membrane surrounding the compound-enriched core. This model is combined with a sustained release profile because of the increased diffusion distance as well as the hindering effect of the surrounding solid lipid shell.

### **3. Methods**

When a new SLNs system is developed, it is particularly important to examine the different properties. This would determine if the fabricated nanoparticles exhibit the suitable properties for the intended use. Moreover, an adequate characterization provides a forecast of the system quality during the storage time (Pardeike 2009; Alhaj and Rasade 2009). The basic properties, which are commonly determined to investigate the overall applicability of the SLNs, are described in details as follows:

#### **3.1 Particle size and size distribution**

The particle size of the SLNs is an important parameter, which affects their other properties, as well as their physical stability (Garud et al. 2012). Various factors influence the particle size of the SLNs. These are the lipid matrix used, the active compound to lipid ratio, the surfactant or surfactant blend being employed, the viscosity of both lipid and aqueous phases, and the production parameters (Bleve et al. 2011). For example, the use of a high lipid concentration is combined with a high viscosity of the dispersed phase. Thus, the efficiency of the homogenization decreases and the particle size would be larger as a result (Vitorino et al. 2011). The photon correlation spectroscopy (PCS; also known as dynamic light scattering, DLS) is one of the most powerful and commonly used techniques for the determination of the particle size (Garud et al. 2012; Mukherjee et al. 2009). This technique is based on the interaction of the particle with an incident light beam (Fanun 2010). The

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Brownian motion of the individual particles in the fluids results in a dynamic scattering of the laser light (Abhang et al. 2015; Jahagirdar et al. 2015). The intensity of the scattered light detected at a fixed angle provides the mean size and size distribution of the particles in the sample (Fanun 2010). Furthermore, this method is favorable because of the speed of analysis, the lack of required calibration and its sensitivity to submicrometer particles (Abhang et al. 2015). It is suitable for the measurement of particles in the range of a few nanometers to about 3 microns (Mukherjee et al. 2009; Jahagirdar et al. 2015) and, hence, an adequate tool to characterize the size of SLNs.

In addition, PCS determines another important parameter of nano- and submicron liquid dispersions, i.e. the particle size distribution (Akbari et al. 2011). This can be defined as the average number of particles within a given size class of width for a unit volume of suspension (Reynolds et al. 2010). In the field of nanotechnology, particle size distribution is a parameter that is as important as the particle size (Fanun 2010). It is generally evaluated based on the polydispersity index (PDI), which takes several factors into account. These are the particle size, the refractive index of the solvent, the measurement angle and the variance of the distribution (Koppel 1972). The value of PDI changes in the range of 0-1. A lower value indicates a monodisperse distribution (a narrower size distribution), whereas a higher value indicates a polydisperse distribution (a broader size distribution) (Fanun 2010; Gaumet et al. 2008; Romero-Pérez et al. 2009).

### **3.2 Zeta potential**

Zeta potential indicates the electric potential in the interfacial double layer of a fluid, which is attached to the dispersed particles (Delgado et al. 2007). In other words, zeta potential is measured as the charge of the particle surface (Müller et al. 2000), reflecting the strength of the electrical repulsion between particles (Bleve et al. 2011; Mahajan et al. 2014). Thus, it allows the prediction about the potential storage stability of the colloidal dispersions (Al-Haj und Rasedee 2009; Jahagirdar et al. 2015). The higher the absolute value of zeta potential, the higher the stability of the system to aggregation. In general, a zeta potential value of higher than +30 mV or lower than -30 mV indicates an adequate stability of the system (Müller et al. 1996; Riddik 1968), whereby the charge repulsion between the particles is sufficient for the prevention of aggregation (Couvreur et al. 2002). Furthermore, a zeta potential of greater than +60 or lower than -60 mV indicates an excellent stability (Riddik 1968; Freitas and Müller 1998). The zeta potential of a nano-colloidal system is influenced by the charge of the different components including, in particular, the surfactant molecules located at the interface with the dispersion medium (Couvreur et al. 2002). Some surfactants contribute a positive zeta potential to the nanodispersed system. Other surfactants contribute

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a negative one. Zeta potential is commonly determined using the electrophoretic light scattering (Fanun 2010). In this work particle size, PDI and zeta potential were measured by dynamic light scattering on a Zetasizer nano ZS (Malvern Instruments Limited, Worcestershire, UK).

### 3.3 Total content and entrapment efficiency

The total drug content (TC) in the nanoparticle dispersion indicates the encapsulated and nonencapsulated amount of the drug. First, the nanoparticles are disrupted in their dispersion in order to release the amount of drug, which is captured within or adsorbed onto the particles. This is carried out by mixing the taken sample with an appropriate solvent for this purpose. Second, the sample is measured using an adequate analytical technique to determine the TC (Fanun 2010).

In the present work, the TC for the SLNs loaded with EGCG and cholecalciferol was determined according to Noack et al. (2012) and Rohit and Pal (2013) with minor modifications. This was started with the mixing of 100 µl of the SLNs sample with 2 ml of chloroform:MeOH (1:1 v/v). The mixture was vortexed and the absorbance was measured. For the SLNs loaded with EGCG the absorbance was measured using the UV/Vis spectrophotometer at a detection wavelength of 280 nm. For the SLNs loaded with cholecalciferol the TC was determined using an Agilent 1100 HPLC system with a quaternary pump and an UV-Vis detector. A CC 250/4 Nucleodur C18, 5 µm column (Macherey Nagel, Germany) was used. An isocratic ten minutes method was used with 90/10 v/v water/Acetonitril; both in HPLC grade, acidified with 0,1 % formic acid. The HPLC conditions were as follows: a flow rate of 1.3 ml/min, an injection volume of 20 µl, a column temperature of 20°C and a detection wavelength of 265 nm. Determinations were conducted in triplicates.

The concentration was calculated using the corresponding calibration curve established with at least six concentrations of the investigated bioactive compound (EGCG/cholecalciferol) in chloroform:MeOH (1:1 v/v). The TC was calculated using the following equation (V):

$$TC, \% = \frac{\text{calculated amount /ml of SLN dispersion}}{\text{total theoretical amount added /ml of SLN dispersion}} \times 100 \quad (V)$$

The entrapment efficiency (EE) is one of the most important physicochemical characteristics of the bioactive nanocarriers. It indicates the amount of the encapsulated drug as a

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percentage of the total drug content in the nanodispersion (Fanun 2010). Many methods have been developed to evaluate the EE, where the performance of the method used depends on the properties of the bioactive compound encapsulated. Available methods include the dialysis bag diffusion, the gel filtration, the ultrafiltration (UF), and the ultracentrifugation (UC). Among these methods, UF is the most widely used technique for determining the EE of nanoparticles (Yue et al. 2009).

### 3.3.1 EE for EGCG

The encapsulation efficiency of the EGCG in the nanoparticles was determined using the UF-centrifugation technique following a method previously mentioned with minor modifications (Kotikalapudi et al. 2012; Noack et al. 2012; Rohit and Pal 2013). 2 ml of the diluted sample of EGCG-loaded SLNs was placed in the outer chamber of Centrisart tube (Sartorius, Germany, molecular weight cutoff 100,000 Da). The tube was centrifuged at 4000 rpm for 15 min. SLNs containing the encapsulated EGCG remained in the outer chamber. The obtained supernatant containing the nonencapsulated EGCG moved into the sample recovery chamber through a filter membrane. The absorbance of the supernatant was measured spectrophotometrically at a wave length of 280 nm. The concentration of nonencapsulated EGCG was calculated from the corresponding calibration curve established with six EGCG concentrations in distilled water. The entrapment efficiency was calculated indirectly from the difference between the total amount of EGCG available in the SLNs dispersion and the amount of nonencapsulated EGCG that presented in the supernatant using the following equation (VI):

$$EE, \% = \frac{TC - C_{free}}{TC} \times 100 \quad (VI)$$

Where  $C_{free}$  = amount of EGCG in the clear supernatant (nonencapsulated EGCG).

To ensure that the quantity of nonencapsulated EGCG has fully passed the filter material and moved into the supernatant, a sample of an aqueous EGCG-solution, having a comparable concentration of EGCG as the EGCG-SLNs sample, was centrifuged using the same tubes and at same parameters. The concentration of EGCG, before and after centrifugation, was measured spectrophotometrically using the calibration curve. Furthermore, a sample of blank SLNs was mixed with a comparable amount of nonencapsulated EGCG. The sample was centrifuged and the concentration of EGCG before and after centrifugation was measured as described above. This was performed to check if the presence of SLNs in the sample above

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the filter could impede the moving of untrapped EGCG through the filter material into the supernatant.

The control experiments described above had shown that nearly 20% and 6% of nonencapsulated EGCG were captured by the filter material or was left on the SLNs layer over the filter membrane, respectively. This was considered during the calculation of the EE.

### 3.3.2 EE for cholecalciferol

The encapsulation efficiency of cholecalciferol was determined using the size exclusion chromatography (SEC) on cross-linked dextran. This enables a direct determination of the EE. The principle is based on the method of Fry et al. (1978) using the mini column centrifugation. 5 ml syringes were filled with the dextran slurry and centrifuged for 3 min at 1000 rcf to remove the excess water. Diluted samples of cholecalciferol-loaded SLNs were pipetted onto the column and the syringes were again centrifuged using the same conditions in order to separate the encapsulated from the nonencapsulated cholecalciferol. The supernatant collected after the second centrifugation contained the encapsulated cholecalciferol. The cholecalciferol content in the supernatant was determined as described above (determination of the total drug content) since the disruption of the particles is needed in order to extract the encapsulated cholecalciferol. The EE of cholecalciferol was calculated using the following equation (VII):

$$EE, \% = \frac{\text{Encapsulated cholecalciferol}}{TC} \times 100 \quad (VII)$$

### 3.4 *In-vitro* release tests

*In-vitro* digestion tests provide valuable informations about the release behavior of the active compound and the potential interactions between the nanocarrier and the active compound (Fanun 2010). In the present work, the release experiments were achieved using a tablet dissolution testing instrument (Pharma Test, DT70 Germany). The experiment was conducted by the addition of the sample of EGCG-SLNs to the corresponding release medium, which was then incubated over the tested period. Prior to the addition of EGCG-samples, the SGF and SIF were incubated at 37 °C for an adequate time for preheating.

SGF was prepared according to (USP 31) by dissolving 0.6 g sodium chloride in 300 ml of distilled water. The pH value was set to 1.2 with hydrochloric acid. 96 mg of pepsin was dissolved in the medium discribed above, namely directly before the beginning of the experiment. The sample of EGCG-SLNs was added to the release medium and whole was

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incubated at 37°C at 150 rpm. For analysis, samples were taken at 0, 5, 10, 20, 30, 60, 90, 120 minutes.

SIF was prepared according to Abdalla et al. (2008), Lee et al. (2012) and Noack et al. (2012) with minor modifications. SIF solution was prepared by dissolving 634 mg bile extract, 294 mg phospholipids (Phospholipon 90G), 2.36 g sodium chlorid and 166.5 mg calcium chloride in 150 ml Sörensen phosphate buffer (PBS) pH 6.8. Furthermore, 2.63 g of pancreatin was separately dissolved in 150 ml PBS. Both solutions (total volume of 300 ml) were incubated over night at room temperature and 150 rpm on an orbital shaker. On the next day, these solutions were centrifuged and the obtained supernatants were mixed directly before starting with the experiment. The equivalent volume of SLNs dispersion was added to the prepared SIF and the whole was incubated at at 37°C at 150 rpm. Samples were taken at 0, 10, 20, 30, 60, 120, 180, 240 minutes.

The amount of EGCG released during the experiments under gastric and intestinal conditions was determined by the fraction of free EGCG presents in the samples. The absorbance was measured using the UV-Vis spectrophotometry at 280 nm. The concentration was calculated by applying the corresponding calibration curve established with six concentration of EGCG in SGF or SIF.

### **3.5 Thermal analysis**

The crystallinity and polymorphism of the SLNs were investigated using the differential scanning calorimetry (DSC). These parameters are of particular importance affecting the encapsulation of active compounds, as well as the rates of release (Mehnert and Mäder 2001). The measurement principle of the DSC is based on the fact, that different lipid modifications exhibit different melting points and different melting enthalpies. The DSC instrument involves two identical sample holders. One is for the cell containing the sampe being investigated. The other is for an empty cell used as reference. During the measurement, both cells are heated and/or cooled at the same rate under symmetric conditions. As long as no structural change occurs in the sample, the temperature in both cells changes linearly to the same extent resulting in a zero baseline. Once the sample undergoes a structural change, a temperature difference is observed in the DSC curve as a signal (peak) representing the heat flow as a function of the time or the temperature. The maximum height of the transition; also called peak maximum, occurs at the phase transition. This peak represents the melting/crystallization temperature of the lipid modification. During endothermic events (melting) the recorder will move upward indicating that more energy is required. In contrast, an exothermic event (crystallization) causes a downward deflection

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indicating that less energy is required from the system to maintain the temperature (Chiu and Prenner 2011; Bunjes and Unruh 2007).

### **3.6 Morphology measurements**

The morphology of the SLNs was visualized using the freeze-fracture transmission electrical microscopy technique (FF-TEM). This technique is based on the physical fracturing of the frozen sample. The structural detail of the fractured sample is visualized by vacuum-diposition of platinum-carbon to make the replica for examination in a TEM: rapid freezing of the sample, fracturing, replication and replica cleaning and visualization of the replica in a TEM (Mittal and Matsko 2012). Sample preparation for this technique involves the freezing and direct viewing of the nanoparticle dispersion (Fanun 2010). In this work, samples were first cryoprotected at -120°C using a BAL-TEC BAF 400 unit. The frozen specimens were broken up with a knife-edge under a low pressure atmosphere at low temperature. The resulting thin layer of replicas, which imprinted the texture of the broken surface, was put on a mesh grid for TEM observation. The very short time needed for the freezing enables the preservation of the original morphology of the SLNs and avoids the formation of any new crystals in the aqueous phase (Varga et al. 2014). This is a particular advantage of this technique since it ensures a realistic image of the sample.

### **3.7 Storage stability**

The stability of SLNs system can be evaluated following the several characteristics during the investigated storage time (Fanun 2010). A common problem associated with nanoparticle systems is the occurrence of aggregation. However, SLNs have additional features, such as the supercooled melts, the presence of different modifications and the non-spherical shapes. These would contribute to or determine the stability of the colloidal lipid suspensions. Major problems, which could happen during the storage of SLNs, are the gelation phenomena, the increase in particle size and the compound expulsion from the nanoparticles. External parameters such as temperature and light appear to be of primary importance for the long term stability (Mahajan et al. 2014). An adequate characterization of the SLNs during the storage time using different techniques is a very important tool, in order to obtain a realistic image of their properties. This is required for an effective control of the system quality (Al-Haj and Rasedee 2009; Pardeike et al. 2009). In the present work, the stability was examined in terms of particle size, PDI, zeta potential, EE, TC and thermal profiles. Changes in these characteristics are associated with the system instability, such as precipitation, creaming, aggregation and drug expulsion.

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In addition, accelerated stability studies were performed in this work. These studies are generally carried out in order to generate the stability results faster, which are regarded to be reliable. The accelerated stability tests are based on the increasing of the stresses upon a product and the attempt to predict whether it will be stable against these stresses under usual conditions for a sufficiently long storage time. Different procedures could be applied for the acceleration of the system aging, including thermal and mechanical (centrifugation) techniques.

The testing of an emulsion stability using the centrifugation technique indicates the instability detected by creaming, i.e. the appearance of the oil phase at the surface, or by sedimentation of components having a higher density. This technique induces an accelerated migration of the particles/droplets and, hence, the potential destabilization process can be evaluated. The system is subjected to centrifugal forces and the transmission of the scattered light is measured. When a local alteration of particles concentration occurs, changes in light transmission are recorded. Zones of a homogeneous particle distribution are correlated with a low light transmission, whereas the local light transmission rises when some clarification occurs due to changes in particle concentration (creaming/sedimentation). Commonly, the transmission of the light at a defined wavelength is taken as a measure of turbidity. It has been experienced, that the measurement of the turbidity at two widely separated wavelengths makes more sense, whereby the result is recorded as a ratio. The following of the changes in the turbidity ratio as a function of aging time (during centrifugation) exhibits the relative system stability more exactly compared with the measuring of the turbidity at a single wavelength (Song et al. 2002). In this work, the turbidity was measured spectrophotometrically at 450 and 850 nm and the ratio was reported as a stability index (R-value), which was calculated using the following equation (VIII):

$$R - value = \frac{\text{Absorbance at 850 nm}}{\text{Absorbance at 450 nm}} \quad (VIII)$$

The lower the value of stability index the more stable is the system, whereas the higher this value is, the less is the stability indicating a wider particle size distribution (Kaufman and Garti 2007).

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## 4. Manuscript 1: The experimental design for the preparation of SLNs

### Development and characterization of solid lipid nanoparticles (SLNs) made of cocoa butter: A factorial design study

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#### 4.1 Abstract

The present study is motivated by the development of solid lipid nanoparticles (SLNs) for food applications. A focus of the study is the use of a factorial design to optimize the preparation variables. SLNs were prepared by hot homogenization at 60°C. Cocoa butter was used to form the lipid core and the surfactant blend used to emulsify and stabilize the system was a mixture of sodium stearyl-2-lactylate (SSL) and mono- and diglycerides of fatty acids (MDG). The particle characteristics and stability of obtained SLN-suspensions were investigated. Moreover, the effect of various cooling conditions on the properties of SLNs and the storage stability during a period of three months were examined. Results proved that cocoa butter is suitable to prepare SLNs with a food-grade quality where the optimized preparation variables resulted in a particle size of 112.7 nm.

#### 4.2 Introduction

Solid lipid nanoparticles (SLNs) were developed as an alternative carrier system showing many interesting advantages over the other colloidal carriers. In particular, they are prepared from physiological lipids (Aditya and Ko, 2015; Mehnert and Mäder, 2001) and show high chemical and physical stability, improved bioavailability, sustained release, and protection of sensitive incorporated molecules against environmental effects (Awad et al., 2008; Patidar et al., 2010). This makes the SLNs especially valuable to manufactures of functional foods as a novel way to introduce lipophilic but chemically sensitive bioactive compounds (Weiss et al., 2008). Due to the enhanced surface area of nanoparticles per mass unit, they are considered to show a higher biological activity compared to larger sized particles of the same chemical

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composition (Rai et al., 2015; Sozer and Kokini, 2009), which is of particular importance for food applications and offers promise of improving delivery properties, solubility, prolonged residence time in the gastrointestinal tract and efficient absorption of the bioactive compounds through cells (Ravichandran, 2010; Sozer and Kokini, 2009). Common disadvantages of solid lipid nanoparticles are the aggregation and gelation of the suspensions under production and/or storage conditions (Helgason et al., 2008).

Cocoa butter is a vegetable fat which constitutes the main lipid phase in chocolate (Garti and Widlak, 2012; Toro-Vazquez et al., 2005). Despite the simple composition, cocoa butter displays a very complex structural behavior resulting from the complex polymorphism of the representing triacylglycerides. The polymorphism of cocoa butter is well described in the literature and defined by six different polymorphic forms. These are  $\gamma$ (I),  $\alpha$ (II),  $\beta'$ (III),  $\beta'$ (IV),  $\beta$ (V) and  $\beta$ (IV) in increasing order of melting points and stability (Garti and Widlak, 2012; Langevelde et al., 2001; Loisel et al., 1998). Cocoa butter either pure or blended with other lipids was used before as particle core in the preparation of SLNs. In the study by (Kim et al., 2005), the formation of SLNs was achieved by changing the physical properties of curdlan by means of reducing the pH value of the system. This induced the solidification of curdlan around the cocoa butter droplets and thereby resulted in the forming of the SLNs. Qian et al. (2013) also investigated the preparation of SLNs using cocoa butter compared to hydrogenated palm oil or a mixture thereof using the hot homogenization. This study has focused on the effect of thermal treatment of the SLN samples after production on their physical stability and encapsulated bioactive compounds during storage.

