Nanoemulsions as delivery systems of food ingredients: Improving food safety and functionality

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Dipòsit Legal: L.412-2014
http://hdl.handle.net/10803/134733

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Doctoral Thesis
Lleida, 2014

The present project has been carried out in the pilot plant and laboratory of the research unit of Novel Technologies for Food Processing in the Food Technology Department of the University of Lleida under the supervision of Prof. Olga Martín Belloso and Prof. Robert Soliva Fortuny. The current work is part of the project 'Development of nanostructured edible coatings as carriers of active compounds' (AGL2009-11475) funded by the 'Ministerio de Ciencia e Innovación'.

The fellow has received funding to carry out the PhD studies from the following entities:

- March 2009 - December 2009: University Doctoral Grant UdL-Competitive Projects, University of Lleida.
- January 2010 - September 2010: Catalan Government Doctoral Grant FI-DGR 2010, Generalitat de Catalunya, Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR).
- October 2010 - March 2013: Spanish Government Doctural Grant FPU, Ministerio de Ciencia e Innovación.

The study of the bioactive nanoemulsions containing β-carotene was carried out in the laboratory of Food Biopolymers and Colloids in the Food Science Department of the University of Massachusetts, in the United States, under the supervision of Prof. D. Julian McClements. The fellow received an internship grant from the 'Ministerio de Ciencia e Innovación' to study abroad.

Acknowledgements-Agradecimientos-Agraïments

En primer lugar quiero agradecer a mi directora, la Prof. Olga Martín Belloso, todo el apoyo que he recibido durante la realización de mi tesis. Desde que fue mi profesora en la carrera, se forjó una buena relación profesional, y me ilusionó con la idea de hacer la tesis. Todavía me acuerdo de sus palabras cuando yo le pregunté qué iba a representar para mi tener una tesis doctoral. Me dijo que terminaría dominando el método científico, y que ese espíritu crítico lo aplicaría en todos los ámbitos de mi vida, tanto profesional como personal. Y fue así. Durante todos estos años he madurado y aprendido mucho sobre ciencia, el mundo académico y sobre mi misma. Gracias Olga.

En segon lloc al meu altre director de tesi, el Dr. Robert Soliva Fortuny. Gràcies pels teus valuosos consells i l'acompanyament en aquest procés d'aprenentatge. Ets un investigador brillant a qui admiro profundament.

También a la Dra. M. Alejandra Rojas Graü, que para mi ha ejercido un papel fundamental en la realización de esta tesis. Ale, de ti he aprendido a ser perseverante y tenaz. Pero sobretodo valoro tu apoyo y tu amistad en todo momento.

A todos mis compañeros del grupo de Nuevas Tecnologías por compartir tantísimos momentos juntos. En especial a Mariana, que es mi gran amiga y con quien compartí mis inicios en el mundo de la ciencia. Aunque ahora haya mucha distancia entre nosotras, siempre has estado cerca de alguna manera. También a Alejandra, con quien he compartido nano-experimentos y una buena amistad. A la Blanca, una genial companya d'oficina. También al resto de compañeros de laboratorio así como las personas que han venido de estancia. También a Gemma, Magda y Manel por la ayuda técnica durante la parte experimental.

I also would like to thank Prof. D. Julian McClements for receiving me in his research unit at the University of Massachusetts and for the great guidance. I had an absolutely positive and rewarding experience. Also many thanks to all my lab mates at UMass for facilitating my work in the lab and helping me at any time.

I am also deeply thankful to Karin Winter and her family for hosting me in Amherst, for being caring, supporting and encouraging. You are an extraordinary woman from who I learnt so much.

I also would like to thank Krzysiek, who has been my great friend and strong support during the end of my PhD. Now it's your turn to graduate. I will support you as well.

Als meus amics. Sobretot a la Blanca, per seves paraules d'ànim en tot moment. Però no menys especialment a la resta, Maria, Lorena, Núria, Lluís, Seró, Alex, per fer que els caps de setmana valguin la pena. També a tota la resta d'amistats que han contribuït d'alguna manera en ajudar-me a arribar fins aquí i per tots el moments compartits. Especialment al Ruben, qui ha estat el meu company durant molts anys, gràcies per tot el teu suport i els bons moments que hem viscuts plegats.

I finalment, he d'agrair a la meva família tota l'ajuda i recolzament durant tots aquests anys. Pare, sense tu jo no hagués arribat fins aquí. T'estic agraïda de tot cor per l'esforç i sacrifici que sé que has fet per mi, i per guiar-me i donar-me suport en tot moment. Sempre m'has ajudat a prendre decisions valentes i m'has recolzat a seguir aquest camí, gràcies per estar sempre a la meva vora. A la Gemma, per ser la millor germana del món. I per últim, a la meva mare, a qui trobo a faltar cada dia, però que sento a prop a la vegada. Gràcies per donar-me els valors dels quals avui disposo. Els duré amb mi sempre.