SSL is an anionic, food grade surfactant, which forms ordered crystalline aggregates upon dispersion in water and, thus, naturally exhibits pickering properties (Kurukji et al., 2013). The emulsifying and stabilizing properties of SSL in O/W food emulsions were confirmed by several researchers (Flores et al., 2007; Zinoviadou et al., 2011). MDG is a nonionic, food grade surfactant, which is a mixture of mono- and diglyceryl esters of saturated and unsaturated long chain fatty acids (Food and Drug Administration (FDA), 2006). In addition to the good emulsifying properties of MDG (Hasenhuettl and Hartel, 2008), the presence of higher amount of mono- and diglycerides in the lipid phase (cocoa butter) used in the preparation of SLNs results in significantly smaller particles (Mehnert and Mäder, 2001) and causes, moreover, a disorganization of the lipid matrix. This enables the production of a particle core with more voids for a higher bioactive compounds entrapment (Gomes et al., 2013; Weiss et al., 2008). The aim of the present work was to investigate the influence of different preparation variables (i.e. the surfactant concentration, the cocoa butter concentration, the homogenization pressure and the number of homogenization cycles) on the physical properties of solid lipid nanoparticles made of cocoa butter as well as to optimize

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the experimental conditions in order to obtain a minimum droplet size and size distribution and a maximum zeta potential by using the experimental design methodology. Besides, the influence of different cooling temperatures and conditions were examined. Using a full factorial design the influence of preparation variables and their interactions on the properties of SLNs dispersion, prepared by hot homogenization using cocoa butter as lipid core, lipophilic and hydrophilic surfactants and water for the continuous phase, was investigated.

## **4.3 Materials and Methods**

### **4.3.1 Materials**

Cocoa butter in small disc shaped chips was purchased from Pati-Versand.de (Herzlake, Germany). Sodium stearoyl-2-lactylate (SSL, E481) and mono- and diglycerides of fatty acids (MDG, E471), respectively, which were a kind gift from Palsgaard® (Juelsminde, Denmark). Tween® 20 and Tween® 80 were purchased from Carl Roth (Karlsruhe, Germany). Sunflower lecithin Lecistar S was obtained from Sternchemie (Hamburg, Germany).

### **4.3.2 Preparation of SLNs**

Solid lipid nanoparticles were prepared using the hot homogenization method (Mehnert and Mäder, 2001; Sagis, 2015). Cocoa butter was initially treated by melting at 80°C for 30 min and cooling to room temperature to delete any thermal memory (Malssen et al., 1996).

For the preparation of SLNs formulation, the appropriate amount of treated cocoa butter was melted at 60°C using a water bath. Lipophilic surfactants were added to the melted cocoa butter. The required amount of water at the same temperature containing hydrophilic surfactants was added to the lipophilic phase. Next, the total mixture was stirred at a high speed at the equivalent melting temperature using an Ultra Turrax (Ystral, D-79282, Ballrechten, Dottingen, Germany) for 3 min at 19000 rpm. Lastly, the obtained pre-emulsion was homogenized using a high pressure homogenizer (Niro Soavi S.P.A., NS100 1L 2K, S N.4810, Parma, Italy) at pressures between 500 and 1500 bar for 1 to a maximum of 10 homogenization cycles yielding a hot O/W nanoemulsion. The hot emulsion was cooled in order to solidify the core of obtained SLNs and the effect of different cooling conditions was investigated. All formulations were filled in standard test tubes with screw cap (12 ml sample volume) and stored in the dark at -2°C until the next day for analysis.

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### **4.3.3 The effect of individual formulation variables**

The effects of homogenization pressure and cycle number as well as different cooling conditions were first studied individually using the same compositions and concentrations of materials needed for the SLNs preparation. The performing of these one-factor-at-a-time (OFAT) experiments is useful in order to define the ranges of chosen variables to be investigated by the next experimental design methodology.

### **4.3.4 The Experimental design**

The main purpose of the experimental design is to establish a causal relationship between independent and dependent process variables and on the SLN dispersions properties as well as to minimize the required experiment number. I.e., all observations obtained are used for estimating the effects of each factor and the interactions between factors are estimated simultaneously.

A two levels full factorial design with three centerpoints was employed to investigate the influence of four variables in order to optimize the solid lipid nanoparticle preparation. Surfactant concentration, cocoa butter concentration, homogenization pressure and number of homogenization cycles were selected as process variables. Particle size, polydispersity index and zeta potential were considered as a function of investigated variables since they reflect the emulsion quality and physical stability. The required number of experiments for an experimental design with two levels is in general  $2^k$ , where  $k$  is the number of variables. Four variables were used in the present study, therefore the necessary number of experiments was  $2^4 = 16$ . The particle size, PI and ZP values obtained from the 20 experiments including four experiments at the center points are illustrated in (Table 4-3).

### **4.3.5 Particle size, PDI and zeta potential**

The mean particle diameter, PDI and zeta potential of obtained SLNs were measured using Zetasizer Nano-ZS (Malvern, USA) based on laser light scattering technique (refractive index 1.54, 0.8872 cp, at 25°C). Samples were diluted with distilled water. Each sample was measured in triplicate and the mean value was calculated and used for the factorial design.

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#### **4.3.6 Thermal measurements**

Thermal properties of SLN dispersions were measured using differential scanning calorimetry (DSC Q2000, TA instruments, Germany). Samples of 5 mg for bulk cocoa butter as well as 10-15 mg for SLNs were placed in hermetic aluminum pans and sealed. A reference of a sealed empty hermetic pan was used and all samples were undergone a thermal program between 0 - 65°C using a scan rate of 5°C/min for both heating and cooling.

#### **4.3.7 Physical stability of SLNs**

The stability study was carried out for SLNs dispersions filled in transparent test tubes as described above and stored in the dark at ~2°C. During the investigated storage period of three months SLNs dispersions were checked for their stability by measuring their physicochemical properties (particle size, PDI and zeta potential) in time intervals of two weeks.

#### **4.3.8 Data analysis**

The results of the experimental design were analyzed using Design Expert 6.0.11 Software employing the statistical method: analysis of the variance (ANOVA) in order to determine the statistical significance of the effect of each variable as well as their interactions on responses. Evaluation parameters include coefficient of determination ( $R^2$ ), F values of model, curvature and lack of fit and adequate precision. The  $P$ -value < 0.05 indicates a significant result. Statistical analysis for data obtained from the one-factor-at-a-time (OFAT) experiments was done using the software GraphPad prism (GraphPad, version 6, USA). Two-way ANOVA using Tukey's multiple comparisons test was conducted to calculate the significance of differences at  $p < 0.05$ .

### **4.4 Results and discussion**

#### **4.4.1 Surfactant blend**

Table (4-1) represents a range of surfactants/surfactant blends used for SLNs fabrication. The selection of these surfactants/surfactant blends was based on earlier works done in the field of SLNs. In addition, a surfactant blend consisting of SSL and MDG was examined with the aim to be introduced as a new surfactant system for the preparation of SLNs.

All these surfactants enabled the production of SLNs from cocoa butter using the hot homogenization showing a particle size  $\leq 315.7$  nm,  $PDI \leq 0.364$  and a negative zeta potential ( $\geq -57.3$ ). A comparison between the results of particle size, PDI and zeta potential shows that the SLNs prepared using the surfactant blend (SSL+MDG) had the smallest particle size and PDI value, as well as the highest absolute value of zeta potential. Results of particle size obtained with Tween 20 were different from those reported by Qian et al. (2013), where the SLNs prepared from a lipid mixture (50%:50%) of cocoa butter and hydrogenated palm oil using 1.5% Tween 20 by the hot homogenization at 620 bar for three cycles, exhibited a particle size of approximately 170 nm. In the study by Manea et al. (2014) the particle size and the PDI value of SLNs prepared by the high shear homogenization with a combination of Tween 20 and lecithin were significantly smaller than that obtained using the Tween 80 in combination with lecithin. In general, similar results were observed in the present study when comparable combinations were used but the size difference was not as significant as that reported by Manea et al. (2014). SSL was earlier used for the preparation of O/W emulsions using the high pressure homogenization at 1000 bar for a single cycle, where a particle size of approximately 200 nm (PDI: 0.2-0.3) was achieved (Kurukji et al., 2013). In the present study, a higher particle size resulted when SSL was used. However, these SLNs showed a significantly higher absolute value of zeta potential confirming the strong stabilizing capacity of SSL.

**Table 4-1:** Average particle diameter of SLNs as a function of the typ of surfactant. SLNs were prepared at 600 bar for three homogenization cycles. Concentrations of cocoa butter and surfactant blend used in these experiments were constant at 5% and 1.5%, respectively, n=3. Values with different letters indicate differences significant at  $p < 0.05$ .

Surfactant*	Particle size, nm	PDI	ZP, mV
<b>Tween20</b>	315.7 <sup>a</sup>	0.283 <sup>a</sup>	-37.0 <sup>a</sup>
<b>Tween80</b>	299.7 <sup>b</sup>	0.364 <sup>a</sup>	-37.2 <sup>a</sup>
<b>18%Tween80+82%Lecithin</b>	273.1 <sup>c</sup>	0.304 <sup>a</sup>	-33.2 <sup>b</sup>
<b>16%Tween 20+84%lecithin</b>	251.9 <sup>d</sup>	0.247 <sup>a</sup>	-32.8 <sup>b</sup>
<b>SSL</b>	282.2 <sup>e</sup>	0.267 <sup>a</sup>	-57.3 <sup>c</sup>
<b>54%SSL+46%DMG</b>	234.4 <sup>f</sup>	0.210 <sup>a</sup>	-55.2 <sup>c</sup>

\*Surfactant blends were made in accordance with the required HLB of cocoa butter (6)

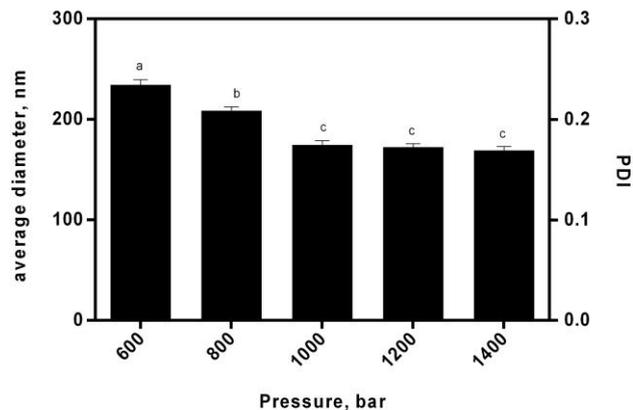
For further experiments in this study the surfactant blend of 54% SSL and 46% MDG was used. The obtained SLN dispersions exhibited a particle size in the range of 110.4–261 nm. All these samples were homogenous milky white, odorless and liquid. The pH value was nearly 7.

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## 4.4.2 One-factor-at-a-time (OFAT) experiments

### 4.4.2.1 Effect of homogenization pressure

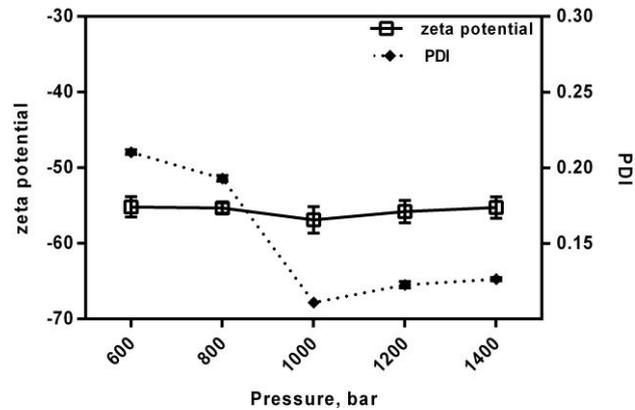
The effect of homogenization pressure on the particle properties is described in (Figures 4-1 and 4-2).



**Figure 4-1:** Average particle diameter of SLNs as a function of homogenization pressure done for three homogenization cycles. Concentrations of cocoa butter and surfactant blend used in these experiments were constant at 5% and 1.5%, respectively,  $n=3 \pm \text{STD}$ . Values with different letters indicate differences significant at  $p<0.05$ .

The particle size decreased significantly with increasing homogenization pressure up to 1000 bar (Figure 4-1). This finding is confirmed by other studies (Floury et al., 2000) and associated with the significantly increased cavitation as well as shear forces and interfacial forces when increasing the homogenization pressure. This leads to further breakup of the dispersed phase into smaller particles (Datta and Tomasula, 2015; Floury et al., 2000). Furthermore, increasing the homogenization pressure causes an important increase in the temperature of fluids being homogenized, which is attributed to the enhanced velocity of the fluid flow (Floury et al., 2000). As a result, a part of this mechanical energy is dissipated as heat in the fluid and, thus, an increase in fluid temperature is obtained (e.g. at 1000 bar the formulation temperature increased from 60°C before homogenization to 66°C after three homogenization cycles). A higher fluid temperature is associated with a decreased viscosity of both the lipid and the aqueous phases of fluids being homogenized under turbulent flow conditions, which enables the production of smaller droplets as a result of reduced interfacial tension between lipid and aqueous phases. At homogenization pressure above 1000 bar the particle size did not show any further reduction in the present study. In other studies, the application of higher pressures had led to a slight increase in the particle size of the SLNs.

This was attributed to droplet coalescence caused by excess kinetic energy, which is introduced by high homogenization pressure and could overcome the electrostatic repulsion forces between particles (Floury et al., 2000; Schwarz et al., 1994).

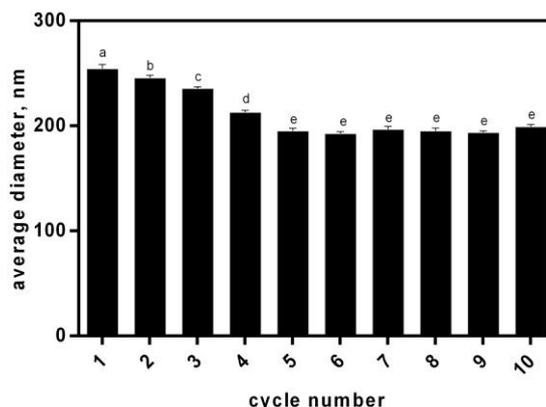


**Figure 4-2:** PDI and zeta potential of SLNs as a function of homogenization pressure done for three homogenization cycles. Concentrations of cocoa butter and surfactant blend used in these experiments were constant at 5% and 1.5%, respectively,  $n=3 \pm \text{STD}$ .

In this work, no statistically significant differences in PDI were noticed with homogenization pressures above 1000 bar (Figure 4-2). This is in accordance with the obtained particle size above. The almost constant values of particle size and PDI indicated no coalescence when the homogenization pressure was raised above 1000 bar. Also zeta potential was constant when homogenization pressure was changed in the range of 600 – 1400 bar.

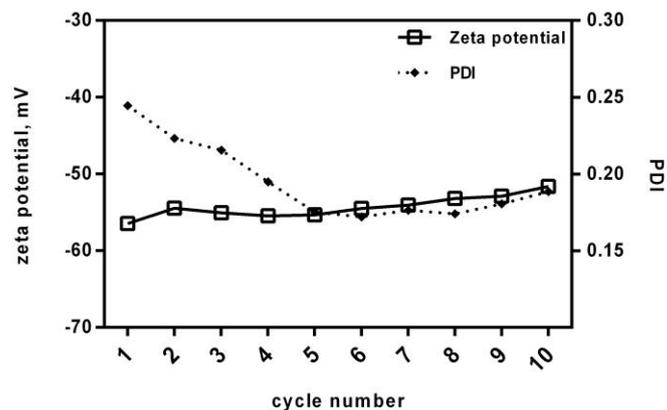
#### 4.4.2.2 Effect of the number of homogenization cycle

The effect of the number of homogenization cycles in the range of 1 to 10 is illustrated in the (Figures 4-3 and 4-4).



**Figure 4-3:** Average particle diameter of SLNs as a function of homogenization cycle number at 600 bar. Concentrations of cocoa butter and surfactant blend used in these experiments were constant at 5% and 1.5%, respectively,  $n=3 \pm \text{STD}$ . Values with different letters indicate differences significant at  $p<0.05$ .

The particle size dropped significantly when the number of homogenization cycles increased up to 5 cycles (Figure 4-3). This effect was in agreement with the results of other authors and could be explained by the increased energy input for emulsification with increasing the number of homogenization cycle at high homogenization pressure (Floury et al., 2000). A further increase of the number of homogenization cycles was not effective since the particle size was almost constant, which meant that the kinetic energy formed during further homogenization cycles had no significant effect. This is not in agreement with the results of a comparable study, where the higher number of homogenization cycles had a negative effect on the nanoemulsion stability. This was suggested to be caused by overcoming the electrostatic repulsion between particles, which then come close together to form bigger particles and start to flocculate (Schwarz et al., 1994).



**Figure 4-4:** PDI and zeta potential of SLNs as a function of the number of homogenization cycle at 600 bar. Concentrations of cocoa butter and surfactant blend used in these experiments were constant at 5% and 1.5%, respectively,  $n=3 \pm \text{STD}$ .

Similar to the changes observed for the particle size, the PDI also decreased correspondingly with the increasing cycle number (Figure 4-4). However, these changes were statistically not significant. The absolute value of the zeta potential decreased also steadily along these experiments but this reduction was statistically significant up to 2 homogenization cycles.

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#### 4.4.2.3 Effect of the Cooling conditions

In order to evaluate the effect of cooling conditions on the SLNs properties prepared in this work, five different cooling temperatures were applied and the corresponding particle characteristics were determined and illustrated in Table (4-2).

**Table 4-2:** Effect of different cooling temperatures and/or methods on particles properties of SLNs prepared at 600 bar for 5 homogenization cycles using 5% and 1.5% of cocoa butter and surfactant blend, respectively, n=3 ± STD.

Cooling temperature (°C)	method	Size (nm)	STD	PI	STD	ZP	STD
1	thermostat	156.1	2.804	0.143	0.003	-56.7	0.473
2	thermostat	180.5	2.390	0.170	0.004	-56.2	0.625
2	refrigerator	195.4	3.361	0.210	0.004	-55.6	1.210
5	thermostat	197.6	2.155	0.189	0.004	-55.1	0.611
10	thermostat	211.1	2.303	0.264	0.003	-54.7	0.723
RT	-	232.7	6.385	0.248	0.003	-51.2	0.416

The results described in (Table 4-2) show that the cooling temperature of 1°C resulted in a smaller particle size compared to the higher cooling temperatures of 2, 5, 10 and room temperature. Overall, the changes in the particle size were significant ( $p < 0.05$ ) except for the results at 2°C (refrigerator) and 5°C (thermostat), since there were almost no significant differences in the particle size obtained under these conditions. Cooling under the refrigeration conditions was not as efficient as by thermostat. Besides, the cooling at very low temperatures (under 0°C in refrigerator) resulted in a high increase in particle size (>400 nm), and PDI ( $PDI > 0.5$ ) and a decrease in the absolute value of zeta potential ( $Zp > -30$  mV). This could be explained by the brittleness of the lipid and, hence, the disruption of the particles is favoured at very low temperatures (Torchilin, 2014), which might be correlated with induced aggregation under such cooling conditions. The changes in the PDI corresponded with those observed in the particle size, but were not statistically significant ( $p < 0.05$ ), while the absolute value of zeta potential changed significantly at room temperature and 4°C (refrigerator) when compared to the results obtained using other cooling temperatures.

#### 4.4.3 Full factorial design experiments

The experimental design as described before was applied in order to optimize the process and mixture variables for the SLNs preparation. Each variable was investigated at two levels

(-1, +1) as well as at (0) for center points, as shown in (Table 4-3). Experiments with a concentration of the surfactant blend of less than 1.5% were not possible since the amount was not sufficient to obtain an efficient emulsification (data are not shown). Considering the the limitations in the food sector regarding the maximum acceptable amounts of the emulsifiers employed, the experimental field of emulsifier concentration used in this study was defined between 1.5 and 2%. When the surfactant concentration was higher than 1.5% foaming occurred during the preparation process, which resulted in unstable homogenization pressure and caused a loss of ingredients. Cocoa butter concentrations used in the following preparation of SLNs ranged between (5–10)%, based on the amount of lipid being commonly used in the preparation of SLNs (Kumar and Karimulla, 2012; Pardeike et al., 2009). The range for the homogenization pressure as well as the number of homogenization cycles was chosen based on the previous results obtained from the OFAT experiments where significant changes in particle properties were observed.