Nanoemulsions as delivery systems of food ingredients: Improving food safety and functionality

Summary

Consumers are currently demanding safe and healthy foods as similar to the natural as possible, free from synthetic additives. For this reason, there is a growing interest among both the scientific and industrial community regarding the design of new delivery systems of food ingredients to optimize their functionality. In this sense, nanoemulsions may be excellent carriers of lipophilic active compounds in food products. Therefore, the aim of the present work was to unravel the factors affecting the physicochemical characteristics and the functionality of nanoemulsions containing antimicrobials (essential oils, EOs) or bioactive compounds (β-carotene).

Nanoemulsions containing lemongrass essential oil (LEO) with a droplet size below 10 nm were obtained by ultrasonication (100 μm for 180 s) or microfluidization (150 MPa for 3 cycles) treatments. Despite ultrasounds were effective to obtain nanoemulsions with a small droplet size, they negatively affected the antimicrobial activity of essential oils. On the contrary, nanoemulsions produced by microfluidization exhibited a faster and higher antimicrobial activity than the respective conventional emulsions at the same EO concentration. Moreover, the EO and surfactant concentrations mainly determined the emulsion droplet size. Even though the antimicrobial activity of nanoemulsions was mostly defined by the LEO concentration, the addition of stabilizing agents, such as Tween 80 or sodium alginate, improved the dispersion of EOs, thus enhancing the antimicrobial properties. Regarding the type of EO, lemongrass, clove, thyme or palmarosa-loaded nanoemulsions exhibited the highest antimicrobial activity. Furthermore, the use of antimicrobial nanoemulsions in the formulation of edible coatings effectively maintained the quality of fresh-cut Fuji apples during 2 weeks of storage time. In this regard, edible coatings formed from nanoemulsions exhibited a faster and greater inactivation of *Escherichia coli* though storage compared with conventional emulsions. Also, nanoemulsion-based edible coatings with LEO at 0.5% or 1% (v/v) completely inhibited the natural microbiota of fresh-cut *Fuji* apples for 2 weeks.

Regarding the bioactive nanoemulsions, the emulsion droplet size, lipid carrier composition and concentration significantly influenced their biological fate under simulated digestion conditions. In fact, a strong relationship was observed between the oil digestibility and the β-carotene bioaccessibility. On one hand, the smaller the droplet size, the higher the oil digestibility and the higher the β-carotene bioaccessibility. In this sense, the β-carotene bioaccessibility increased from 34% in emulsions with large droplet size up to 59% in nanoemulsions with small droplet size. On the other hand, increasing the content of long chain triglycerides (LCT) and decreasing the medium chain triglycerides (MCT) in the lipid phase of nanoemulsions increased the oil digestibility and, in turn, the β-carotene bioaccessibility. Nevertheless, nanoemulsions with high MCT content showed enhanced β-carotene bioaccessibility after increasing the concentration of the lipid phase up to 4% (w/w).

The results obtained in the present work evidence that the antimicrobial activity or the β-carotene bioaccessibility could be modulated by controlling the factors affecting nanoemulsions properties. Therefore, the current research represents a new insight to the incorporation of active ingredients in food products, towards a more rationalized and optimized use of food additives.

Nanoemulsiones como sistemas de liberación de ingredientes alimentarios: mejora de la seguridad y funcionalidad de los alimentos

Resumen

Actualmente, los consumidores piden productos seguros y saludables lo más parecidos posible a los naturales, libres de aditivos sintéticos. Por este motivo, existe un creciente interés por parte de la comunidad industrial y científica por el diseño de nuevos sistemas de liberación de ingredientes alimentarios para la optimización de su funcionalidad. Las nanoemulsiones pueden ser excelentes portadores de compuestos activos lipófilos en los alimentos. Así, el objetivo del presente trabajo fue estudiar los factores que afectan las propiedades físico-químicas y de funcionalidad de nanoemulsiones conteniendo antimicrobianos (aceites esenciales, AEs) o compuestos bioactivos (β-caroteno).

Se obtuvieron nanoemulsiones de AE de citronela con tamaños de partícula inferiores a los 10 nm mediante ultrasonidos (100 μm durante 180 s) o microfluidización (150 MPa durante 3 ciclos). A pesar de que los ultrasonidos fueron efectivos para la formación de nanoemulsiones con tamaño de partícula en el rango nanométrico, afectaron negativamente a la actividad antimicrobiana de los aceites esenciales. Por el contrario, las nanoemulsiones producidas mediante microfluidización presentaron una mayor y más rápida inactivación microbiana en comparación con las emulsiones convencionales con la misma concentración de AEs. Además, las concentraciones de AE y surfactante fueron las que determinaron principalmente el tamaño de partícula de las nanoemulsiones. Aunque la concentración de AEs en la formulación determinó en mayor parte la a actividad antimicrobiana de las nanoemulsiones, la adición de agentes estabilizantes, como Tween 80 o alginato de sodio, mejoraron la dispersión de los AEs, mejorando sus propiedades antimicrobianas. Con respecto al tipo de AEs, las nanoemulsiones conteniendo AE de citronela, clavo, tomillo o palmarosa presentaron la mayor actividad antimicrobiana. Además, el uso de nanoemulsiones antimicrobianas como recubrimientos comestibles mantuvieron la calidad de manzana Fuji fresca cortada durante 2 semanas. Asimismo, los recubrimientos comestibles formados a partir de nanoemulsiones mostraron una mayor y más rápida inactivación de *Escherichia coli* durante el tiempo de almacenamiento en comparación con los recubrimientos formados