The factorial model with a 2FI (two-factor-interaction) order was the best fitting model for all experimental data obtained and the appropriate transformation for all three responses was established by analysing each response separately, in order to meet the acceptable statistical parameters when assessing the experimental design. The model assumptions were better satisfied for inverse transformation of obtained data, which conditionally include no 0 value. The coefficient of determination ( $R^2$ ) value for all three responses: Particle size, PDI and ZP and was: 0.99, 0.95 and 0.93, respectively. This  $R^2$  value indicates a good fitting between independent variables and responses.

The obtained equations for these responses using the factorial 2FI model in term of coded factors are as following:

$$1.0/(\text{Particle size, nm}) = +6.749 \times 10^{-3} + (1.023 \times 10^{-4})A - (5.104 \times 10^{-4})B + (8.565 \times 10^{-4})C + (5.091 \times 10^{-4})D - (1.656 \times 10^{-4})BC - (4.835 \times 10^{-5})BD + (1.711 \times 10^{-4})CD$$

$$1.0/(\text{Pdl}) = +6.74 - 0.44 B + 0.36C + 0.51 D - 1.126 \times 10^{-3} BC - 0.16BD + 0.40CD - 0.11BCD$$

$$1.0/(\text{ZP, mV}) = -0.018 + 1.957 \times 10^{-4} A - 7.696 \times 10^{-4} B + 2.031 \times 10^{-4} C + 1.516 \times 10^{-4} D + 1.297 \times 10^{-4} AB + 5.383 \times 10^{-5} BC + 6.733 \times 10^{-5} BD - 1.208 \times 10^{-4} CD$$

Where A, B, C and D describe the independent variables: surfactant concentration (%), cocoa butter concentration (%), homogenization pressure (bar) and cycle number, respectively.

**Table 4-3:** The full factorial design with the resulting properties of obtained SLNs, n=3

Run	Variables				Responses		
	*Surfactant (%)	*cocoa butter (%)	*Pressure (bar)	*Cycle number	Particle size (nm)	PDI	ZP (mV)
1	-1	-1	-1	-1	175.0	0.147	-55.8
2	1	-1	-1	-1	162.4	0.150	-56.0
3	-1	+1	-1	-1	194.9	0.173	-49.6
4	+1	+1	-1	-1	191.8	0.170	-51.4
5	-1	-1	+1	-1	135.3	0.156	-56.5
6	+1	-1	+1	-1	132.6	0.161	-57.9
7	-1	+1	+1	-1	157.6	0.168	-52.1
8	+1	+1	+1	-1	155.8	0.161	-53.6
9	-1	-1	-1	+1	156.0	0.143	-56.7
10	+1	-1	-1	+1	150.1	0.146	-56.8
11	-1	+1	-1	+1	172.0	0.169	-51.6
12	+1	+1	-1	+1	165.8	0.163	-53.8
13	-1	-1	+1	+1	112.7	0.112	-56.9
14	+1	-1	+1	+1	110.4	0.117	-57.4
15	-1	+1	+1	+1	135.2	0.136	-52.3
16	+1	+1	+1	+1	132.5	0.140	-54.8
17	0	0	0	0	148.9	0.144	-54.1
18	0	0	0	0	145.3	0.140	-54.9
19	0	0	0	0	146.1	0.147	-55.2
20	0	0	0	0	146.7	0.145	-55.0

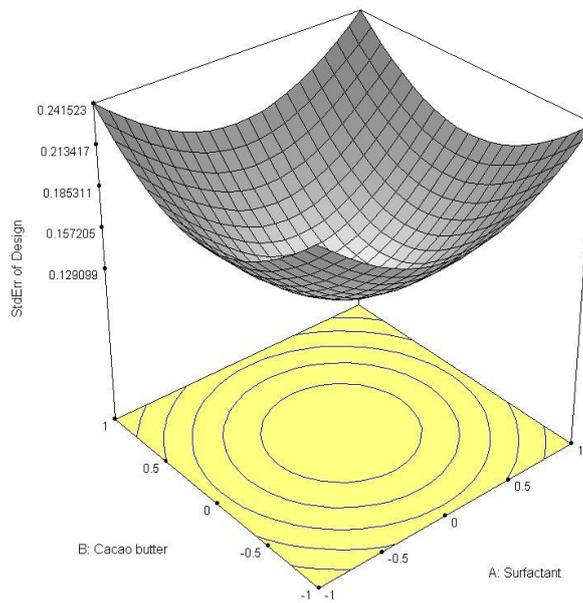
Values were measured at pH of nearly 7 (pH of SLNs dispersion). \*Surfactant (%): -1=1.5, 0=1.75, +1=2; cocoa butter (%): -1=5, 0=7.5; +1=10; pressure (bar): -1=600, 0=800, +1=1000, cycle number (n): -1=3, 0=4, +1=5

**Table 4-4:** ANOVA for designed experiments showing statistical results for particle size

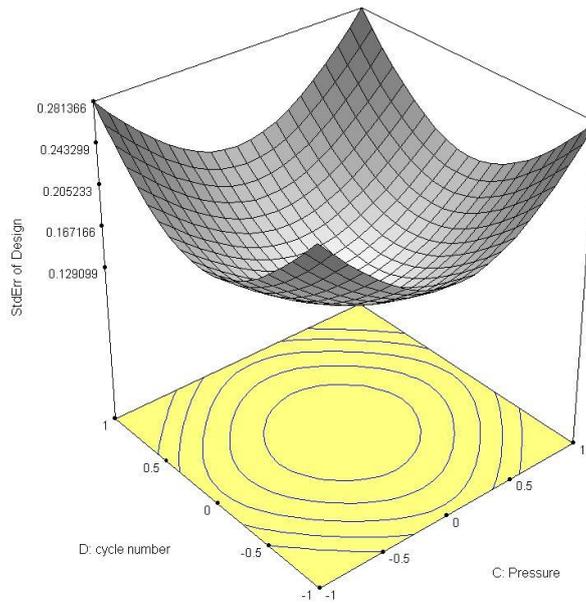
Source	Sum of squares	Df	Mean square	F-value	P-value
Model	6.35x10 <sup>-5</sup>	7	7.08x10 <sup>-6</sup>	726.88	<0.0001
A	5.02x10 <sup>-7</sup>	1	5.02x10 <sup>-7</sup>	40.23	<0.0001
B	1.25x10 <sup>-5</sup>	1	1.45x10 <sup>-5</sup>	1002.18	<0.0001
C	3.52x10 <sup>-5</sup>	1	3.52x10 <sup>-5</sup>	2821.77	<0.0001
D	1.24x10 <sup>-5</sup>	1	1.24x10 <sup>-5</sup>	996.92	<0.0001
BC	1.32x10 <sup>-6</sup>	1	1.312x10 <sup>-6</sup>	105.52	<0.0001
BD	1.12x10 <sup>-7</sup>	1	1.12x10 <sup>-7</sup>	8.99	0.0043
CD	1.40x10 <sup>-6</sup>	1	1.40x10 <sup>-6</sup>	112.56	<0.0001
Curvature	4.90x10 <sup>-8</sup>	1	4.90x10 <sup>-8</sup>	3.93	0.0532
Residual	5.99x10 <sup>-7</sup>	48	1.25x10 <sup>-8</sup>		
Lack of fit	1.78x10 <sup>-7</sup>	8	2.23x10 <sup>-8</sup>	2.11	0.0569
Pure Error	4.21x10 <sup>-7</sup>	40	1.05x10 <sup>-8</sup>		
Corrected total	6.45x10 <sup>-5</sup>	59			

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The statistical analysis of the design data (Table 4-4) confirmed that the proposed model was significant ( $P < 0.05$ ) having a very high coefficient of determination ( $R^2 = 0.99$ ), which indicated a best fit between variables and response. The "*predicted  $R^2$* " of 0.9857 was in reasonable agreement with the "*Adjusted  $R^2$* " of 0.9893. Moreover, the "*Adequation of Precision*" of 82.58 indicated an adequate signal and, hence, this model can be used to navigate the design space. The insignificance of the lack of fit implied that the model fits the experimental data and the independent variables have considerable effects on output responses. The standard error for the design as a function of the composition as well as the process variables is shown in (Figures 4-5 and 4-6), respectively. As expected, the standard error increases at the minimum and maximum limits of the systems.



**Figure 4-5:** Surface plot of the standard error for Design as a function of composition variables



**Figure 4-6:** Surface plot of the standard error for Design as a function of process variables

#### 4.4.3.1 Effects of independent variables on particle size

The particle size was strongly affected by the changing levels of investigated variables (Table 4-3). The most significant effect on the average particle size was caused by the homogenization pressure. The number of homogenization cycles and the cocoa butter concentration also showed significant effects. The effects of homogenization pressure, as well as the cycle number regarding the decrease of the particle size at higher levels were expected from the OFAT experiments and can be explained as above. The interaction effects between the homogenization pressure and the cocoa butter concentration are shown in the (Figure 4-7(A)). A higher cocoa butter concentration caused an increase in the particle size, which was in agreement with other findings and can be explained by the increased viscosity of the dispersed phase and the effect of droplet coalescence (Floury et al., 2000; Mehnert and Mäder, 2001; Schubert, 2003). The dispersed phase viscosity influences the mean droplet size considerably because it contributes to the stability towards droplet breakage. At higher dispersed phase viscosity the droplet breakage probability becomes lower due to enhanced cohesive forces (Kraume et al., 2004; Sathyagal et al., 1996; Tadros et al., 2004). This enables the production of smaller droplets with a narrower droplet size distribution. Additionally, it was shown that at a lower viscosity the droplet sizes were more uniformly distributed (Sathyagal et al., 1996). Moreover, when the fat content increased the surfactant amount available in the system decreased proportionally. Thus, the emulsifying capacity, as well as the stabilizing benefits also decreased. This induced the oil droplet coalescence and led to an increased droplet diameter. These observations confirmed the earlier findings by

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other authors (Floury et al., 2000). Moreover, when the lipid concentration increased with a constant concentration of surfactants the particles tended to aggregate because of insufficient amount of surfactants to coat all particles (Kim et al., 2005). Furthermore, the tendency of lipid nanoparticles to coalesce at high lipid concentration was reported in another study (Shah and Pathak, 2010). A small negative effect of an increased surfactant concentration on the particle size was observed, as the higher concentration led to a further reduction of the particle size, which is explained by the decrease of the interfacial tension (Tadros et al., 2004). This effect was quite small indicating that the usage of 1.5% of surfactants was sufficient to produce fine particles and cover the surface of the nanoparticles effectively (Mehnert and Mäder, 2001) and, thus, prevent the agglomeration during processing.

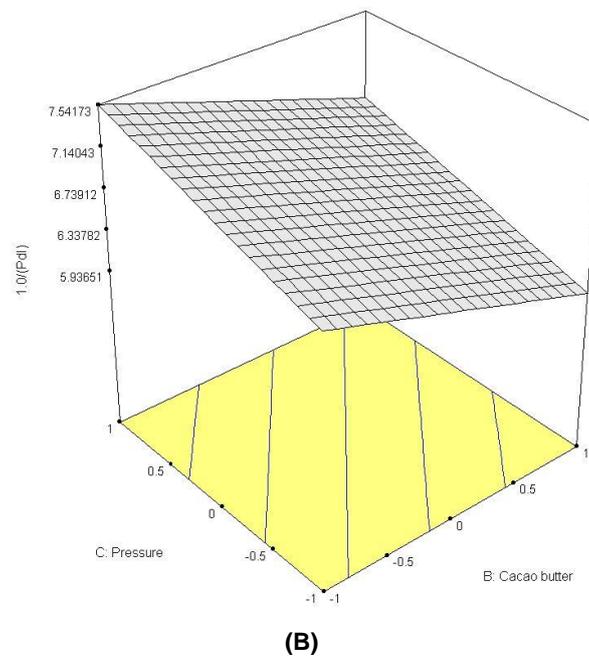
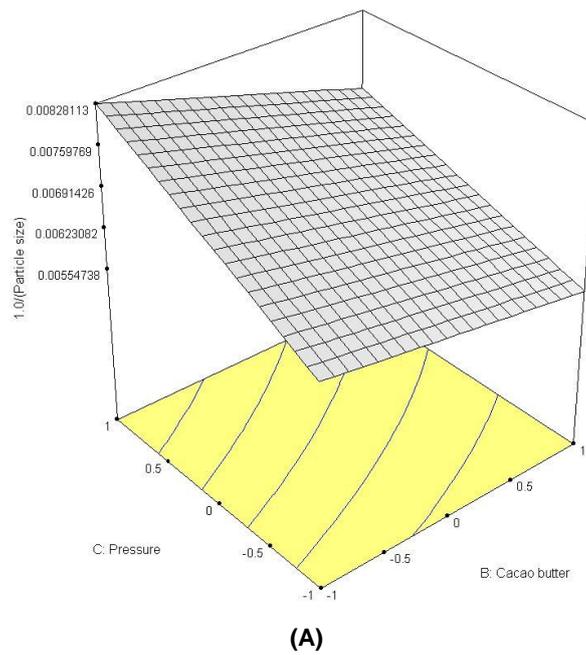
#### **4.4.3.2 Effects of independent variables on polydispersity index (PDI)**

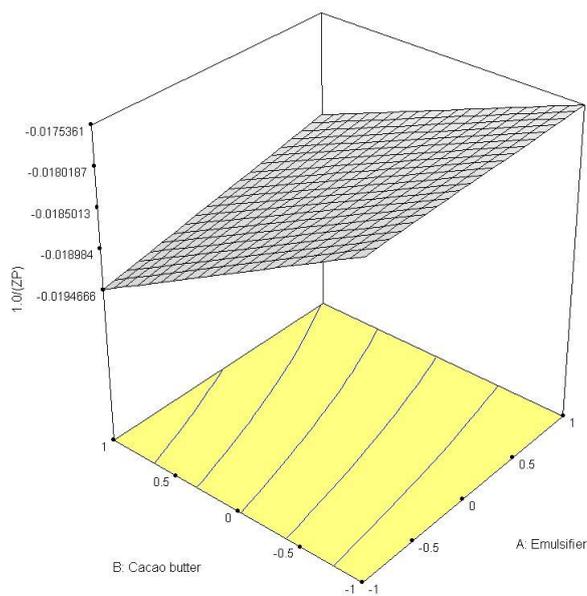
Table (4-3) shows that the PDI value changed within a narrow range when the investigated variables were changed. The PDI value of all formulations ranged from 0.112 to 0.173, which indicates a narrow size distribution and homogeneity, and reflects a high stability of the obtained SLNs (Khare et al., 2016). Significant effects on the PDI were caused by the homogenization pressure and the cocoa butter concentration, as well as the homogenization cycle number and their interactions. Figure (4-7(B)) shows the interaction effects between the homogenization pressure and the cocoa butter concentration. The increase in the cocoa butter concentration led to a broader particle size distribution as a result of an increased dispersed phase viscosity by affecting the rate of droplet breakage (Floury et al., 2000; Kraume et al., 2004; Mehnert and Mäder, 2001; Schwarz et al., 1994). In the range employed for this experimental design no significant effect of the surfactant concentration on the PDI was observed. In other findings as that by (Vijayan et al., 2013), where Tween 80 was used as surfactant in comparable concentrations to those applied in the present study, the increase in Tween 80 concentration resulted in a slightly smaller PDI value.

#### **4.4.3.3 Effects of independent variables on zeta potential**

Zeta potential changed also narrowly when changing the investigated variables (Table 4-3). Zeta potential value of all formulations was  $\leq -50\text{mV}$  indicating a good stability. While the surfactant concentration, as well as the homogenization pressure and the number of homogenization cycles have shown a limited positive effect on the zeta potential. The cocoa butter concentration had a significant negative effect at the higher level. This could be

attributed to the insufficient amount of surfactant molecules adsorbed on the monolayer around the lipid droplets, resulting in a less negative surface charge at the shear plane, which was reflected by the reduction in zeta potential (Sun and Gunasekaran, 2009). The interaction effects between the cocoa butter concentration and the surfactant concentration are shown in Figure (4-7(C)).



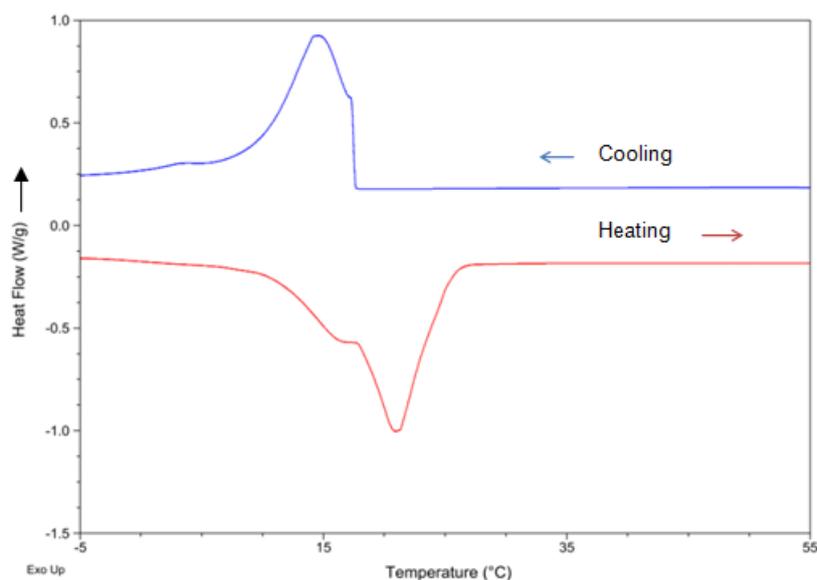


(C)

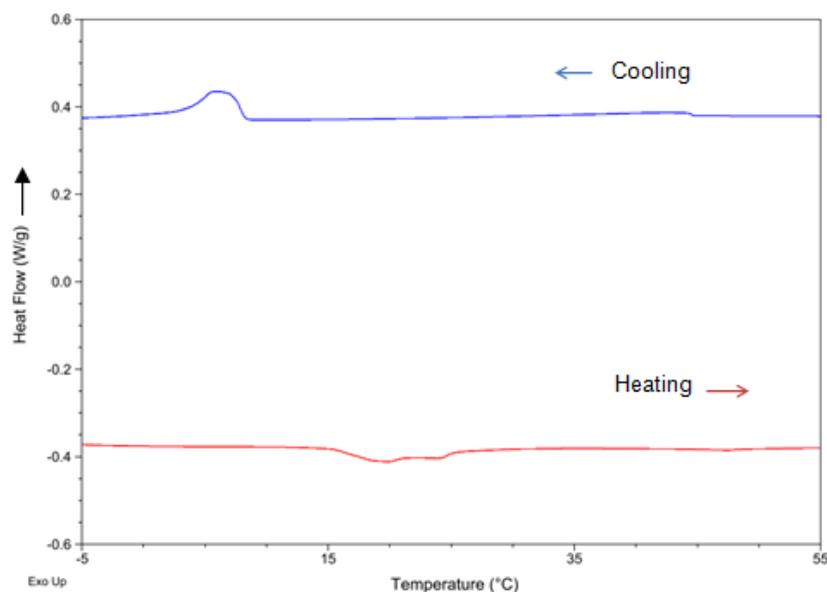
**Figure 4-7:** Surface plots of the effects of the variables on the different responses (**A:** particle size; **B:** PDI; **C:** zeta potential)

#### 4.4.4 Crystal modification

DSC thermograms of bulk cocoa butter and SLNs dispersions were recorded during heating and cooling in the range (5 to 65 to 5°C) in order to compare the polymorphic form of cocoa butter in bulk and as core of nanoparticles in the presence of MDG and SSL under preparation conditions employed in this work (Figures 4-8 and 4-9).



**Figure 4-8:** DSC heating and cooling curves of bulk cocoa butter,  $n=3$  ( $\pm$  STD: 0.16 for the melting peak; 0.087 for the recrystallization peak)

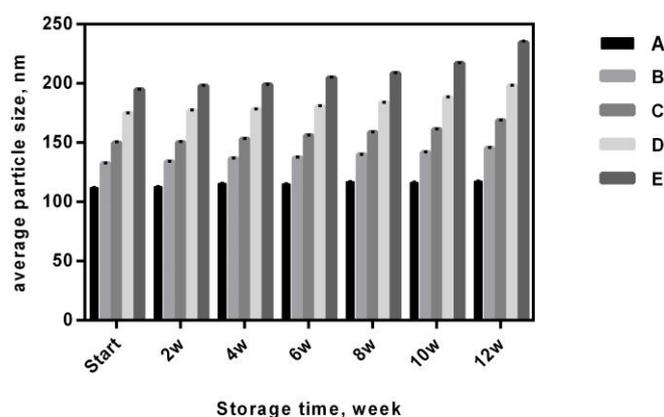


**Figure 4-9:** DSC heating and cooling curves of SLNs prepared at 1000 bar and 5 homogenization cycles using 5% and 1.5% of cocoa butter and surfactant blend and cooled at 1°C, n=3 ( $\pm$  STD: 0.075 for the first melting peak; 0.040 for the second melting peak; 0.025 for the recrystallization peak).

For bulk cocoa butter (Figure 4-8), a single melting peak was observed at 20.7°C which indicates that cocoa butter existed in the  $\alpha$ -modification before the processing. In contrast, the melting thermogram of SLNs (Figure 4-9) showed two broad peaks in the range of 18.8 to 24.1°C. This indicates that the crystal form of the cocoa butter core in SLNs was a mixture of the two unstable modifications  $\alpha$  and  $\beta'$ (III) (Loisel et al., 1998; Manning and Dimick, 1985; Wille and Lutton, 1966). The formation of  $\alpha$ - and  $\beta'$ -crystal modification is a combined result of the complex triglycerides composition of cocoa butter and the cooling of the SLN formulations at adequately low temperature (Long et al., 2006; Weiss et al., 2008). The surfactant blend might also have its influence on the crystal modification obtained: it was reported that the presence of diglycerides included in the surfactant blend induced the occurrence of  $\beta'$ -crystals and enhanced the number of lattice imperfection (Long et al., 2006). When comparing the cooling thermograms it can be concluded that the processing of the cocoa butter into SLNs caused a delay in the recrystallization which occurred at about 9°C below that of cocoa butter in bulk. These observations might be explained by the presence of an enormous number of nanosized particles that responded individually upon melting and recrystallization. This resulted in a sum of different individual melting/recrystallization temperatures, which is shown in a broad single or several peaks (Bunjes and Unruh, 2007; Higami et al., 2003; Mehnert and Mäder, 2001). Besides, the presence of surfactants and the preparation conditions play an additional role in the occurrence of such changes (Bunjes et al., 2002; Bunjes et al., 2003; Mehnert and Mäder, 2001).

#### 4.4.5 SLNs stability

To judge the emulsion stability, it is necessary to follow the changes in droplet size, size distribution and zeta potential as well as the optical appearance during an extended period of time (Figure 4-10). For the stability study, samples were taken from five formulations prepared by the designed experiments: 13(A), 6(B), 10(C), 1(D), 3(E) (Table 4-3), which differed in their initial particle size.



**Figure 4-10:** Changes in the average particle size of SLN during a storage time of three months at 2°C considering the initial size (A:112.7 nm, B:132.6 nm, C: 150.1 nm, D: 175.0 nm, E: 195 nm),  $n=3\pm\text{STD}$ .

In all formulations tested the absolute zeta potential value obtained was higher than 50 mV indicating a stable dispersion (Guy Broze, 1999; Radaic et al., 2014).

The storage stability of SLN formulations during three months was studied by following the changes in their properties depending on the initial average particle size, PDI and zeta potential. As seen in (Figure 4-10), almost no changes were seen in the formulation with the initial particle size of 112.7 nm. Changes became more obvious at higher initial size, i.e. the size of particles with 195 nm at the beginning increased about 20% within 12 weeks. The changes in the PDI were comparable to those obtained with particle size and the absolute zeta potential value of this formulation decreased slightly over the storage time. These results confirm the earlier findings that the shelf life of SLNs depends on initial particle size and PDI since the smaller particle size correlates with reduced droplet aggregation, gravitational separation and creaming rate (Floury et al., 2000; McClements, 2011). The lower stability of bigger particles is attributed to the effect of gravity, which increases with increasing particle size and lead in this case to a higher creaming velocity (McClements, 2011; Tadros et al., 2004).

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## 4.5 Conclusion

SLNs consisting of a cocoa butter core were successfully prepared in order to be applied for delivering bioactive substances through food products. Cocoa butter with its melting range close to body temperature has shown good results regarding particle properties and storage stability. The surfactant blend consisted of SSL and MDG was effective in producing small nanoparticles and stabilizing the system during the storage time. Lower cooling temperatures showed a positive effect on the particle size characteristics, whereas very low temperatures caused adverse effects. Using a 2<sup>4</sup> full-factorial design optimal preparation conditions were determined and the interactions between preparation variables and process conditions were estimated. SLNs preparation using 5% of cocoa butter and 1.5% of surfactants at 1000 bar and five homogenization cycles resulted in a low particle size of 112.7 nm, which were stable for three months. An increase in the surfactant concentration to 2% did not result in a significant reduction of the particle size (110.4 nm). Hence, the SLNs formulation with the particle size of (112.7 nm), which was obtained under the same conditions except the use of 1.5% of surfactant blend seems to be from the economic point of view more preferable. Moreover, the use of lower percentages of surfactants in the food manufacturing is of general interest.

## Acknowledgement

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## 5. Manuscript 2: Encapsulation of EGCG in SLNs

### Encapsulaion of (–)-epigallocatechin-3-gallate (EGCG) in solid lipid nanoparticles for food applications

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(submitted to Food Research International, FOODRES-S-17-00630, March 2017)

#### 5.1 Abstract

A nanoparticulate delivery system was prepared in order to develop a food grade carrier for the major bioactive constituent in green tea; (-)-epigallocatechingallate (EGCG), protecting it against degradation during storage and digestion under simulated gastrointestinal pH conditions. EGCG-loaded solid lipid nanoparticles (EGCG-SLNs) were produced by the hot homogenization method. The lipid matrix used in the production consisted of pure cocoa butter. A combination of sodium stearyl-2-lactylate (SSL) and mono- and diglycerides (MDG) was applied as a surfactant blend. The nanoparticles loaded with different concentrations of EGCG had an average particle size in the range of 108-122 nm. A maximal encapsulation efficiency of 68.5% was obtained. The produced food grade SLNs successfully protected the encapsulated EGCG along the storage period as well as under the adverse conditions at neutral pH values.

#### 5.2 Introduction

Polyphenols are naturally occurring compounds, which are found abundantly in food plants (Pandey & Rizvi, 2009; Quinones, Miguel, & Aleixandre, 2012). Due to their potential health benefits in human (Cencic & Chingwaru, 2010; Vauzour, Rodriguez-Mateos, Corona, Oruna-Concha, & Spencer, 2010), it is of interest to look for novel strategies to introduce polyphenols in functional foods and pharmaceutical products (Bilia, Isacchi, Righeschi, Guccione, & Bergonzi, 2014; Granja, Pinheiro, & Reis, 2016; Grumezescu, 2016a; Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). (-)-Epigallocatechin-3-gallate (EGCG, Figure 4-2) is the most abundant and biologically active polyphenol found in green tea. The total amount of catechins in one brewed cup of green tea consists of 50–80% of EGCG, which makes approximately 200–300 mg (Klinski, 2013; Singh, Shankar, & Srivastava, 2011). EGCG is known as an antioxidant compound, which exhibits health-promoting and therapeutic properties, and plays an effective role in the prevention of several chronic diseases, especially cancers (Granja et al., 2016; Klinski, 2013). Owing to its strong anti-

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inflammatory and antioxidant activity, as well as the ability to reduce the level of cholesterol and normalize glucose metabolism and T-cell immunity, EGCG offers a promising therapeutic potential for the treatment of health problems, such as arthritis, neurodegenerative, cardiovascular diseases, obesity, diabetes and autoimmune diseases (Grumezescu, 2016a; Jantan, Ahmad, & Bukhari, 2015; Upadhyay & Dixit, 2015).

Unfortunately, there are some drawbacks associated with EGCG, which negatively affect its functional benefits. These are, generally, the short half-life and the high sensitivity to light and heat (Grumezescu, 2016b; Munin & Edwards-Lévy, 2011). Furthermore, EGCG is instable in neutral and alkaline medium (Kanwar et al. 2012) and, therefore, undergoes an extensive gastrointestinal degradation inside the human body. This poor stability of EGCG results, besides the poor membrane permeability and intestinal transporter-mediated efflux, in an inefficient systemic delivery and very low oral bioavailability of EGCG despite its high water solubility (Kanwar et al., 2012; Klinski, 2013; Siddiqui et al., 2009). Hence, researchers made efforts to develop novel methods for increasing the stability of EGCG as a tool for enhancing its oral bioavailability. A number of such methods have been previously reported (Ghadiri et al., 2012; Klinski, 2013; Siddiqui et al., 2009; Smith et al., 2010; Smith et al., 2013).

(Smith et al., 2010) and (Zou et al., 2014) tried to overcome the drawbacks combined with the instability by the encapsulation of EGCG in nanodelivery systems, such as SLNs and liposomes, respectively. It has been demonstrated that the encapsulation in SLNs provides protection and better control over the release kinetics, as well as specific targeting of the encapsulated compounds. This significantly improves the physicochemical stability of the encapsulated compounds by decreasing their reactivity against the outside environmental factors (Pool et al., 2012; Weiss et al., 2008). Bioactive loaded-SLNs consist of a core of a crystallized biodegradable lipid (or lipid blend) with the encapsulated compound (Attama, Momoh, & Builders, 2012). Two general models of bioactive loaded-SLNs are distinguished (Üner & Yener, 2007; Zur Mühlen, Annette and Mehnert, Wolfgang, 1998):

- 1) The solid solution model: where the bioactive compound is molecularly dispersed in the lipid matrix.
- 2) The core-shell model, where two types are to be considered: the bioactive-enriched shell and the bioactive-enriched core, indicating the location of the encapsulated bioactive.

Although the development of SLNs was thought for lipophilic compounds, there is sufficient data in the literature which confirms the possibility of the encapsulation of both hydrophilic

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and lipophilic molecules in the SLNs with an improved bioavailability (Ghadiri et al., 2012; Manea, Andronescu, & Meghea, 2014).

In the present work the encapsulation of EGCG within SLNs was investigated. Unlike the other works in this field, where SLNs were prepared from triglycerides or polymers, the presented SLNs were prepared from a food-grade hard fat (cocoa butter). Preparation was carried out according to an organic solvent-free process using the hot homogenization method. The obtained SLN dispersions were characterized in terms of particle size, zeta potential, entrapment efficiency, release behaviour, thermal analysis and particle morphology.

### **5.3 Materials and methods**

#### **5.3.1 Materials**

Cocoa butter in small disc shaped chips was purchased from Pati-Versand.de (Herzlake, Germany). Mono- and diglycerides of fatty acids (MDG, E471) and sodium stearyl-2-lactylate (SSL, E481) were kindly a gift from Palsgaard® (Juelsminde, Denmark). Sodium chloride (content  $\geq 99.5\%$ ) was purchased from Carl Roth GmbH, (Karlsruhe, Germany). Green tea polyphenols extract 95% (obtained from green tea leaves, catechins content  $>75\%$ , EGCG  $>65\%$ ; according to HPLC analysis) was purchased from cfm Oskar Tropitzsch e. K. (Marktredwitz, Germany). The term EGCG used in the present study refers to the green tea extract. Lecithin (Phospholipon® 90G) was purchased from Phospholipid GmbH (Köln, Germany). Calcium chloride (content  $\geq 93\%$ ), bile extract from porcine pancreas, pancreatin from porcine pancreas (activity equals to 8x USP specification) and bovine pepsin from porcine gastric mucosa were purchased from Sigma Aldrich (Steinheim, Germany). Methanol and acetonitrile (both in HPLC-Grade,  $\geq 99.9\%$ ) and chloroform (UV/IR-Grade,  $\geq 99.8\%$ ) were purchased from Carl Roth (Karlsruhe, Germany). All materials were used as received without any further treatment, except the cocoa butter, which was melted at 80 °C for 30 minutes in order to avoid any potential effect of the thermal memory.

#### **5.3.2 Preparation of EGCG-SLNs**

EGCG-loaded SLNs (EGCG-SLNs) were prepared by the high pressure homogenization method with slight modification. Briefly, 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5 %w/w of green tea polyphenols (related to the cocoa butter concentration used for the preparation) were dissolved in 12 ml water. This solution was then emulsified into the organic phase containing

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5% of melted cocoa butter (60 °C) with the needed amount of MDG using a high speed mixer (Ultra-Turrax Ystral, Germany) at 11000 rpm for 5 min. The resulting blend was re-emulsified into the aqueous phase containing the required amount of SSL and having the same temperature (60 °C). The total mixture was processed with the high speed stirrer at 19000 rpm for 3 minutes in order to create the pre-emulsion. This was followed by the homogenization using a high pressure homogenizer (GEA NIRO-SOAVI S.P.A., Italy) at 1000 bar for five cycles. The resulting hot nanoemulsions were cooled in the thermostat at 1 °C (12ml sample volume in standard test tubes with screw cap) for one hour and stored at 2 °C in the dark for 24 hours before the further analysis.

### **5.3.3 Characterization of EGCG-SLNs**

#### **5.3.3.1 Particle size and zeta potential**

Particle size analysis was performed using a Zetasizer NanoZS (Malvern, USA) based on dynamic light scattering (DLS), (refractive index 1.54 for solid lipid particles (Bunjies und Koch, 2005), at 25 °C), which yields the average particle size, the polydispersity index (PDI) and the zeta potential. Samples were diluted 1:200 times beforehand using distilled water and directly measured. Measurements were conducted in triplicate.

#### **5.3.3.2 Total EGCG content (TC)**

The total amount of EGCG per unit volume present in the SLNs formulation was determined according to (Noack, Oidtmann, Kutza, & Mäder, 2012) and (Rohit & Pal, 2013). 100 µl of SLNs dispersion were disrupted in 2 ml of chloroform:methanol (1:1 v/v) mixture and the absorbance was measured using UV/Vis spectrophotometer (Helios Gamma UV-Vis, Thermo Spectronic, Cambridge, UK) at 280 nm, which was determined by UV scan. The concentration of EGCG was assessed using the calibration curve established with six concentrations of EGCG in chloroform:methanol (1:1 v/v) mixture. A similarly processed blank SLNs sample was taken as control. The total EGCG content was determined by using the equation given below.

$$Total\ content\ TC = \frac{\text{calculated amount /ml of SLN dispersion}}{\text{total theoretical amount added /ml of SLN dispersion}} \times 100$$

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### 5.3.3.3 EGCG entrapment efficiency (EE)

The entrapment efficiency was determined according to (Kotikalapudi, Adepu, VijayaRatna, & Prakash V Diwan, 2012; Noack et al., 2012) and (Rohit & Pal, 2013). The determination was carried out indirectly by analysing the concentration of free EGCG (unentrapped) in the clear aqueous medium separated by ultra-filtration using Vivaspin-2 tubes with PES membrane (Sartorius, Germany, molecular weight cutoff 10,000 Da). 2 ml of the diluted sample of EGCG-loaded SLNs was placed in the outer chamber and centrifuged at 4000 rpm using swinging bucket rotor for 15 min. The ultrafiltration tubes were rinsed with clear water before adding the sample. EGCG entrapped in the SLNs remained in the outer chamber, whereas the free EGCG migrated into the filtrate. A calibration curve was established using six concentrations of the EGCG in distilled water. The supernatant was measured spectrophotometrically at a wave length of 280 nm and the concentration of the unentrapped EGCG was calculated using the corresponding calibration curve. The entrapment efficiency was calculated from the difference between the total amount of EGCG available in SLNs dispersion and the amount of free EGCG that presented in the supernatant using the following equation:

$$EE\% = \frac{TC - C_{free}}{TC} \times 100$$

Where  $C_{free}$  = amount of EGCG in the clear supernatant.

In addition, two further samples were prepared as follows: a known amount of nonencapsulated EGCG was dissolved in (a): distilled water, and (b): diluted blank SLNs. Both samples were centrifuged using the same tubes and at same parameters as described above. These samples were measured spectrophotometrically before and after centrifugation and the EGCG concentration was calculated using the calibration curve. The purpose of this procedure was to ensure that the quantity of nonencapsulated EGCG has fully passed the filter material and moved into the supernatant (sample a), and to check if the presence of SLNs in the sample above the filter could impede the moving of unentrapped EGCG through the filter material into the supernatant (sample b). The results obtained from this procedure were considered during the calculation of the EE.

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### 5.3.4 *In-vitro* release experiments

*In-vitro* release experiments were carried out in simulated gastric fluid (SGF, pH 1.2) and intestinal fluid (SIF, pH 6.8) using a tablet dissolution testing instrument (Pharma Test, DT70, Germany). The SLNs formulation with the highest entrapment efficiency (1% w/w) was used. SGF was prepared according to (USP 31) by dissolving 0.6 g sodium chloride in 300 ml of distilled water. The pH value was set to 1.2 with hydrochloric acid. 96 mg of pepsin was dissolved in the prepared medium directly before the beginning of the experiment. The equivalent volume of SLNs dispersion was added to the release medium and incubated at 37°C and 150 rpm. For analysis samples were taken at 5, 10, 20, 30, 60, 90, 120 minutes.

SIF was prepared according to (Abdalla, Klein, & Mäder, 2008; Lee, Yim, Choi, van Anh Ha, & Ko, 2012) and (Noack et al., 2012) with slight modification. 300 ml of SIF solution was prepared by mixing the following two solutions: 634 mg bile extract, 294 mg phospholipids (Phospholipon 90G), 2.36 g sodium chlorid and 166.5 mg calcium chloride were dissolved in 150 ml Sörensen phosphate buffer (PBS) pH 6.8. Furthermore, 2.63 g of pancreatin was separately dissolved in another 150 ml PBS. Both solutions were incubated over night at room temperature and 150 rpm on an orbital shaker. The first solution was centrifuged and the supernatant was mixed with the pancreatin solution just before starting the experiment. The equivalent volume of SLNs dispersion was added to the prepared SIF and incubated at 37°C at 150 rpm. Samples for analysis were taken at 5, 10, 20, 30, 60, 120, 180, 240 minutes.

The concentration of EGCG released during the experiments under gastric and intestinal conditions was determined by the measuring of the fraction of free EGCG in the samples using UV-Vis spectrophotometry at 280 nm and applying the corresponding calibration curves.

### 5.3.5 Thermal measurements

The calorimetric measurements of cocoa butter as bulk material, unloaded SLNs and EGCG-loaded SLNs were performed by the differential scanning calorimetry (DSC, Perkin Elmer, Germany). 5 mg of bulk cocoa butter and 10-15 mg of SLN dispersion were weighted in hermetic aluminium pans. The pans were sealed, placed in the DSC and the thermal analysis was started. A sealed empty hermetic pan was used as reference. The scan rate employed was 5°C/min for both heating and cooling in the temperature range from 0 to 65°C.

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### 5.3.6 Morphology measurements

The surface morphology of blank and EGCG-loaded SLNs was observed by freeze-fracture transmission electron microscopy (FF-TEM) (Klang, Matsko, Valenta, & Hofer, 2012; Zhou et al., 2009). The effect of the concentration of EGCG loaded into the SLNs was also examined using EGCG-SLNs samples with two different concentrations of EGCG (1% and 2%(w/w)).