a partir de emulsiones convencionales. Por otro lado, recubrimientos comestibles formados a partir de nanoemulsiones conteniendo AE de citronela a 0,5% o 1% (v/v) inhibieron completamente el crecimiento de la microbiota natural de las manzanas frescas cortadas durante 2 semanas.

Con respecto a las nanoemulsiones con compuestos bioactivos, el tamaño de partícula así como la composición y concentración de la fase lipídica fueron factores que afectaron significativamente a su comportamiento bajo condiciones de digestión *in vitro*. De hecho, se observó una relación directa entre la digestibilidad del aceite y la bioaccesibilidad del β-caroteno. En este sentido, la bioaccesibilidad del β-caroteno incrementó del 34%, tras la digestión *in vitro* de las emulsiones con mayor tamaño de partícula, al 59% en las nanoemulsiones con tamaño de partícula nanométrico. Por otro lado, un incremento en el contenido de triglicéridos de cadena larga y un descenso en el de triglicéridos de cadena media en la fase lipídica de las nanoemulsiones incrementó la digestibilidad del aceite y, a su vez, en la bioaccesibilidad del β-caroteno. Además, un aumento en la concentración de aceite de hasta el 4% (w/w) en las nanoemulsiones con un elevado contenido de triglicéridos de cadena media en la fase lipídica, mejoró significativamente la bioaccesibilidad del β-caroteno.

Los resultados obtenidos en el presente trabajo evidencian la posibilidad de modular la actividad antimicrobiana o la bioaccesibilidad del β-caroteno mediante el control de los factores que afectan las propiedades de las nanoemulsiones. De este modo, la presente tesis doctoral representa un punto de partida para mejorar la incorporación de ingredientes en los alimentos, hacia un uso más racional y optimizado de los aditivos alimentarios.

Nanoemulsions com a sistemes d'alliberament d'ingredients alimentaris: millora de la seguretat i funcionalitat dels aliments

Resum

Actualment, els consumidors demanen aliments segurs i saludables el més semblants possible als naturals, evitant l'ús d'additius sintètics. Per aquest motiu, hi ha un interès creixent per part de la comunitat industrial i científica pel disseny de nous sistemes d'alliberament d'ingredients alimentaris que permetin optimitzar-ne la funcionalitat. Les nanoemulsions poden ser excel·lents portadors de compostos actius lipòfils en els aliments. Així, l'objectiu del present treball fou estudiar els factors que afecten les propietats fisicoquímiques i de funcionalitat de les nanoemulsions com a portadores d'antimicrobians (olis essencials, OE) o compostos bioactius (β-carotè).

Es van obtenir nanoemulsions d'OE de citronel·la amb una mida de partícula inferior als 10 nm a través de l'aplicació d'ultrasons (100 μm durant 180 s) o de microfluidització (150 MPa durant 3 cicles). Malgrat que els ultrasons foren efectius per a la formació de nanoemulsions amb una mida de partícula en el rang nanomètric, van afectar negativament a l'activitat antimicrobiana dels OEs. En canvi, les nanoemulsions obtingudes per microfluidització van presentar un major i més ràpida inactivació microbiana en comparació amb emulsions convencionals amb la mateixa concentració d'OE. A més, les concentracions d'OE i surfactant foren les que van determinar principalment la mida de partícula de les nanoemulsions. Tot i que l'activitat antimicrobiana de les nanoemulsions fou determinada en major mesura per la concentració d'OE en la formulació, la incorporació d'agents estabilitzants, com el Tween 80 o l'alginat de sodi, va millorar la dispersió dels OEs, i per tant les seves propietats antimicrobianes. En relació al tipus d'OE, les nanoemulsions amb OE de citronel·la, clau, timó o palma-rosa foren les que van presentar una activitat antimicrobiana més elevada. D'altra banda, l'ús de nanoemulsions com a recobriments comestibles va permetre mantenir la qualitat de poma Fuji fresca tallada durant 2 setmanes. Tanmateix, els recobriments comestibles formats a partir de nanoemulsions van mostrar una major i més ràpida inactivació d'*Escherichia coli* durant el temps d'emmagatzematge en comparació amb els recobriments formats a partir d'emulsions convencionals. A més, els recobriments comestibles formats a partir de nanoemulsions

amb OE de citronel·la al 0,5% o 1% (v/v), van inhibir completament el creixement de la microbiota natural de les pomes fresques tallades durant 2 setmanes.