For freeze-fracturing, the samples were first cryoprotected by mixing 2 mL of the sample with 1 mL of glycerol (87%, wt/wt), then cryofixed by immersion of small droplets into melting Freon 22 (-160°C). Specimens were freeze-fractured at -120°C with a BAL-TEC BAF 400 unit (Balzers, Liechtenstein). Freeze-fractured specimens were replicated by layering Pt/C and C by electron-gun evaporation. The replicas were cleaned in concentrated sodium hypochlorite and in acetone with repeated intermediate rinses in distilled water. Specimen replicas were examined with FEI Tecnai 10 (Eindhoven, Netherlands) TEM operating at 80 kV.

### 5.3.7 EGCG Stability study

The effect of the encapsulation of EGCG in the developed SLNs on its stability was studied by following the absolute amount of EGCG in the SLNs dispersion during the storage at 2°C. The results were compared with those obtained for two aqueous solutions containing the same initial content of EGCG. The first control solution was prepared by dissolving EGCG in distilled water. The second control solution was prepared by dissolving EGCG in distilled water containing the same amount of SSL, which was used in the preparation of SLNs and homogenized at the same production parameters. This control was done to evaluate the influence of SSL on the EGCG stability. Sampling was conducted in time intervals of 5 days. The total amount of EGCG in the SLNs dispersion was determined by extracting the encapsulated EGCG using the method mentioned above and the concentration was determined using an Agilent 1100 HPLC system with a quaternary pump and an UV-Vis detector. A CC 125/4 Nucleodur Sphinx RP, 5 µm column (Macherey Nagel, Germany) was used. A gradient twelve minutes method was applied using water (A) and acetonitril (B) acidified with 0,1 % formic acid (the following gradient steps were used: 1 min: 5% B; 10-10.50 min:95 %B; 11-12 min: 5%B). The HPLC conditions were: flow rate of 1 ml/min, injection volume of 10 µl, column temperature of 19°C and a detection wavelength of 280 nm. Analyses were conducted in triplicates.

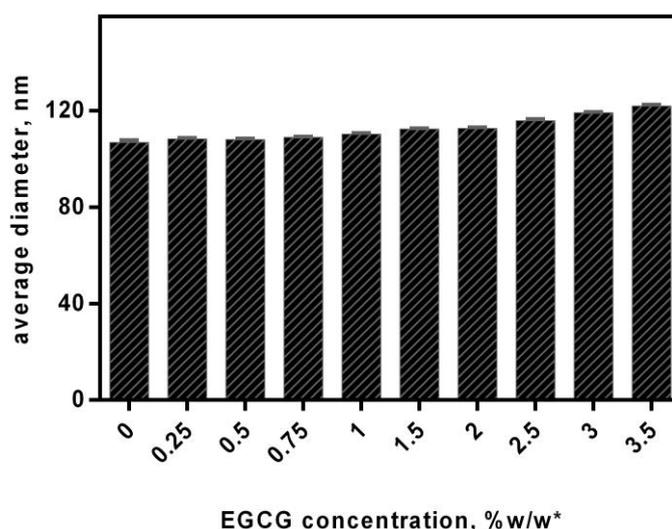
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## 5.4 Results and discussion

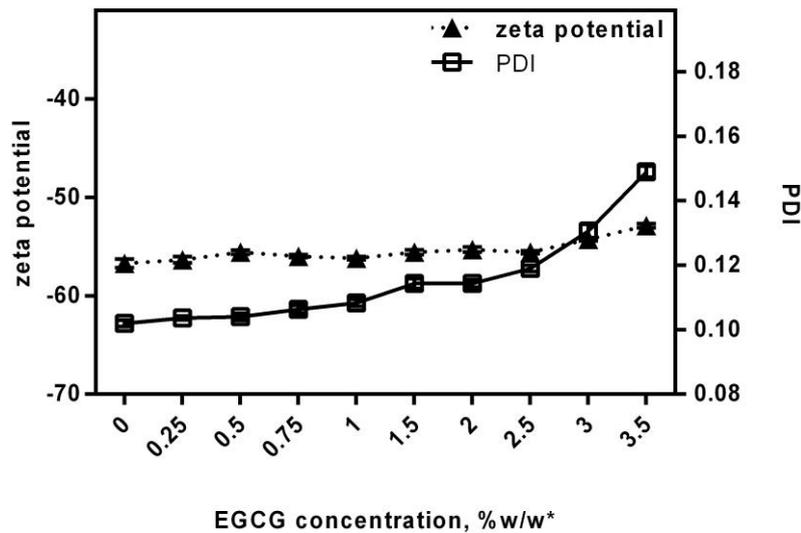
### 5.4.1 Particle size measurements

All SLNs dispersions obtained from this study showed a milky appearance. The colour differed from milky white for blank SLNs to light brown for EGCG-SLNs. The colour became darker with increasing the concentration of EGCG used in the preparation. No aggregation or phase separation was observed during the preparation. The average size, PDI and zeta potential of the SLNs dispersions are shown in the (Figures 5-1 and 5-2). An average diameter in the range of 106.8 to 120 nm (Figure 5-1) with monodisperse size characteristics (PDI: 0.102-0.149) was obtained. The zeta potential value was in the range of -56.7 to -52.9 mV (Figure 5-2). Up to 1% w/w of EGCG (related to cocoa butter concentration) was encapsulated in the SLNs without any significant increase in the particle size. Higher EGCG concentrations resulted in an increased particle size for the SLNs dispersion prepared using 3.5% w/w of EGCG, where the size increased about 14.2%.

The slight size increase of SLNs loaded with EGCG compared with unloaded SLNs is attributed to the incorporation of EGCG in the outer shell of the solid cocoa butter core of SLNs due to the high solubility of EGCG in water. This phenomenon is commonly associated with the drug-enriched shell model (Üner & Yener, 2007; Zur Muhlen, Schwarz, & Mehnert, 1998; Zur Mühlen, Annette and Mehnert, Wolfgang, 1998).



**Figure 5-1:** Average particle diameter of EGCG-loaded SLNs as a function of the EGCG concentration in %w/w\* related to the concentration of cocoa butter. All SLNs were prepared at 1000 bar for 5 homogenization cycles using 5% and 1.5% of cocoa butter and surfactant blend, respectively, n=3±STD.



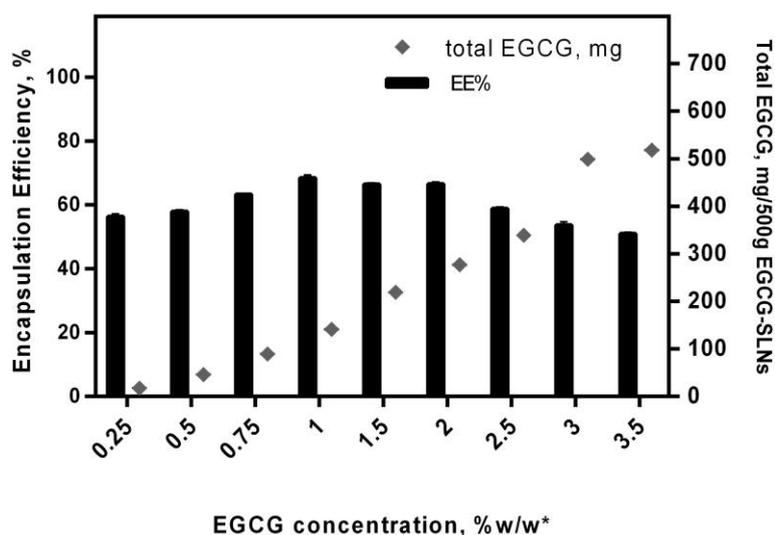
**Figure 5-2:** PDI and zeta potential of SLNs as a function of the EGCG concentration in %w/w\* related to the concentration of cocoa butter. All SLNs were prepared at 1000 bar for 5 homogenization cycles using 5% and 1.5% of cocoa butter and surfactant blend, respectively, n=3±STD.

EGCG-SLNs showed a relatively narrow size distribution and a high negative zeta potential value between -50 and -60mV indicating a high physical stability. This means, the electrical repulsion between the particles was high enough to prevent particle aggregation during the preparation steps (Bleve, Pavanetto, & Perugini, 2011; Freitas & Müller, 1998). However, a concentration of EGCG above 2.5% w/w led to a marked increase in the PDI. Similar observation was reported by Lin, Feng, Lai, Lin, & Chen (2014).

#### 5.4.2 Entrapment efficiency

The processing and analyzing of samples a and b had shown that nearly 20% and 6% of nonencapsulated EGCG were captured by the filter material or was left on the SLNs layer over the filter membrane, respectively. The calculated EE was between 50 and 70 % and increased with increasing the EGCG concentration from 0.25 to 1% w/w (Figure 5-3). A similar increase of the EE as a function of EGCG concentration was reported for peptides/chitosan nanoparticles (Hu, Ting, Zeng, & Huang, 2013). At 1% EGCG w/w, a saturation of the system was reached (EE: 68.5%). Similar EE was obtained for encapsulation of other hydrophilic compounds like Paromomycin in SLNs (Ghadiri et al., 2012). The use of higher EGCG concentrations (up to 2% w/w) resulted in a slight decrease in the EE. Further, higher EGCG concentration (>2% w/w) resulted in a significant decrease in the EE.

A reduction of the EE was also described for EGCG-SLNs with more than 3 % drug load (Frias, Neves, Pinheiro, & Reis, 2016; Radhakrishnan et al., 2016), whereby the EE reached was higher and ranged between 89 and 67 % for EGCG concentration of 3 and 5 %, respectively. This could be caused by the highly effective pharmaceutical grade surfactants used. In addition, it was suggested that a high EE could be caused by interactions of EGCG with the surface active lipids used (Frias et al., 2016). EGCG was found to bind and solubilize membrane phospholipids (Sun, Hung, Chen, Lee, & Huang, 2009). Such an interaction could also explain the observed initial increase of the EE in the present study, because an interaction is affinity driven and dependent on the ratio of the interacting compounds.



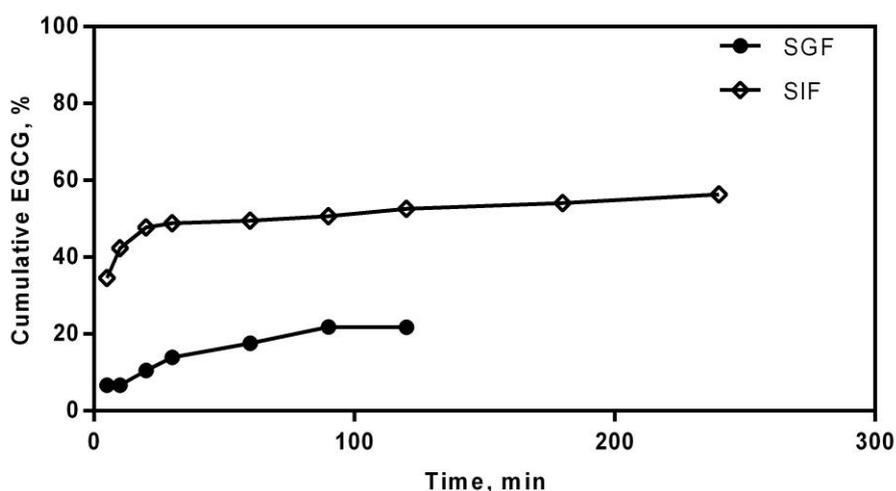
**Figure 5-3:** Entrapment efficiency and total EGCG content in EGCG-loaded SLNs as a function of the EGCG concentration in %w/w\* related to the concentration of cocoa butter, n=3±STD.

The decrease in the EE when the concentration of EGCG increased could be explained by the reduced available space within the lipid particles for EGCG to be entrapped. In this case, the unloaded EGCG moved to the aqueous phase due to its high water solubility (Kim, Na, & Choi, 2005).

### 5.4.3 *In-vitro* release profiles of EGCG-SLNs

The release profiles of EGCG from the nanoparticles under gastric (SGF) and intestinal (SIF) conditions during a period of two and four hours, respectively, are illustrated in (Figure 5-4). In these experiments, the SLNs dispersion used contained 1% w/w EGCG. Directly after the introducing of SLNs into SGF, a detectable aggregation was noted. The EGCG-SLNs sample showed a slight release of EGCG in SGF. Considering the initial value for release of 6.7% caused by nonencapsulated EGCG, approximately 15% of EGCG was released from EGCG-SLNs at the end of the incubation time, i.e. the total amount of free EGCG in SGF was 21.7%. However, the released amount was less than expected when comparing it with the EE obtained, which suggested that >30% of EGCG was located in the aqueous phase (Figure 5-3). This can be explained by the aggregation of the SLNs (pH 7) observed under the acidic condition in the SGF at pH 1.2. The aggregates partially prevented the free EGCG from the moving into the SGF medium and thus, a lower concentration of free EGCG was measured.

In contrast, a higher amount of EGCG was released during the incubation in SIF at pH 6.8. This might be due to the effects of bile salts and phospholipids present in SIF, which increased the solubility of encapsulated agents and, thus, caused a faster release of EGCG in the intestinal medium (Noack et al., 2012). The presence of pancreatin decreases the protection capacity of the nanoparticles as a result of the digestion of the lipid core under these conditions.



**Figure 5-4:** EGCG release (%) from loaded SLNs (1% w/w EGCG) under gastric conditions (pH=1.2, containing pepsin) and under intestinal conditions (pH=6.8, containing pancreatin) at 37°C, n=3±STD.

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The high initial concentration of released EGCG in SIF might be caused by the EGCG molecules located at the surface of the nanoparticles, which could freely diffuse and be in incessant contact with the release medium (Noack et al., 2012; Parhi & Suresh, 2010). About 34.6% of free EGCG was detected after 5 minutes in SIF. This value correlates well with the determined EE (Figure 5-3). After the first 5 minutes, a prolonged release over the incubation time was achieved. This was expected and indicates the release of encapsulated EGCG from the SLNs. This release is caused by the large surface area of the SLNs due to their small size in the nanometer range, the high diffusion coefficient of EGCG due to small molecular size, the low viscosity in the matrix and the short diffusion distance (i.e. release from outer surface region of the nanoparticle). 21.8% of encapsulated EGCG in SLNs was released at the end of the incubation in SIF, where a total amount of free EGCG (inclusive the initial release) of 56.4% was measured.

A drug-enriched shell model, as expected for the present EGCG-SLNs, also contributes to the observed release (Üner & Yener, 2007; Zur Muhlen et al., 1998; Zur Mühlen, Annette and Mehnert, Wolfgang, 1998). Furthermore, the fast release rate is also attributed to the liquid state of the cocoa butter core at the releasing temperature of 37°C and, therefore, the high mobility of EGCG in the lipid matrix (Kim et al., 2005). In addition, release by erosion of the nanoparticle shell is possible (Fangueiro et al., 2014). Complementarily, the stability of free EGCG was spectrophotometrically followed during the incubation in SIF and a linear decrease in the EGCG concentration of approximately 50% per hour was found. A degradation of 90% EGCG was also reported in PBS (pH 7.4, 37°C) after two hours incubation (Zhang, Nie, & Wang, 2013). In addition, the SIF was after 240 minutes incubation clear, which suggested that all components of SLNs were almost completely digested at this time point and the whole amount of EGCG released. This means the proportion of EGCG released from SLNs after 240 minutes is higher than 56.4% as the free EGCG is simultaneously decomposed in the SIF medium.

#### **5.4.4 Crystal modification of SLNs**

Table (5-1) illustrates the recorded DSC heating and cooling curves of bulk cocoa butter, blank SLNs dispersion (without EGCG) and EGCG-SLNs (1% and 2% w/w related to the cocoa butter). The heating curve of cocoa butter as bulk material revealed one distinct peak with a melting point at 20.7°C. This melting temperature can be attributed to the subcell  $\alpha$ -polymorphic form of cocoa butter (Manning & Dimick, 1985; van Malssen, van Langevelde, Peschar, & Schenk, 1999). In contrast, the nanoparticle samples displayed a broad melting curve with several distinct peaks. These differences can be explained by the colloidal nature

of the SLNs affecting some of the physicochemical properties, especially in terms of melting and recrystallization temperatures (Bunjes & Unruh, 2007). These very fine particles varying in size within the colloidal range behave individually and melt at different temperatures, which results in a stepwise melting process rather than a continuous event and, thus, shows multiple DSC melting profiles (Higami, Ueno, Segawa, Iwanami, & Sato, 2003) such as those observed in this study.

On the other hand, multiple peaks shown in the melting curves of SLNs indicated the presence of different crystal modifications. During the melting process, the first endothermic peak appeared around 19 °C was attributed to the metastable  $\alpha$ -polymorphic form of cocoa butter. The second peak was observed around 24 °C and corresponded to the  $\beta'$ -polymorphic form of cocoa butter (Manning & Dimick, 1985; van Malssen et al., 1999; Wille & Lutton, 1966). Therefore, it could be concluded that a disorganized crystalline structure was formed inside the particles. This is favorable in nanoparticle formation, since a microstructural disorganization results in the presence of voids. This increases the particle capacity to incorporate bioactive molecules. Moreover, disordered structure exhibits a reduced expulsion of encapsulated compounds, as well as a less aggregation and gelation during storage (Gomes, Simplicio, Souto, Cardoso, & Pinho, 2013). A comparison between the blank SLNs and EGCG-SLNs showed that the melting points of the EGCG-loaded SLNs were similar to those of the blank SLNs, since they were prepared using the same method and the particle size in these samples was in the same range as well (106-122 nm).

**Table 5-1:** Melting and recrystallization temperatures of EGCG-SLNs loaded with two different concentrations of EGCG.

Sample	DSC- Heating curve		DSC- Cooling curve	
	Melting peak	STD	Recrystallization peak	STD
<b>Bulk cocoa butter</b>	20.7	0.160	13.63	0.087
<b>Blank SLNs</b>	1. 18.80	0.075	4.2	0.025
	2. 24.10	0.040		
<b>1% w/w EGCG-loaded SLNs</b>	1. 18.44	0.070	4.20	0.015
	2. 23.87	0.050		
<b>2% w/w EGCG-loaded SLNs</b>	1. 19.05	0.055	4.21	0.021
	2. 23.50	0.091		

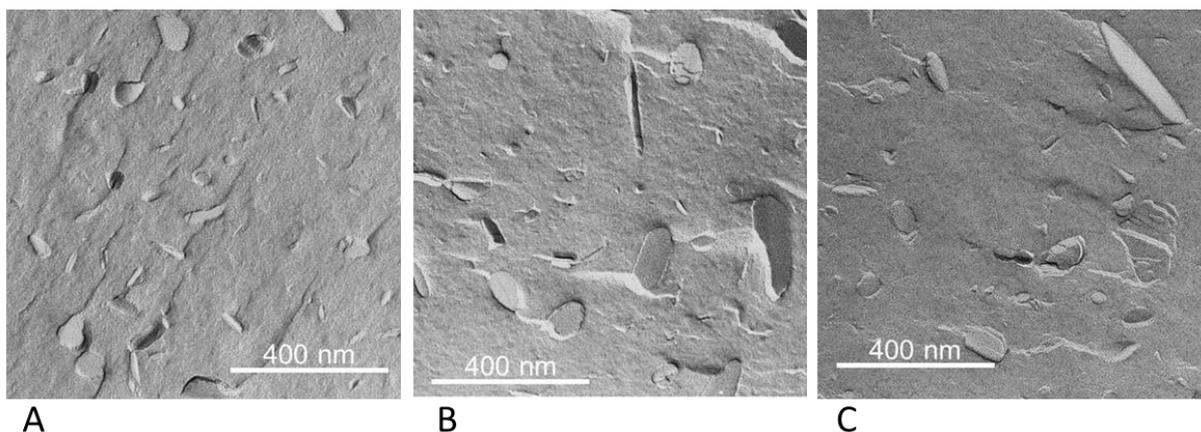
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Unlike melting transitions, the DSC cooling curve showed a single distinct exothermic peak. Compared to bulk cocoa butter, the recrystallization of SLNs occurred at a lower temperature, whereby the molten SLNs recrystallized approximately 9°C below the recrystallization temperature of bulk cocoa butter. The delay in recrystallization can be attributed to the small size effect (Bunjes, Koch, & Westesen, 2000; Bunjes & Unruh, 2007; Higami et al., 2003). Comparing the blank SLNs with the EGCG-SLNs, the single exothermic peak during the cooling did not vary. Furthermore, the surfactants used in the formation of SLNs system may, in general, induce a depression of the recrystallization point, which increases with increasing surfactant concentrations (Zur Mühlen, Annette and Mehnert, Wolfgang, 1998).