Pel que fa a les nanomeulsions amb compostos bioactius, la mida de partícula així com la composició i concentració de la fase lipídica foren factors que van afectar significativament el seu comportament durant la digestió *in vitro*. De fet, es va trobar una relació directa entre la digestibilitat de l'oli i la bioaccessibilitat del β-carotè. En aquest sentit, la bioaccessibilitat del β-carotè va incrementar-se del 34%, després de la digestió *in vitro* de les emulsions amb una mida de partícula més gran, al 59% en aquelles nanoemulsions amb una mida de partícula nanomètrica. D'altra banda, un major contingut en triglicèrids de cadena llarga i un menor contingut en triglicèrids de cadena mitja en la fase lipídica de les nanoemulsions va augmentar la digestibilitat de l'oli, i a la vegada la bioaccessibilitat del β-carotè. A més, un increment en la concentració d'oli de fins al 4% (w/w) en les nanoemulsions amb un elevat contingut en triglicèrids de cadena mitja en la fase lipídica va millorar significativament la bioaccessibilitat del β-carotè.

Els resultats obtinguts en el present treball evidencien que es poden modular l'activitat antimicrobiana i la bioaccessibilitat del β-carotè controlant els factors que afecten les propietats de les nanoemulsions. Per tant, la present tesi doctoral representa un punt de partida per a la incorporació d'ingredients en els aliments de manera més eficient, per a un ús més racional i òptim dels additius alimentaris.

TABLE OF CONTENTS

INTRODUCTION

INTRODUCTION

Review

Edible nanoemulsions as carriers of active ingredients: a review

Laura Salvia-Trujillo, Robert Soliva-Fortuny, M. Alejandra Rojas-Graü, D. Julian McClements, Olga Martín-Belloso

Comprehensive Reviews of Food Science and Food Safety (submitted)

Abstract

There is a rapid increase of the interest on edible nanoemulsions as delivery systems of food ingredients due to their unique properties. Oil-in-water nanoemulsions are systems consisting on lipid droplets, with sizes below 100 nm, dispersed within a continuous aqueous phase. In this sense, nanoemulsions can act as carriers of lipophilic active substances, such as antimicrobials or health promoting compounds, to be delivered in foods. Their small droplet size results in a high active surface area, which confers them a high reactivity with biological cells and macromolecules. Therefore, in the case of antimicrobial loaded nanoemulsions they might be able to inactivate food-borne pathogens in a high extent. On the other hand, nanoemulsions containing bioactive compounds are expected to increase their digestibility and bioavailability. However, little is know about the effective implications of using functional nanoemulsions compared with emulsions of a larger droplet size. In this review, we aim to go through the most important factors affecting the formulation of nanoemulsions as well as to review the main fabrication methods and characterization techniques. Moreover, an overview of the most significant studies is presented, reporting concluding data to elucidate their real benefits over conventional emulsions.

Keywords

Nanoemulsions, lipid nanoparticles, delivery, carrier, food ingredients

Table of contents

1 Introduction

Nowadays, consumers are demanding safe and healthy food products but they require them to be as natural as possible. So, new trends in the food market are leading towards a more rational use of food ingredients, such as preservatives or bioactive compounds, minimizing the concentration of synthetic additives or even replacing them with natural substances. To achieve this goal, the design of new carriers of active ingredients to improve the delivery in foods is required. In this sense, nanotechnology is bursting into the food sector with several applications since it offers new insights to develop safer and healthier foodstuffs (Chaudhry et al., 2008; Durán & Marcato, 2013; Sozer & Kokini, 2009). The properties of materials that are on the nanometer (10^{-9} m) scale provide ground-breaking scientific, technological and commercial applications. Nanoscale control of food molecules allows the modification of macroscale characteristics of foods such as processability, texture, sensory attributes, shelf-life and functionality (Moraru et al., 2003). The use of nanotechnological advances has a direct impact in several areas, such as waste-water treatment, developing nanosensors for food safety and traceability, improved food packaging systems and higher efficient incorporation of food ingredients and additives (Imran et al., 2010; Sozer & Kokini, 2009). Both the scientific and industrial communities are putting a lot of attention on developing new nano-sized delivery systems of active compounds. In fact, according to the Helmut Kaiser Consultancy, the nanofood market has increased from a value of US$ 2.6 billions in 2003 to US$ 5.3 billions in 2005; and the United States Department of Agriculture (USDA) forecasts that the global impact of nanoproducts will be US$ 1 trillion annually by 2015 (Durán & Marcato, 2013).