In conclusion, DSC results showed that the incorporation of EGCG did not significantly affect the melting/recrystallization profiles of the SLNs. This in turn may provide a confirmation of the above results, that the EGCG molecules located in the outer layer of the cocoa butter core and, therefore, did not alter the thermal properties. In contrast, when the compound is molecularly dispersed inside the lipid matrix of the particle core, a shift in the melting and/or recrystallization compared with the equivalent blank SLNs should be observed according to (Manea et al., 2014) and (Omwoyo et al., 2014).

#### **5.4.5 SLNs Morphology**

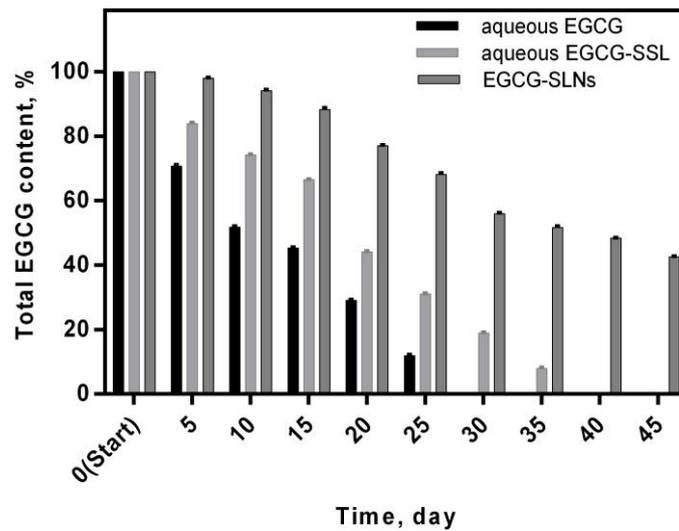
FF-TEM was used to observe the particle morphology of blank SLNs and EGCG-SLNs and also to check the presence of any aggregates. The TEM images (Figure 5-5) displayed that the SLNs dispersions, loaded and unloaded with EGCG, revealed nanoparticles of two shapes, spherical and oval/lamellar. The spherical shape related to the presence of  $\alpha$ -crystals (Weiss et al., 2008), whereas the oval/lamellar shape was attributed to the presence of  $\beta'$ -crystals when considering the melting profiles obtained by DSC. No aggregations were observed indicating that SLNs dispersions were stable. Furthermore, the TEM measurements showed that there was not any notable difference in the particle size and/or shape between blank and EGCG-SLNs independently of the EGCG concentration. This provided a confirmation of the results obtained by DLS.



**Figure 5-5:** TEM images of SLNs: Blank (A), loaded with 1% w/w EGCG (B), loaded with 1.5% w/w EGCG(C)

#### 5.4.6 Stability profile of EGCG in SLNs

To evaluate the storage stability of EGCG in the investigated nanoparticulate system, the total EGCG content in the EGCG-SLNs dispersion and that in an aqueous system of nonencapsulated EGCG was followed over a period of three months at 2°C. To clarify whether an improved stability of EGCG in the encapsulated form was achieved by the SLNs rather than the presence of surfactants, EGCG was added to another aqueous solution containing dispersed SSL and the total EGCG content in this medium (called aqueous EGCG-SSL) was in parallel followed during the storage experiment described above. The initial EGCG content was the same for all three systems (considered as 100%) and the pH value was 6.8. EGCG-SLNs sample used in this experiment was the one prepared with 1% w/w EGCG. The aqueous solution of SSL was first homogenized under the same conditions applied in this study in order to exclude any possible effects of the homogenization process on the obtained results. The calculated EGCG content for all three systems at every time point indicated the stability of EGCG.



**Figure 5-6:** EGCG stability in three different formulations (aqueous EGCG, aqueous EGCG-SSL and EGCG-SLNs containing 1% w/w EGCG) in water during 45 days at pH 6.8 and 2 °C in dark, n=3 STD.

The encapsulation of EGCG in SLNs has been shown to enhance the stability of EGCG against the degradation caused by the environmental factors (Figure 5-6). The EGCG content in the distilled water dropped rapidly during the storage time. At the time point (25 days), only about 12 % of EGCG was present in this medium. After 30 days, no EGCG could be detected. Other studies determined EGCG stability in PBS buffer (pH 7.4) at 4 °C of approximately 2 days (Zhang, Nie, Martinez-Zaguilan, Sennoune, & Wang, 2016). The higher stability of EGCG in the present study was attributed to the use of distilled water instead of the buffer. For the aqueous SSL-EGCG, the stability of EGCG was significantly higher compared to that obtained with the aqueous system. After 15 days, a phase separation was observed in this medium and the stability decreased from this time point significantly. Only about 8% of EGCG could be measured in this medium on the storage day 35. A possible explanation is that the SSL aggregates dispersed in the aqueous phase (Kurukji, Pichot, Spyropoulos, & Norton, 2013) provided a slight protection of the EGCG molecules involved therein and, thereby, an enhanced stability was achieved. After the phase separation, this medium behaved like the aqueous EGCG. The stability achieved with the EGCG-SLNs was clearly improved in comparison to the aqueous solution and SSL-dispersion. At the end point of the storage time (45 days), a total EGCG content of 42,6% was still found in this system.

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## **5.5 Conclusion**

EGCG-SLNs with a particle size of  $\leq 122$  nm and a narrow size distribution were successfully produced using a solvent-free high pressure homogenization technique. A relatively high EE was achieved. The results suggested that the encapsulated EGCG molecules were located in the outer shell of the particle core, which is to be expected when hydrophilic compounds, such as EGCG, are encapsulated in a lipid-based system. However, the investigated SLNs showed a good enhancement of the EGCG stability against the environmental factors. A considerably prolonged release of the encapsulated EGCG from SLNs under gastric and intestinal conditions could be proved. Thus, an improved bioavailability can be expected. These results indicate that the produced SLNs might be a suitable nanodelivery system to introduce and protect EGCG in food products. Further investigations of their compatibility with the food systems would be valuable.

## **Acknowledgements**

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## 6. Manuscript 3: Encapsulation of cholecalciferol in SLNs

### 6.1 Abstract

Solid lipid nanoparticles (SLNs) loaded with cholecalciferol (vitamin D<sub>3</sub>) were prepared by high pressure homogenization technique using cocoa butter for the particle core. The system was stabilized by a surfactant blend consisting of sodium stearyl-2-lactylate (SSL) and mono- and diglycerides of fatty acids (MDG). The high pressure homogenization was performed at 60°C applying 1000 bar for five cycles. The obtained nanosystem was cooled using a thermostat at 1°C and then characterized in terms of particle size and zeta potential using a Zetasizer, whereas the encapsulation efficiency (EE) was determined by the size exclusion chromatography (SEC). The long term stability was investigated by following the turbidity of the nanodispersion during an accelerated aging under centrifugal forces. In addition, a differential scanning calorimetry (DSC) was used to study the thermal profiles of the cholecalciferol-loaded SLNs, as well as to follow the stability of the system during a storage time of four months. The encapsulation of cholecalciferol did not affect the average size of the SLNs compared to the blank system, which was almost constant at approximately 112 nm. The EE achieved was in the range of 67.3-82.9%. The system showed a good long term stability, which was confirmed by the accelerated stability test and the DSC measurements.

### 6.2 Introduction

Cholecalciferol is one of the two major forms of the prohormone “vitamin D”, also known as vitamin D<sub>3</sub>. It is synthesized in the skin following the exposure to ultraviolet radiation (Eitenmiller et al. 2008; Ross 2011), or consumed in the diet from some animal-based foods. Cholecalciferol itself is biologically inactive. In the human body, it is converted to the metabolically active form of vitamin D, i. e., calcitriol through two enzymatic hydroxylation reactions in liver and kidney (Ross 2011). The calcitriol exhibits a number of actions including the regulation of serum calcium and phosphate homeostasis, which in turn maintains the bone health. This makes vitamin D essential for the prevention of rickets in children and osteomalacia in adults (Eitenmiller et al. 2008; Ward et al. 2007). Furthermore, calcitriol has shown some immunomodulatory and antiproliferative effects giving it a potential role in the prevention and/or therapy of cancers (Masuda and Jones 2006), type 1 diabetes (Harris 2005), cardiovascular disease and several infections (Ross 2011).

Due to the lipophilic nature of the dietary vitamin D (including cholecalciferol), it is absorbed in the small intestine (Haddad et al. 1993). The amount of dietary vitamin D is normally

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expressed in International Units (IU) or in microgram ( $\mu\text{g}$ ), where the biological activity of 1  $\mu\text{g}$  represents 40 IU (Ross 2011). The daily adequate intake (AI) of vitamin D has recently been defined by the EFSA with 15  $\mu\text{g}$  for healthy individuals > 1 year old (including pregnant and lactating women) and 10  $\mu\text{g}$  for infants aged 7-11 months (EFSA 2016). Unfortunately, the recommended daily intake of vitamin D is often not covered. On the one hand, vitamin D produced via sunlight is affected by some factors, such as northern latitude, clothes and the utilization of sunblock (Ward et al. 2007; Chesney 2003). On the other hand, naturally occurring food sources of vitamin D are few (e.g., fatty fish, fish liver oil and egg yolk) (Ross 2011). These limitations have led to the development of food products, which are fortified with vitamin D in order to face the health problems associated with the vitamin D deficiency. Such examples are: fortified milk and dairy products like yogurts and some cheeses, and also ready to eat cereals etc. (Jarvis 2010). However, vitamin D is very sensitive to oxidation, light- and heat-induced conformational changes, as well as attacks by free radicals. Such factors affect vitamin D and result in transformation products, which have less biological activity than vitamin D (Hollis 2008). The encapsulation of cholecalciferol offers an ideal solution for these drawbacks. Most innovative methods used for the encapsulation of sensitive bioactive compounds such as vitamin D are based on the formulation of nanoparticulate systems. Their efficiency to encapsulate cholecalciferol has been examined by several researchers. Related studies were based on whey protein isolate (Abbasi et al. 2014), alginate (Li et al. 2011); and maize protein (Luo et al. 2012). Additionally, the encapsulation of ergocalciferol in (SLNs) has been investigated by using solid lipid nanoparticles consisting of a tripalmitin core (Patel and San Martin-Gonzalez 2012).

In the present study, cholecalciferol was encapsulated in SLNs system, where the lipid core consisted of cocoa butter. The surfactant used was a mixture of sodium stearoyl-2-lactylate (SSL) and mono- and diglycerides of fatty acids (MDG). The preparation of the SLNs was performed by hot homogenization. A further focus of this study was to investigate the long term stability of the SLNs in the presence and absence of cholecalciferol. This was achieved by following the accelerated creaming during centrifugation. In addition, the stability of cholecalciferol-loaded SLNs was controlled using the DSC analysis during the storage period.

## **6.3 Materials and methods**

### **6.3.1 Materials**

Cholecalciferol (Vitamin D<sub>3</sub>) of a pharmaceutical secondary standard was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Cocoa butter in small disc shaped

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chips was purchased from Pati-Versand.de (Herzlake, Germany). The surfactant blend used was a mixture of sodium stearyl-2-lactylate (SSL, E481) and mono- and diglycerides of fatty acids (MDG, E471), respectively, which were kindly a gift from Palsgaard® (Juelsminde, Denmark). Methanol, acetonitrile (both of HPLC-Grade  $\geq 99.9\%$ ), and absolute ethanol ( $\geq 99.8\%$ ) and chloroform (UV/IR-Grade  $\geq 99.8\%$ ) were purchased from Carl Roth (Karlsruhe, Germany).

### **6.3.2 Preparation of cholecalciferol-loaded SLNs**

SLNs using 1.5% of surfactants and 5% of cocoa butter were prepared by hot homogenization (Mehnert and Mäder 2001; Sagis 2015). 0, 0.1, 0.2 and 0.4 %w/w of cholecalciferol (related to the cocoa butter fraction) was used in the encapsulation. Briefly, 524.5  $\mu\text{l}$  of 50, 100 and 200 mg/ml of cholecalciferol in ethanol was added to the melted cocoa butter at 60°C containing DMG. The required amount of water, containing SSL and having the same temperature, was added to the lipophilic phase. Next, the total mixture was stirred using an Ultra Turrax (Ystral, D-79282, Ballrechten, Dottingen, Germany) for 3 min at 19000 rpm. The obtained pre-emulsion was homogenized using a high pressure homogenizer (Niro Soavi S.P.A., NS100 1L 2K, S N.4810, Parma, Italy) at 1000 bar for five homogenization cycles yielding a hot O/W nanoemulsion. The hot nanoemulsion was cooled at 1°C in a thermostat for 1 hour in order to solidify the core of obtained nanoparticles. All formulations were stored in the refrigerator at  $-2^\circ\text{C}$  until the next day for analysis.

### **6.3.3 Characterization of cholecalciferol-loaded SLNs**

#### **6.3.3.1 Particle size and zeta potential measurements**

The particle size, PDI and zeta potential of SLNs prepared in this work were assessed by dynamic light scattering (DLS) using the Zetasizer Nano-ZS (Malvern Instruments, USA) at 25°C (refractive index 1.54, 0.8872 cp). Samples were diluted 1:200 times using distilled water and directly measured. All samples were conducted in triplicate.

#### **6.3.3.2 Total cholecalciferol content**

The total content (TC) of cholecalciferol was determined using an Agilent 1100 HPLC system with a quaternary pump and an UV-Vis detector at 265 nm wavelength. A CC 250/4 Nucleodur C18 (5  $\mu\text{m}$ ) column (Macherey Nagel, Germany) was used. The isocratic mobile

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phase consisted of 90:10 v/v methanol/acetonitrile; both in HPLC grade, acidified with 0.1 % formic acid. The HPLC conditions were as follows: a flow rate of 1.3 ml/min, an injection volume of 20  $\mu$ l, a column temperature of 20°C and a detection wavelength of 265 nm. Determinations were conducted in triplicate.

### 6.3.3.3 Cholecalciferol entrapment efficiency

The entrapment efficiency (EE) of cholecalciferol was determined by size exclusion chromatography (SEC) on crosslinked dextran using the mini column centrifugation based on the method of (Fry et al. 1978). 5 ml syringes were filled with the dextran slurry and centrifuged at 1000 rcf for 3 minutes to remove the excess water. 500  $\mu$ l of diluted samples of cholecalciferol-loaded SLNs were pipetted onto the column and the syringes were again centrifuged under the same conditions in order to separate the encapsulated from the nonencapsulated cholecalciferol. The supernatant collected after the second centrifugation was analyzed as described above to determine the concentration of the encapsulated cholecalciferol. The encapsulation efficiency was calculated using the following equation:

$$EE, \% = \frac{\text{Encapsulated cholecalciferol}}{TC} \times 100 \quad (I)$$

### 6.3.4 Accelerated stability test

The measurement of the turbidity of the emulsions has shown to be a useful tool for the assessment of the long term stability of the system. While the investigated system is normally let for some time for aging before its turbidity is measured, the presented SLNs were subjected to centrifugal forces in order to accelerate the aging process. SLNs with and without cholecalciferol were diluted with distilled water 1:1. Then, 1.4 ml of the dilution was filled in a disposable cuvette and the turbidity was directly measured spectrophotometrically (Spectrophotometer, Helios Gamma UV-Vis, Thermo Spectronic, Cambridge, UK) at 450 and 850 nm. All samples were subjected to centrifugation (Allegra X-30R centrifuge, Beckman Coulter, Germany) at 4000 rcf and 25°C. The absorbance of all samples was measured at 450 and 850 nm in time intervals of 30 minutes during a period of two hours. All measurements were conducted in triplicate. The results are reported in terms of the stability index (R-value), which is determined from the following equation (Kaufman and Garti 2007; Song et al. 2002):

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$$R - value = \frac{\text{Absorbance at 850 nm}}{\text{Absorbance at 450 nm}} \quad (II)$$

### 6.3.5 Thermal measurements of cholecalciferol-loaded SLNs

The DSC measurements were done for SLNs loaded with 0.1, 0.2, 0.4%w/w of cholecalciferol in order to investigate the effect of the existence and the loading concentration of cholecalciferol on the thermal profiles of the SLNs. Furthermore, the thermal measurements of the SLNs dispersion loaded with 0.4%w/w of cholecalciferol were assessed with the DSC over a storage time of four months. The EE of this dispersion was also parallelly checked as described before. For the measurement, approximately 10 mg of the SLNs dispersion was placed in hermetic aluminum pans (Tzero), which were then tightly sealed. An empty sealed pan was used as a reference. The pans were put into the calorimeter (DSC-Q2000, TA instruments, Germany) and heated up to 100°C at a scan rate of 5°C/min and subsequently cooled down to -10°C at the same rate.

### 6.3.6 Statistical analysis

All experiments were done in triplicate and the results obtained from the measurements were analyzed by the statistical software GraphPad Prism (version 6, San Diego, USA) using analysis of variance ANOVA. Tukey's multiple comparisons test was applied at the significant level of  $p < 0.05$ .

## 6.4 Results and discussion

### 6.4.1 Particle size and zeta potential

The encapsulation of cholecalciferol into the SLNs in the range of 0.1-0.4%w/w did not significantly affect the particle size, PDI or zeta potential (Table 5-1). All these parameters remained constant, which reflects that no aggregation occurred due to the presence of cholecalciferol molecules in the SLNs system. In the literature, the encapsulation of 5 to 20% ergocalciferol in SLNs using a comparable production approach resulted in a linear decrease of the particle size with increasing the concentration of ergocalciferol (Patel und San Martin-Gonzalez 2012). This was explained by means of a decreased viscosity of the dispersed lipid phase due to the plasticizing effect of ergocalciferol. A good plasticizing effect was also described for cholecalciferol (Kangarlou i wsp. 2008). However, such influence of the

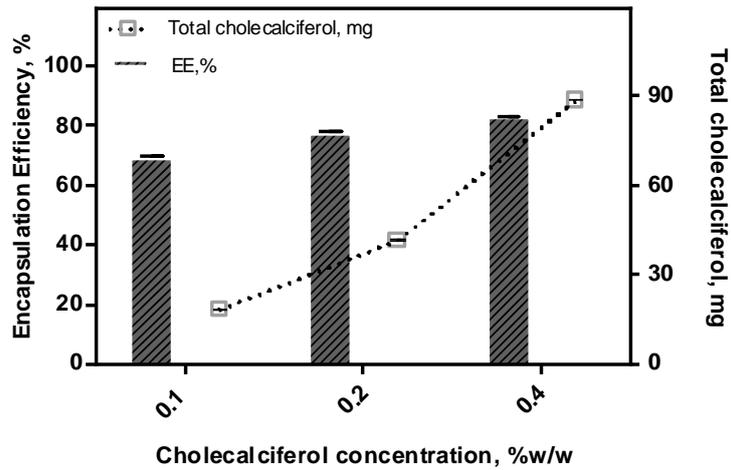
increased concentration of cholecalciferol on the particle size was not observed in the present study. This is probably due to the low concentration of the cholecalciferol used here. Constant particle size and zeta potential further suggest that cholecalciferol was encapsulated within the crystallized lipid core and not on the particle surface, which might have increased the particle size and altered the zeta potential.

**Table 6-1:** Average size, PDI and zeta potential of SLNs, unloaded and loaded with different concentrations of cholecalciferol, as a function of the cholecalciferol concentration in %w/w\* related to the concentration of cocoa butter. All SLNs were prepared at 1000 bar for 5 homogenization cycles using 5% and 1.5% of cocoa butter and surfactant blend, respectively, n=3±STD.