The use of antimicrobials, antioxidants, vitamins, flavorings or colorants in the food industry still presents a challenge due to their poor water-solubility and stability in food formulations. These compounds are barely used directly and often are incorporated in other lipid carriers. In this sense, several nano-sized delivery systems of active ingredients are incorporated as functional materials in foods (J Weiss, Takhistov, & McClements, 2006). Nano-sized structures such as nanodispersions of oil-in-water (o/w) are regarded as useful tools with a great potential in the food sector to incorporate food ingredients. Nanoemulsions are systems defined as oil droplets, with particle sizes comprised between 10 and 100 nm, dispersed in an aqueous media (Mason, Wilking,

Meleson, Chang, & Graves, 2006; McClements & Rao, 2011; Tadros, Izquierdo, Esquena, & Solans, 2004). Nevertheless, when the droplet size is below 10 nm they are defined as microemulsions (McClements & Rao, 2011). Due to the rapid growth of the publications dealing with the formulation of nanoemulsions, a clarification of the term 'nanoemulsion' to distinguish them from microemulsions has been necessary. Lately, two reviews have been published regarding this topic (Anton & Vandamme, 2011; McClements, 2012). Besides their droplet size, the major difference between nanoemulsions and microemulsions relies in their thermodynamical stability. Even though nanoemulsions present a high long-term kinetic stability (several months), they are thermodynamically unstable. The free energy of the nanoemulsion system is higher than the respective lipid or aqueous phases separately, whereas microemulsions have a lower free energy. Therefore, the droplet characteristics (size and shape) of nanoemulsions do not depend on the system external conditions, so they remain stable at different temperatures or after dilution.

So far, several advantages have been attributed to nanoemulsions over conventional emulsions. Due to their reduced droplet size they are considered to be more stable in terms of coalescence, gravitational separation or particle aggregation (Mason et al., 2006). Moreover, they barely scatter the light being almost transparent systems, which makes them suitable to be incorporated in clear drinks and foods (McClements, 2002). Often, their particular properties as well as type of lipid and aqueous phases might be modulated to achieve systems with novel textures and rheological properties. In addition, there are evidences that the reduction of droplet size and the subsequent increase in surface area of nanodroplets might increase the functionality of the bioactive compound. Moreover, it has been recently pointed out that nanoemulsions may enhance the transport of active ingredients through biological membranes, thus intensifying the bioavailability of bioactive compounds (Acosta, 2009) or the bactericidal activity of antimicrobials (Donsì, Annunziata, Sessa, & Ferrari, 2011). Even though the described benefits of using nanoemulsions as ingredient carriers, the reported data are mainly dealing with model systems. However, their application in foodstuffs remains limited due to the lack of scientific evidence about whether they preserve or not their properties once incorporated in formulated products. Therefore, in this review article we aim to overview the main factors affecting the formulation, production and characterization of food-grade nanoemulsions as well as to provide an insight into their potential applications in food products.

2 Formulation

The formulation of nanoemulsions consists on mixing at least three components being oil and aqueous phases plus a stabilizer. The characteristics and concentration of the components in the nanoemulsion system will determine its final properties.

2.1 Lipid phase

The lipid phase in oil-in-water nanoemulsions generally acts as a carrier of lipophilic active compounds and is the dispersed phase in the continuous aqueous phase. Usually, the lipophilic active ingredients are solubilized in the oil phase prior to the formation of nanoemulsions. The oil phase can be formulated with different non-polar compounds, such as triglycerides, mineral oils or essential oils (McClements & Rao, 2011; McClements, 2011). The bulk physicochemical characteristics of the oil, such as viscosity, density, refractive index or interfacial tension have a direct impact on the formation of emulsions and also nanoemulsions (McClements, 2005). It has been described that the lower the viscosity of the oil phase, the smaller the droplet size of the nanoemulsions (Jafari, Assadpoor, He, & Bhandari, 2008; Qian & McClements, 2011; Seekkuarachchi, Tanaka, & Kumazawa, 2006; Wooster, Golding, & Sanguansri, 2008). It has been described that oil droplets with higher viscosity need higher residence times inside homogenizers to be deformed and disrupted later on, thus being more difficult to break up (Walstra, 1993). In this sense, essential oils or flavor oils tend to produce emulsions with smaller droplet size, due to their low viscosity, whereas long chain triglycerides tend to produce emulsions with bigger droplet size. However, at the same time, nanoemulsions containing flavor oils present lower long-term stability due to destabilization phenomena, such as Ostwald ripening or coalescence (McClements & Rao, 2011).

Besides the physicochemical characteristics of nanoemulsions, the oil type also influences the nutritional profile and digestibility of the lipid nanoparticles and therefore the functionality. In the case of flavor oils or short chain triglycerides, that are non-digestible oils, they pass through the gastrointestinal tract without being hydrolyzed. However, in the case of digestible oils, long chain triglycerides are reported to lead to higher bioaccessibility of bioactive compounds in comparison with medium or short chain triglycerides (Qian, Decker, Xiao, & McClements, 2012b; L Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013b).

2.2 Aqueous phase

The aqueous phase of oil-in-water emulsions plays a determining role in the physicochemical properties of nanoemulsions. The aqueous phase of food emulsions might contain a variety of water-soluble constituents, including minerals, acids, bases, flavors, preservatives, vitamins, sugars, surfactants, proteins or polysaccharides (McClements, 2005). After adding one or several ingredients to water, the characteristics of the nanoemulsion may change substantially. For instance, the addition of thickening agents that increase the continuous aqueous phase viscosity, are reported to cause a decrease in the droplet size of nanoemulsions, due to an increased shear stress in high pressure homogenizers (Qian & McClements, 2011). In addition, due to their smaller droplet size, nanoemulsions have a higher oil droplet surface area, which is highly exposed to lipid oxidation that takes place at the oil-water interface where the lipid hydroperoxides may be broken down to free radicals (McClements & Decker, 2000). Therefore, in the case of nanoemulsions, the use of water-soluble antioxidants might be strongly recommended in order to prevent lipid oxidation, and protect at the same time the lipophilic active ingredients that might be encapsulated in the lipid core.

2.3 Stabilizers

Besides the lipid and aqueous phases, the formulation of nanoemulsions requires the use of a stabilizer to prevent the breakdown of the nanoemulsion structure once it is formed. Nevertheless, nanoemulsions are reported to be more stable to gravitational separation or aggregation than conventional emulsions (Tadros et al., 2004; Wooster et al., 2008). There are several mechanisms, such as interfacial chemistry methods and/or physical methods, to prevent nanoemulsions destabilization phenoma, such as gravitational separation coalescence, aggregation, flocculation or Ostwald ripening (McClements & Rao, 2011). On one hand, the stabilization by interfacial methods implies the use of components that can create a shell in the oil-water interface that fixes the droplet size and shape. On the other hand, physical methods consist on stabilizing lipid droplets by avoiding the movement of droplets in the continuous phase and preventing them from approaching each other.

Emulsifiers

Emulsifiers or surfactants are surface-active amphiphilic molecules, meaning that they present a hydrophilic and a lipophilic part in their molecular structure. Thus, one part

has an affinity for nonpolar media and another part has an affinity for polar media. A good indicator for the affinity of the surfactant to the lipid phase is the hydrophilic-lipophilic balance (HLB), which is a numerical factor to describe the ratio of hydrophilic or lipophilic groups. HLB numbers >10 have an affinity for water (hydrophilic) and number <10 have an affinity of oil (lipophilic) (Hasenhuettl & Hartel, 2008). Surfactants are able to adsorb at the oil-water interface of droplet surfaces during emulsification, thus protecting droplets against re-coalescence or aggregation (Kralova & Sjöblom, 2009; McClements, 2005). Moreover, emulsifiers lower the interfacial tension of oil and can facilitate the oil droplets disruption during mechanical mixing processes. In the case of nanoemulsions, since the oil droplet size is smaller than in conventional emulsions, the surfactant concentration is a critical factor. Typically a higher concentration of emulsifier has to be used when formulating nanoemulsions since enough surface-active molecules are needed to completely cover the oil particles surface and avoid destabilization phenomena. It has been reported that oil droplet size decreases in nanoemulsions when increasing the surfactant concentration (Qian & McClements, 2011). Nanoemulsions with oil droplet diamaters bellow 200 nm can be obtained when using a surfactant-to-oil ratio (SOR) between 1 and 2 (Rao & McClements, 2011). Also the type of emulsifier to be used has to be carefully selected according to the lipid phase characteristics. Proteins and small molecule surfactants are widely used for food emulsions and nanoemulsions stabilization, due to their ability to adsorb at the oil-water interface (Bos & Van Vliet, 2001). Among proteins, casein and β-lactoglobulin are reported to be suitable for nanoemulsion fabrication, leading to nanoemulsions with oil droplet sizes around 100-200 nm (Qian & McClements, 2011). Nevertheless, proteins are considered to produce nanoemulsions with larger oil droplet sizes, since the size of the protein molecule itself contributes significantly to the oil droplet diameter once it is adsorbed at the interfacial layer. Moreover, they are adsorbed to the oil-water interface slowlier than small molecule surfactants during mechanical emulsification (Karbstein & Schubert, 1995; Stang, Karbstein, & Schubert, 1994). Therefore, small molecule surfactants are regarded to be more suitable than proteins for nanoemulsion formulation. Typically, low molecular weight emulsifiers are classified according to their electrical characteristics and therefore the interfacial charge that confer to oil droplets, being ionic, non-ionic or zwitterionic surfactants (McClements, 2005). Ionic surfactants used for food applications generally lead to negatively charged oil droplets, such as sodium dodecyl sulfate (SDS), sodium lauryl sulfate (SLS), citric