Cholecalciferol concentration (%w/w)	Average size, nm		PDI		Zeta potential	
	Mean value	STD	Mean value	STD	Mean value	STD
0	112.40	0.557	0.112	0.0010	-56.9	0.265
0.1	112.43	0.306	0.112	0.0010	-56.3	0.361
0.2	112.33	0.252	0.113	0.0015	-56.7	0.153
0.4	112.50	0.265	0.112	0.0017	-56.8	0.208

#### 6.4.2 Cholecalciferol content and entrapment efficiency

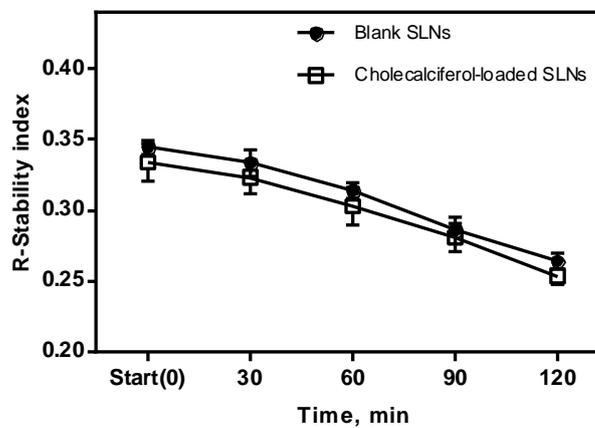
The total cholecalciferol content as well as the EE increased significantly with increasing the concentration of cholecalciferol used for the encapsulation (Figure 6-1). This means, the voids involved in the crystal structure of the particle core were not completely filled with cholecalciferol molecules and the encapsulation of higher concentration was still possible. The EE obtained was high and ranged between 67.3 and 82.9%. Moreover, the constant particle size obtained from DLS measurements suggested that the cholecalciferol molecules mostly existed within the particle core rather than adsorbed on the particle surface.



**Figure 6-1:** Entrapment efficiency and total cholecalciferol content of cholecalciferol-loaded SLNs as a function of the cholecalciferol concentration in %w/w\* related to the concentration of cocoa butter used for the preparation of 500g of SLN formulation, n=3±STD.

#### 6.4.3 Stability index of unloaded and cholecalciferol-loaded SLNs

(Figure 6-2) shows the results of the spectral absorbance of the diluted SLNs at 850 nm and 450 nm during the accelerated creaming in terms of the calculated R-value.



**Figure 6-2:** The change of the stability index of blank SLNs and SLNs loaded with 0.4% w/W of cholecalciferol as a function of the centrifugation time, n=3±STD.

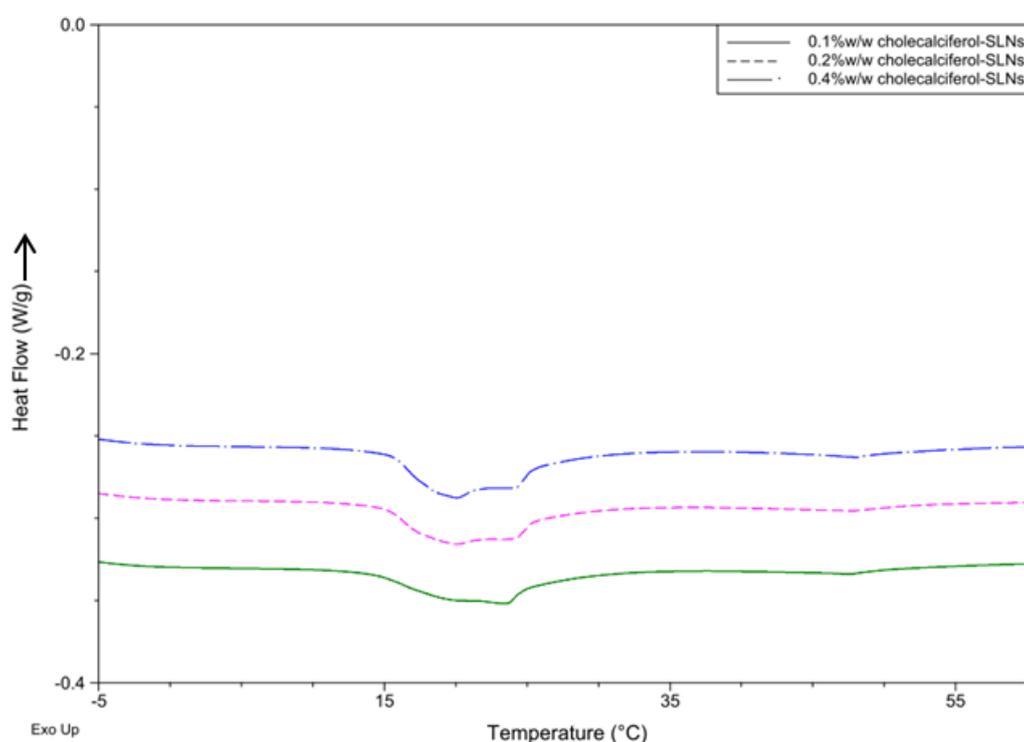
SLNs, unloaded and loaded with cholecalciferol, exhibited a low initial value of the stability index, which correlates with a high creaming stability of the system (Kaufman and Garti 2007). The results are also in accordance with the low PDI findings from DLS, since the stability index correlates with the particle size distribution, whereby the intensity of light scattering increases with decreasing particle diameter (Kaufman and Garti 2007; Song et al.

2002). This means, the lower the initial stability index, the more stable is the system reflecting a narrow range of small particles. Furthermore, the R-value for both SLNs dispersion decreased linearly as a function of the centrifugation time. The explanation for such decrease is, that the total particle concentration in the samples became lower with the time as a result of the breaking process of the dispersion (occurrence of creaming) (Kaufman and Garti 2007; Song et al. 2002). Thus, the slow decrease of the R-value for both samples indicated a good long term stability of the System (Song et al. 2002). A slightly lower initial R-value for the system contained cholecalciferol was observed (not statistically significant).

#### 6.4.4 Crystal modification of cholecalciferol-loaded SLNs

DSC measurements were conducted for SLNs loaded with cholecalciferol (Figure 6-3 and 6-4).

##### 6.4.4.1 Effect of cholecalciferol loading concentration



**Figure 6-3:** DSC heating curves of SLNs as a function of the loading concentration of cholecalciferol.

The heating curves recorded for SLNs with 0.1, 0.2, 0.4%w/w cholecalciferol (Figure 6-3) showed a melting event in the range of 18.9 to 24.3°C indicating a mixed structure of  $\alpha$ - and

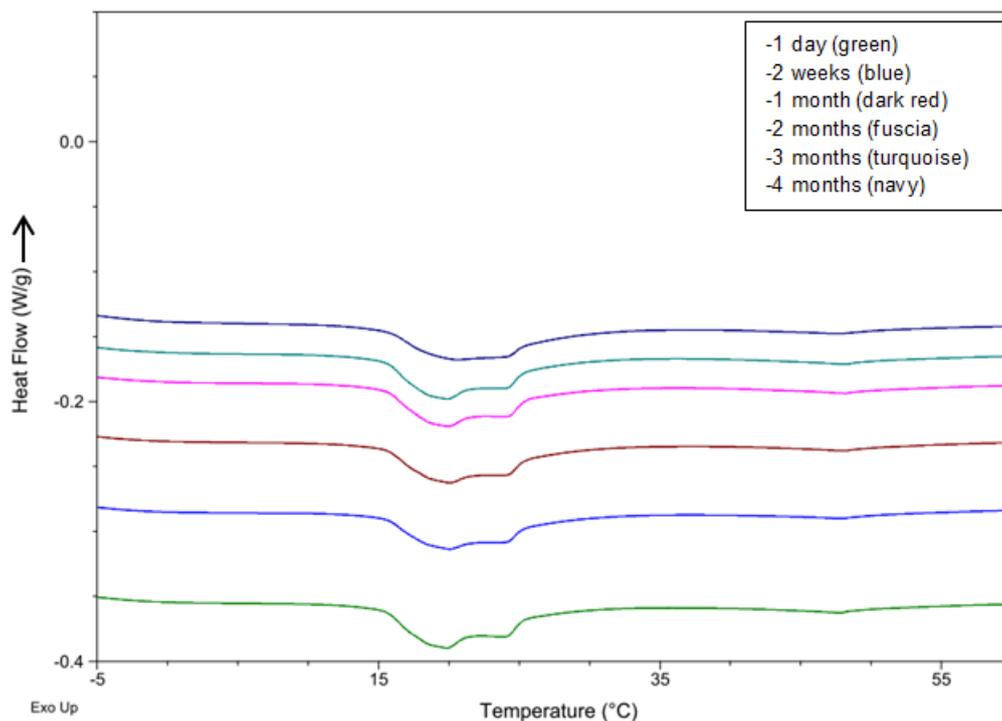
$\beta'$ -sub-cell forms (Manning and Dimick 1985; Wille and Lutton 1966). No significant differences were observed in the melting range with increasing the loading concentration of cholecalciferol. However, the higher the loading concentration applied, the more observable was the peak of the  $\alpha$ -modification (around 18.9°C), which is an indication for an increased encapsulation capacity for the bioactive compound (Mehnert und Mäder 2001; Müller et al. 2000). Furthermore, when comparing the melting enthalpies of these three dispersions as well as of the equivalent blank SLNs (Table 6-2), it was shown that the melting enthalpy increased gradually with increasing the loading concentration of cholecalciferol. These results suggested that the existence of cholecalciferol enhanced the disorganization of the crystal structure of cocoa butter core, which resulted in a higher extent of the  $\alpha$ -modification and, thus, a higher amount of cholecalciferol was retained in the SLNs. This was similar to other findings by Patel and San Martin-Gonzalez (2012) and Siekmann and Westesen (1994).

**Table 6-2:** Melting enthalpy of 0.4%w/w cholecalciferol loaded SLNs as a function of the loading concentration of cholecalciferol, n=3±STD

<b>Cholecalciferol, %w/w</b>	<b>Melting enthalpy, J/g</b>	<b>STD</b>
<b>0</b>	1.8	0.097
<b>0.1</b>	2.0	0.108
<b>0.2</b>	2.2	0.113
<b>0.4</b>	2.5	0.093

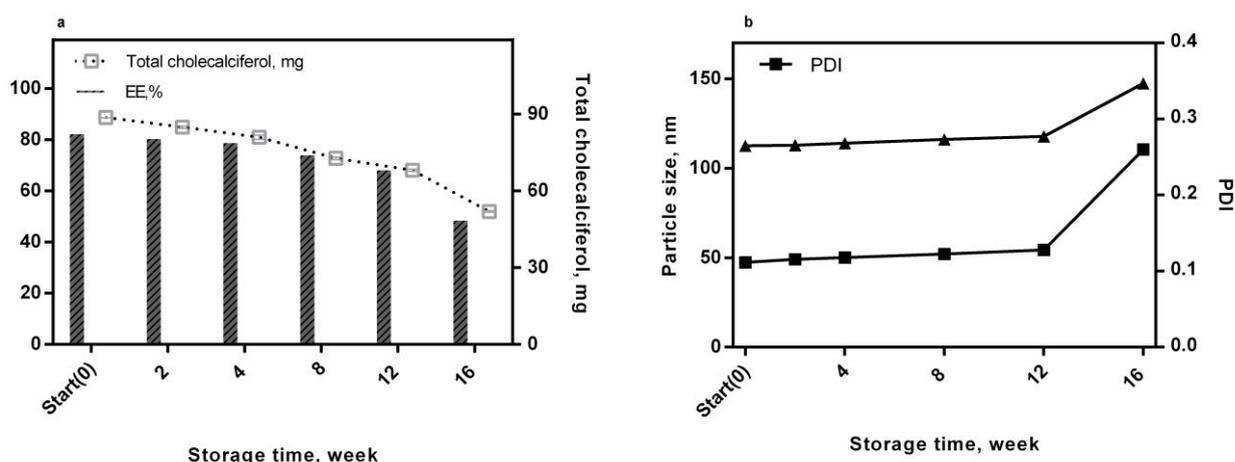
#### **6.4.4.2 Effect of storage time**

In order to investigate the stability of cholecalciferol-loaded SLNs, DSC analysis was performed for the nanodispersion loaded with 0.4%w/w cholecalciferol during a storage time of four months. In addition, TC, EE and particle size characteristics were also followed.



**Figure 6-4:** DSC heating curves of SLNs loaded with 0.4%w/w of cholecalciferol as a function of the storage time.

Figure (6-4) shows that up to three months, almost no changes were observed in the thermal profile of the system. After four months, the thermal profile changed significantly, which was reflected by the reduced extent of  $\alpha$ -modification. These measurements were coupled with the determination of the TC and EE as described before (Figure 6-5).



**Figure 6-5:** Entrapment efficiency and total content of encapsulated cholecalciferol (a) as well as particles size and PDI of SLNs loaded with 0.4%w/w of cholecalciferol as a function of the storage time (b).

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The results of TC and EE were similar to those obtained from the DSC analysis confirming that the system was good stable for three months. The disruption of the dispersion observed after four months could be attributed to the polymorphic transition of the lipid core, where the unstable crystal forms recrystallized in the next more stable form in the order ( $\alpha \rightarrow \beta' \rightarrow \beta$ ) (Qian et al. 2012; Weiss et al. 2008). Although no melting peak for the  $\beta$ -modification was observed in this study, the reduction in the peak of  $\alpha$ -modification was very clear. These polymorphic changes lead to the expulsion of cholecalciferol from the SLNs (Müller et al. 2000). Particle size analysis conducted during the storage time showed no significant changes up to three months (Figure 5-5). After four months, the particle size increased significantly ( $147.36 \pm 0.34$ ;  $PDI=0.26 \pm 0.002$ ). This supports again the results before, since the polymorphic transition into the stable form is correlated with the occurrence of a breaking process, i.e., aggregation. Helgason et al. (2008) observed that the needle-like shaped crystals of the  $\beta$ -form are bigger than the spherical ones and, thus, the resulted new surfaces could not be completely covered by the surfactant molecules leading to the aggregation of the system.

## 6.5 Conclusion

This study revealed that the incorporation of cholecalciferol within the SLNs prepared from cocoa butter by high pressure homogenization technique did not affect the particle size properties. High encapsulation efficiencies up to 82.9% were achieved indicating an appropriate crystal structure of the lipid core to retain lipophilic compounds like cholecalciferol. The accelerated stability test confirmed a good long term stability of the system with or without cholecalciferol. Furthermore, the results obtained from the DSC analysis showed that the cholecalciferol-loaded SLNs were stable for three months. The EE was nearly constant during this time period, whereby no increase in the particle size was observed. In conclusion, it should be kept in mind, that the investigated cholecalciferol concentrations were relatively low. Thus, it would make sense to try higher concentrations in order to get more information on the highest EE that could be achieved and the behavior of the system at this level of encapsulation. This includes particle size properties and crystal structure, since these characteristics eventually determine the overall system functionality and/or stability.

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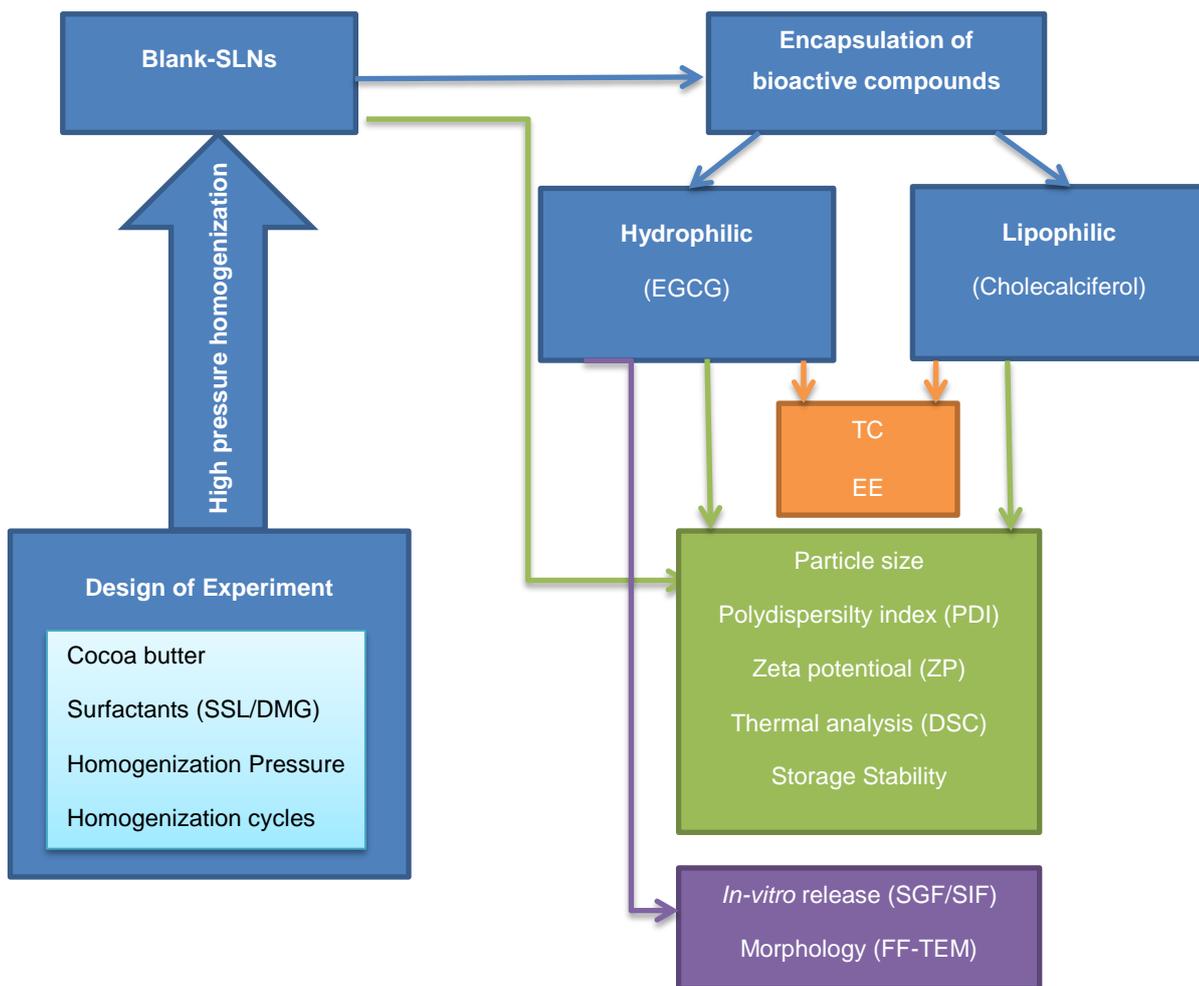
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## 7. General discussion

The approach of the present work focused on the development of a new SLNs system, which could have potential use in the production of functional foods. This involved 1) the optimization of the different preparation variables, 2) the encapsulation of hydrophilic and lipophilic active compounds, 3) the physiochemical characterization and stability testing of loaded and unloaded SLNs (**Figure 7-1**).



**Figure 7-1:** Schematic representation of the overall approach of the present thesis.

The particular aspect of the present work was to develop and demonstrate a new system of SLNs, which is especially designed for uses in the food industry. Several production and analysis techniques have been employed in order to achieve this aim.

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## 7.1 Influence of preparation variables on SLNs characteristics

### 7.1.1 Cocoa butter and surfactant blend

The present thesis was based on the hypothesis (a), that cocoa butter with its individual composition of triacylglycerides exhibits potential to be used as a particle core of the developed SLNs prepared by the high pressure homogenization technique. Some earlier works in this area (Kim et al. 2005; Qian et al. 2013) have used the cocoa butter for the development of SLNs. Although, the present work aimed at preparing SLNs which are applicable in the food sector by using food-grade raw materials and applying a suitable preparation process without using any solvents. Furthermore, all preparation conditions were studied and precisely defined by a full factorial design. In manuscript 1, the hypothesis (a) was checked and the results obtained were discussed. It has been demonstrated that cocoa butter with its melting point close to body temperature showed a good suitability to be used in the preparation of the SLNs. This was of a great interest, since SLNs are normally prepared from lipid components, which should have a melting temperature above the body temperature (Attama et al. 2012; Jennings et al. 2002). The processing of cocoa butter into SLNs resulted in a mixture of the less stable crystal modifications, i.e.  $\alpha$  and  $\beta'$ , which was investigated using the DSC measurements (**Chapter 4.4.4, Figure 4-9**). The FF-TEM images confirmed that the nanoparticles were a mixture of two shapes; spherical and oval/lamellar indicating the creation of  $\alpha$ - and  $\beta'$ -modifications, respectively (**Figure 5-5,A**).

The surfactant blend of SSL and MDG used in the preparation of the SLNs may contribute to the crystal structure of the cocoa butter core obtained. SSL is known as lactic acid-esterified monoglyceride. This mixture of mono- and diglycerides effectively determined the crystallization behavior of SLNs. Monoglycerides have been shown to promote the nucleation of the  $\alpha$ -crystals (Fredrick et al. 2008), whereas the presence of diglycerides is combined with an induced generation of  $\beta'$ -crystals (Long et al. 2006). Furthermore, the surfactant blend allowed the obtaining of a monodisperse system with an adequate minimum particle size and a high negative zeta potential value (**Figures 4-1 to 4-4, Table 4-3**) and, thus, a good system stability during the storage time (**Figure 4-10**). This confirmed the successful selection of a new food-grade surfactant blend to stabilize the developed SLNs system (hypothesis b), whereas the surfactants commonly used for the preparation of SLNs and emulsions are polysorbates, phospholipids, poloxamers, bile salts, etc. (Bhushan 2014; Torchilin 2014).

### 7.1.2 Homogenization pressure and number of homogenization cycles

The applied production technique used in the present work was the hot homogenization at a temperature of 60 °C. This technique is very favorable because of the easy scale up and the

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short production time. No solvents were used in the preparation of SLNs, giving this work an additional value for application in the food systems. The particle size and the PDI of SLNs decreased significantly when the number of homogenization cycles and/or the homogenization pressure was increased. This desired effect, however, was pronounced up to five homogenization cycles (**Figures 4-3 and 4-4**) and/or 1000 bar (**Figures 4-1 and 4-2**). The explanation of such observations is the combined effect of increased energy input, cavitation, shear forces as well as an enhanced fluid velocity, which resulted in an improved disruption of the particles (Floury et al. 2000; Datta und Tomasula 2015). The particle breakup has reached its limit at the homogenization values discribed above. The zeta potential value remained < -50 in all formulations.

The utilization of a two-level full factorial design has allowed the optimization of the composition variables including the concentrations of cocoa butter and surfactant blend, as well as the process variables including the homogenization pressure and the number of homogenization cycles (hypothesis b). These were set at 5% of cocoa butter, 1.5% of surfactant blend, 1000 bar and five cycle numbers. The SLNs dispersion obtained under these conditions had an average size of 112.7 nm with a PDI of 0.112 and a zeta potential of -56.9 mV indicating a very good quality and stability of the developed system (**Table 4-3**).

### **7.1.3 Cooling rate**

The cooling rate of the SLNs dispersion prepared by the hot-high pressure homogenization is another important factor, which greatly affects the characteristics of the SLNs. This factor is mostly ignored when determining the optimal conditions for the preparation of SLNs. In this work, it has been proven that different cooling rates resulted in significantly different properties of the nanoparticles (hypothesis d). The cooling of SLNs formulations at 1 °C using a water bath (thermostat) has shown the most appropriate properties of the nanodispersions (**Table 4-2**). The other tested cooling temperatures were 2, 5, 10°C and the room temperature. This was explained by the induced formation of  $\alpha$ - and  $\beta'$ -crystals under faster cooling. Furthermore, the fraction of  $\alpha$ - crystal has shown to increase at higher cooling rates (Weiss et al. 2008). However, when SLNs formulation were cooled at lower temperatures (< 0°C), particle size as well as PDI increased to unsuitable values, i.e. >400 nm and >0.5, respectively. Zeta potential of these formulations was >-30 mV indicating an induced aggregation (**Chapter 4.4.2.3**). The induced brittleness of lipids under such conditions has been considered to be the reason behind these undesirable changes (Torchilin 2014).

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## 7.2 EE for encapsulated compounds

### 7.2.1 EGCG

SLNs as nanodelivery systems were first developed for the encapsulation and delivery of hydrophobic compounds being entrapped in the solid hydrophobic core. Later, researchs have shown that also hydrophilic compounds could be encapsulated in SLNs (Attama et al. 2012; Patidar et al. 2010; Tiwari et al. 2015). The results of manuscript 2 of the present thesis have supported this fact again by a successful encapsulation of EGCG in the developed SLNs with an EE of 68.48% (**Figure 5-3**). Similar EE was reported for other hydrophilic compounds (Paromomycin) (Ghadiri et al. 2012b). The stability study of EGCG in the encapsulated form (EGCG-SLNs) has demonstrated that EGCG was not only entrapped by the SSL layer, but was also; at least partially, encapsulated in the lipid phase (**Figure 5-6**). In this experiment, the protection of EGCG provided by the SSL molecules was almost lost on the day 35, whereas a total EGCG content of >40% was still found in the EGCG-SLNs system. A good explanation for the successful encapsulation of such high water-soluble compound in the SLNs core was given in the literature (Aboubakar et al. 1999). The adsorption of the surfactant molecules at the surface of the oily droplets was rapid enough, forming a protection film around the droplets. This mechanism limited the diffusion of the EGCG molecules from the droplet towards the aqueous phase, which is associated with the drug-enriched shell model expected for the EGCG-SLNs. Therefore, a higher entrapment has been achieved. In addition, the preparation method was applied (**Chapter 5.3.2**), has combined the high pressure homogenization technique with utilizing the principle of the double emulsion technique (w/o/w). This may contribute to the successful encapsulation of EGCG in SLNs. However, the formation of a w/o/w nanoemulsion in this study could not be confirmed by the morphological analysis using the FF-TEM.

### 7.2.2 Cholecalciferol

The use of comparable loading concentrations of cholecalciferol resulted in significantly higher entrapment efficiencies than those obtained for EGCG (**Figures 6-1 and 5-3**) (hypothesis e). This is generally related to the high solubility of such lipophilic compounds in the oily core (Fresta et al. 1996). The EE continuously increased when increasing the loading concentration of cholecalciferol (**Figure 6-1**). This indicated an appropriate crystal structure ( $\alpha$ - and  $\beta'$ -subcell) of the cocoa butter core containing enough voids to be filled with more cholecalciferol molecules. The DSC melting curves (**Figure 6-3**) have shown that the

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incorporation of cholecalciferol resulted in an additional disorganization in the crystal structure of the cocoa butter core, which is reflected by a higher extent of the  $\alpha$ -modification. This observation was considered a positive response in this study.

### 7.3 Stability of SLNs

In the present thesis, a good stability of the developed SLNs system was hypothesized (c). This could be demonstrated in terms of the particle size characteristics, the retention of the encapsulated EGCG and cholecalciferol in the system and the *in-vitro* release of the EGCG from the nanoparticles.

#### 7.3.1 Physical stability

The evaluation of the physical stability was performed by monitoring the particle size, size distribution and zeta potential of the system over time under defined conditions.

Blank SLNs dispersions (pH value of nearly 7) obtained from the designed experiments of different initial particle size were stored at 2°C for three months (**Figure 4-10**). For all samples, no aggregation, creaming or gravitational separation of the system was observed during the storage time. The particles of the initial size 112.7 nm exhibited stable size and PDI, whereas a slight increase in both values was observed in the SLNs of a higher initial size. This was in agreement with other findings by (Floury et al. 2000; Tadros et al. 2004; McClements 2011) confirming that the creation of nanoparticles with the smallest possible initial size is very important, in order to achieve an improved shelf life stability. However, the zeta potential of all samples was almost stable during the storage period. These results proved that the surfactant blend of SSL and MDG used for the production of SLNs was effective enough to keep the system stable against any breakdown (hypothesis c). Furthermore, when EGCG or cholecalciferol was incorporated within the system, the particle size characteristics were not significantly changed and no breaking process happened. This demonstrated that these compounds did not exert any negative effect on the physical stability of the SLNs.

In addition, the long term stability of the system containing and/or without cholecalciferol was investigated using the measurement of the turbidity. This measurement was based on the spectral absorbance of a diluted sample at two wavelengths, i.e. 450 and 850 nm. Commonly, the changes of the turbidity of emulsions and similar systems; such as SLNs, as a response of the aging process, is taken as a tool to judge the long term stability of the system. In the present work, the aging process was accelerated by subjecting the samples to

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centrifugal forces. The turbidity as a rate of the absorbance at 850 to that at 450 nm (R-value) was monitored along two hours. The system, with and without cholecalciferol, exhibited a low initial R-value indicating a narrow size distribution (Kaufman and Garti 2007; Song et al. 2002). Moreover, a good long term stability was suggested by the slow reduction of the R-value as a function of the centrifugation time (**Figure 6-2**). This was associated with a slow creaming rate of the system. Overall, these experiments confirmed again the good stabilizing capacity of the surfactant blend as it was hypothesized (c).

### **7.3.2 Retention of encapsulated compounds in the system**

It was hypothesized (e) in section 1 that the stability of EGCG against degradation is improved by the encapsulation in the developed SLNs. This was confirmed by comparing the stability of EGCG in the encapsulated form (EGCG-SLNs) with that in an aqueous EGCG-solution and in a homogenized aqueous solution of SSL (EGCG-SSL). The total EGCG content was followed in these three systems during a storage time of 45 days at 2°C. The stability of EGCG in SLNs was clearly improved compared to the other two solutions (**Figure 5-6**). The reduction in the total drug content is attributed with means of chemical degradation during the storage. The slight initial reduction of the content of EGCG in the SLNs system was mainly caused by the degradation of the nonencapsulated EGCG presented in the aqueous phase of the SLNs dispersion. The further reduction of the content of EGCG in this system was related to the amount of EGCG captured by the SSL layer, which could be observed by the following of the reduction of the content in the aqueous EGCG-SSL system. However, a simultaneous, but slight diffusion of the encapsulated EGCG from the SLNs could also have been happened from the beginning of the storage time up to measuring. From the day 35, where almost no protection of the EGCG by the SSL layer was possible, the EGCG content in the SLNs system indicated the encapsulated EGCG, which is still retained in the SLNs. From this time point, only a slight reduction of the content of EGCG was recorded until the end of the investigated storage time, where 42.6% of EGCG could still be determined.

It has been shown that the DSC analysis of an emulsion at different times may give valuable information about the stability of the system. The changes in the cooling or heating curves are correlated with the occurrence of a breaking process caused by the aggregation or the polymorphic transitions. Polymorphic changes of SLNs systems are combined with the expulsion of encapsulated compounds (Mehnert and Mäder 2001). For cholecalciferol-loaded SLNs, a storage stability experiment was conducted by monitoring the thermal measurements during a storage time in order to observe any changes of the temperature or enthalpy, which

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could indicate some system instability. SLNs dispersion loaded with 0.4%w/w of cholecalciferol was stored at 2°C for four months and the thermal profiles were recorded by the DSC (**Figure 6-4**). Simultaneously, the TC and EE were also determined as described in the manuscript 3 (**Chapter 6.3.3.2 and 6.3.3.3**). Up to 1 month no significant changes in the melting profile were observed, whereas a little change could be recorded at the time point of two and three months. The melting curve obtained after four months changed clearly, where the extent of the  $\alpha$ -modification became significantly low. When comparing these changes with those of the TC and EE, up to 1 month no significant changes were observed in these values. After three months the TC and EE decreased obviously but were still about 76.6% and 70%, respectively. At the end of the storage time, the EE dropped significantly to reach 48.7%. This reduction was caused by the polymorphic transition of the crystal structure, which is combined with the expulsion of the encapsulated molecules. Although no aggregation was seen at this time point, a significant increase in the particle size and PDI was observed. Thus, it was concluded that the general stability of cholecalciferol in the SLNs system could be evaluated using the DSC measurement. The reduction in the TC indicated a decomposition of the free cholecalciferol due to its sensitivity against the environmental factors (Petritz et al. 2006).

### 7.3.3 Stability during the *in-vitro* tests

An important advantage suggested for the SLNs systems is the sustained release of the encapsulated compounds (Attama and Umeyor 2015; Zur Mühlen and Mehnert 1998). The release kinetics of the SLNs is influenced by the localization of the encapsulated compound, which is in turn affected by the composition and the preparation method of the nanocarrier (Fanun 2010; Couvreur et al. 2002).

In the present thesis an improved stability of EGCG encapsulated in the SLNs under gastrointestinal conditions was hypothesized (e). This was checked by the implementation of *in-vitro* release experiments for EGCG-SLNs in SGF (pH=1.2, over two hours) and SIF (pH=6.8, over four hours) containing the relevant enzymes; pepsin and pancreatin, respectively. A maximal burst release of 6.7% and 34.6% in SGF and SIF, respectively, was observed (**Figure 5-4**). However, from this time point a sustained release of the EGCG was achieved under both gastric and intestinal conditions (hypothesis e). This was of high importance, especially in terms of protecting the EGCG molecules against the biodegradation under intestinal conditions due to the high instability of the EGCG in neutral and alkaline medium (Kanwar et al. 2012). The high burst release was explained by different mechanisms (Üner and Yener 2007). On the one hand, the large surface area associated with the nanosized particles increases the diffusion rate of the encapsulated compounds

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towards the release medium. On the other hand, the drug-enriched shell model suggested for the EGCG-SLNs system also influenced the burst release due to the short diffusion distance, since the EGCG molecules were encapsulated in the outer layer of the particle core rather than the center. Moreover, the liquid state of the cocoa butter core under the release temperature of 37°C and, hence, the high mobility of the encapsulated EGCG associated was an important factor being considered when explaining the increased release of EGCG from the system. The simultaneous decomposition of EGCG released from the SLNs during the incubation in the SIF medium resulted in a lower release value being determined.

#### **7.4 Outlook and final remarks**

The overall results obtained from the experiments of the presented work demonstrated the good stability of the SLNs system prepared using the cocoa butter. The developed SLNs were suitable to successfully encapsulate EGCG and cholecalciferol molecules with a high EE and without any negative effects on the system stability. The use of a food-grade surfactant blend consisting of SSL and MDG gave the system a special importance to be introduced in food products.

Since the developed nanoparticles were not tested for their compatibility with food products by the presented work, this would be the very next task of the further works. For this purpose, it might be easier and more effective to apply these SLNs in a lyophilized form rather than using them in the actual dispersed form in an aqueous phase. This procedure would offer several advantages including improved long term stability as a result of the considerably reduced water content (Alihosseini et al. 2015; del Pozo-Rodríguez et al. 2009). However, the conditions applied during the lyophilization process should be controlled in order to avoid any undesirable change to the crystal structure of the cocoa butter core. The particle size obtained in this work (108-122 nm) makes the SLNs not suitable to be introduced into clear food systems. For instance, the introduction of cholecalciferol-loaded SLNs into low-fat milk and dairy products; like yogurts, or other unclear food systems like chocolate drinks, etc. would be a means enriching such products with vitamin D (cholecalciferol).

EGCG and cholecalciferol were selected to examine the suitability of the developed system for the encapsulation of hydrophilic and lipophilic compounds, respectively. Thus, other hydrophilic and lipophilic compounds could, in principle, be successfully encapsulated in this system. For instance, the encapsulation of flavour molecules would offer many advantages, including the protection of sensitive and highly volatile flavours against the degradation caused by environmental factors and processing conditions during the food production and

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the storage period, as well (Madene et al. 2006). This is of great importance for the food industry, since the flavour compounds are generally expensive and using them in a high amount mostly affects the quality of the food. Since the cocoa butter melts already at the body temperature, thus, a sustained release of the flavour compound would occur in the mouth giving this system an additional value for such applications. Flavours encapsulated in SLNs could be effectively employed in the manufacturing of products like candies, chewing gums, etc.

Furthermore, the introduction of the SLNs within a food system must be followed by an adequate sensory evaluation. This is especially important when a new food system is developed, since the sensory properties determine the final product acceptance by the consumers. Finally, it should be kept in mind that the presented SLNs from cocoa butter are suitable to be introduced in such food systems having a temperature lower than the melting point of the SLNs, which was in the range of 18 to 24°C (DSC melting profiles, **Table 5-1 and Figure 6-3**).

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## 9. Summary

Solid lipid nanoparticles (SLNs) were developed using food grade materials, consisting of the cocoa butter core and the surfactant blend of sodium stearyl-2-lactylate (SSL) and mono- and diglycerides of fatty acids (MDG), in order to be suitable for applications in food systems. The preparation procedure was carried out using the high pressure homogenization technique and the optimal production variables were defined using the full factorial design. The effect of the cooling rate applied to the system directly after the homogenization in order to solidify the particle core showed a significant effect. EGCG and cholecalciferol were successfully incorporated within the SLNs

The average size of SLNs obtained from the presented studies ranged between 108.3 and 122 nm combining a good long term stability for both blank and loaded nanoparticles. No significant effects of the encapsulated compounds on the particle size, PDI or zeta potential were shown. The DSC measurements revealed that the crystal structure of the cocoa butter core was a mixture of  $\alpha$ - and  $\beta'$ -modification. This was of critical importance for the developed system suggesting a high suitability for the encapsulation of bioactive compounds. Confirming results were obtained by the high encapsulation efficiency for EGCG and cholecalciferol. Further results showed an improved stability of the EGCG against degradation caused by environmental factors or induced under the simulated digestion conditions applied during the *in-vitro* release experiments. The represented SLNs system retained a considerable recovery for EGCG and cholecalciferol over six and twelve weeks, respectively.

Conclusively, the present work demonstrated that the developed SLNs are suitable to be used for the delivery of EGCG and cholecalciferol into food products.

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## 10. Zusammenfassung

Feste Lipidnanopartikel (Englisch: Solid lipid nanoparticles (SLNs)) wurden aus lebensmitteltauglichen Materialien bestehend aus Kakaobutter und der Tensidmischung von Natriumsteaoryl-2-lacylate (SSL) und Mono- und Diglyceride von Speisefettsäuren (MDG) entwickelt, um für die Anwendung in Lebensmittelsystemen geeignet einsetzbar zu sein. Die Herstellung erfolgte durch das Hochdruckhomogenisierungsverfahren und die optimalen Variablen wurden unter Einsatz eines vollfaktoriellen Versuchsplans festgelegt. Die Abkühlgeschwindigkeit der direkt nach der Homogenisierung resultierten Nanoemulsion, um das Kristallisieren der Partikel-Kern zu ermöglichen, zeigte einen signifikanten Effekt. EGCG und Cholecalciferol wurden in den SLNs erfolgreich verkapselt.

Die durchschnittliche Größe der durch die vorgelegte Arbeit hergestellten festen Lipidnanopartikel lag zwischen 108.3 und 122 nm, wobei eine langfristige Stabilität für unbeladene und beladene Nanopartikel gezeigt wurde. Keine signifikanten Effekte der verkapselten bioaktiven Substanzen auf die Partikelgröße, PDI oder Zeta Potential wurden gemerkt. Laut der DSC Messung, die Kristallinstruktur der Partikel-Kern war eine Mischung von  $\alpha$ - und  $\beta$ '-Modifikation. Dies war von großer Bedeutung für das entwickelte System hinsichtlich der Verkapselung bioaktiver Substanzen, was durch die erzielten Ergebnisse höher Verkapselungseffizienz für EGCG und Cholecalciferol bestätigt wurde. Weitere Ergebnisse zeigten eine verbesserte Stabilität von EGCG gegen Abbau durch Umweltfaktoren oder während in-vitro Verdau tests. Das entwickelte System wies eine hohe Beibehaltung für EGCG und Cholecalciferol über sechs bzw. zwölf Wochen auf.

Zusammengefasst bewiesen die Ergebnisse dieser Arbeit, dass das vorgestellte SLNs-System geeignet für die Anwendung zur Lieferung von EGCG und Cholecalciferol in Lebensmittelsystemen ist.