acid ester of monoglycerides (CITREM) or diacetyl tartaric acid ester of monoglycerides (DATEM), and in general are considered a good option for the production of nanoemulsions (Leong, Wooster, Kentish, & Ashokkumar, 2009; Qian & McClements, 2011; Wooster et al., 2008). Non-ionic surfactants are typically sucrose esters like sorbitan monooleate or sucrose monopalmitate, polyoxyethylene sorbitan esters of monoglycerides (Tweens) or polyoxyethylene ether surfactants (Brij). In general, non-ionic surfactants do not give electrical charge to emulsified lipid particles, but it is described that they might lead to negative electrical charges under certain conditions (Hsu & Nacu, 2003). Non-ionic small molecule surfactants are those more commonly used for nanoemulsion production, rendering particularly small droplet sizes (Choi, Kim, Cho, Hwang, & Kim, 2009; Laura Salvia-Trujillo, Rojas-Graü, Martín-Belloso, & Soliva-Fortuny, 2013). Finally, the last group corresponds to the zwitterionic surfactants, which are molecules with two or more ionizable groups with opposite charges within the same molecule, being the phospholipid lecithin the most significant. However, it has been observed that they should be used in combination with other surfactants in order to form nanoemulsions with small droplet sizes (Hoeller, Sperger, & Valenta, 2009; Trotta, Cavalli, Ugazio, & Gasco, 1996). Besides the capability of surfactants to produce and stabilize nanoemulsions, the composition and characteristics of the interfacial layer have determining implications when considering the loading of active compounds. For instance, it has been recently reported that β-carotene degradation in β-lactoglobulin-stabilised nanoemulsions was significantly slower than in Tween 20-stabilised ones (Qian, Decker, Xiao, & McClements, 2012a). Moreover, certain surfactants such as SDS or lauric arginate (LAE) have shown to exhibit antimicrobial activity themselves when used to stabilize nanoemulsions (Ziani, Chang, McLandsborough, & McClements, 2011).

Texture modifiers

Hydrocolloids have been extensively used in food formulations for their thickening or gelling properties when incorporated into an aqueous phase (Saha & Bhattacharya, 2010). In food emulsions, their main role is to stabilize oil droplets within the continuous phase due to the viscosity increase. Modifying the rheology of the emulsions aqueous phase not only changes their texture and mouth-feel, but also minimizes the droplet movement in the fluid so retarding gravitational separation (creaming or sedimentation) of lipid particles (McClements, 2005). Even though most of the

hydrocolloids used in foods act as stabilizers, only some of them show emulsification properties due to their surface activity on the oil-water interface (Dickinson, 2009; Garti & Leser, 2001). Among them, gum arabic, modified starch, modified celluloses, modified alginate, pectins, some galactomannans or cellulose derivatives are those with surface activity (Dickinson, 2003). Their surface activity on lipid droplets is due to either their non-polar chemical groups or to their protein components attached to the hydrophilic polysaccharide backbone (Dickinson, 2009). Nevertheless, non-adsorbed hydrocolloids may contribute to destabilize nanoemulsons since they may induce bridging phenomena between molecules or depletion flocculation (Dickinson, 2003). Also, hydrocolloids might interact with previously adsorbed species depending on their anionic or cationic charge of the molecule, thus creating multilayers at the oil-water interface (Dickinson, 2003; Goddard, 2002; Guzey & McClements, 2006). Hydrocolloids properties have been used to design emulsion-based delivery systems of food ingredients (McClements, Decker, & Weiss, 2007). However, their effect on the digestibility and bioactivity of the encapsulated lipophilic compound is highly dependent on the type of biopolymer used. It has been reported that the digestibility of emulsified oil droplets was retarded or inhibited depending on the composition of the interfacial layer (Y. Li et al., 2010). Moreover, the presence of hydrocolloids in emulsions and nanoemulsions influences the rates of passage, digestion, absorption, and fermentation in the digestive tract, which may directly determine the nutritional properties of the lipophilic active ingredients (Dickinson & Leser, 2013; Gidley, 2013).

3 Lipid active ingredients

There are many compounds used in food industry, such as certain antimicrobials or bioactive compounds that present low water solubility. Thus, incorporating them in food formulations with high water content represents a challenge. Usually, poor-water soluble compounds are solubilized in an oil phase prior to the emulsification into the food system. One of the main advantages of reducing the emulsion droplet size up to the nanometric range is related to the increase in the solubility of lipophilic compounds, and therefore to the enhancement of their stability in foods. Nevertheless, the ability of the active lipids to penetrate across biological membranes is also enhanced, thus boosting their biological functionality. Therefore, the interest in nanoemulsions as delivery systems of food ingredients is growing among the scientific and industry community. In

the following sections we will present the functional lipophilic compounds that are good candidates to be encapsulated in nanoemulsion systems (**Table 1**).

Table 1. Lipophilic active ingredients that can be incorporated in nanoemulsions

Compound	Types	Functionality
Antimicrobials		
Plant essential oils	Oregano, sage, clove, mint, limonene, etc.	Flavorings and antimicrobial activity
Bioactive compounds		
Fatty acids	ω-3 fatty acids, conjugated linoleic acid, butyric acid	Coronary heart disease, bone health, immune response disorders, weight gain, stroke prevention, mental health, cancer, and visual acuity
Carotenoids	β-Carotene, lycopene, lutein, and zeaxanthin	Cancer, coronary heart disease, macular degeneration, and cataracts
Antioxidants	Tocopherols, flavonoids, polyphenols	Coronary heart disease, cancer, and urinary tract disease
Phytosterols	Stigmasterol, β-sitosterol, and campesterol	Coronary heart disease
Quinones	Coenzyme Q10	Antioxidant activity

Adapted from McClements, Decker & Weiss (2007).

3.1 Antimicrobials

There are many preservative substances used in foods to prevent the microbial growth and food spoilage, such as organic acids, hydrogen peroxide, bacteriocins or chelating agents (Brul & Coote, 1999). All these substances are water soluble, so there is no practical need to incorporate them in nanosized lipid droplets. However, other naturally occurring antimicrobial agents are recently being investigated as a strong alternative to synthetic preservatives. Essential oils (EOs) are hydrophobic substances typically extracted from herbs and spices that are well known for their antimicrobial properties. The antimicrobial action of EOs relies on their hydrophobic nature, which enables them to partition in the lipids of bacterial cell membrane and mitochondria, causing damages to these structures and rendering them more permeable (Burt, 2004; Hammer, Carson, & Riley, 1999). Leakage of ions followed by efflux of cytoplasmatic constituents cause the viability loss of microbial cells (Lambert, Skandamis, Coote, & Nychas, 2001; Walsh et al., 2003). Moreover, other mechanisms may be involved in microbial inactivation by EOs since they could interfere with membrane function, altering its electron transport, nutrient uptake, proteins, nucleic acid synthesis and enzyme activity (Bajpai, Baek, & Kang, 2011; Tiwari et al., 2009). However, the concentration of

certain types of EOs needed to achieve cell death is in practice way above tolerable taste thresholds. It may be expected that nanoemulsified EOs penetrate faster in the microbial membranes due to an increased surface area per weight unit. This would allow reducing the incorporated concentrations to achieve an equivalent or even greater bactericidal effect over conventional emulsions. Several recent research works report the formation of nanoemulsions containing EOs as a strategy to improve their functionality. For instance, Salvia-Trujillo and co-workers (L Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2012; Laura Salvia-Trujillo et al., 2013) obtained nanoemulsions containing lemongrass essential oil with particle sizes of 5 nm. Also, Ghosh and co-workers (Ghosh, Mukherjee, & Chandrasekaran, 2013; Ghosh, Saranya, Mukherjee, & Chandrasekaran, 2013) obtained basil or cinnamon essential oil nanoemulsions with droplet sizes below 100 nm. However, there is very little information available about the real benefits of nanoemulsions over conventional emulsions in terms of antimicrobial action. Nevertheless, this is a hot topic and few recent research papers approach this issue as it is discussed further.

3.2 Bioactive compounds

The incorporation of bioactive compounds in foods to fortify their health promoting properties is a common practice in recent years. The use of nanoemulsions as carriers and delivery systems presents important advantages and thus it is catching the researchers' attention. There are many lipophilic nutraceutical compounds that are susceptible to be incorporated in foods due to their health promoting properties. These comprise a variety of compounds such as carotenoids, omega-3 fatty acids, polyphenols, flavonoids, phytosterols and tocopherols (vitamin E) (McClements et al., 2007). β-carotene, lutein and lycopene, typically found in fruits and vegetables are the more common carotenoids used for food fortification due to their bioactivity. The strongest correlations have been found for lycopene intake and prostate cancer, β-carotene intake and lung cancer, and lutein intake and age-related macular degeneration and cataract (Johnson, 2002; Mayne, 1996). However, carotenoids are highly susceptible to degradation due to light, oxygen or autooxidation (Boon, McClements, Weiss, & Decker, 2010). The action of incorporating carotenoids in nanoemulsions reduces itself their degradation, however, their chemical stabilization can be further improved during storage of nanoemulstions by either incorporating in the system water soluble

antioxidants such as EDTA or ascorbic acid, or oil soluble antioxidants, such as vitamin E or coenzyme Q_{10} (Qian, Decker, Xiao, & McClements, 2012c). On the other hand, among omega-3 fatty acids commonly present in fishery products, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the most biologically active, showing strong benefits against inflammatory, autoimmune and coronary heart diseases (Bucher, Hengstler, Schindler, & Meier, 2002; Harris, 1989; Simopoulos, 2002). Polyphenols and flavonoids are antioxidant compounds present in fruit, vegetables, wine and tea that exhibit low water solubility, and are susceptible to be incorporated in food formulations due to their well-known cardioprotective effect (Heim, Tagliaferro, & Bobilya, 2002). Phytosterols include compounds such as such as stigmasterol, β-sitosterol, and campesterol, found in vegetable oils and known for their ability to inhibit the intestinal absorption of dietary cholesterol (Ostlund Jr., 2004; Wong, 2001). Typically they are incorporated in fat-rich foods, since its dispersion results relatively simple (McClements et al., 2007). Finally, tocopherols, also known as vitamin E, are a highly hydrophobic group of several molecular forms (α, β, γ, and δ) widely used in foods due to their antioxidant protective effect against cardiovascular disease, cancer, chronic inflammation, and neurologic disorders, being α-tocopherol the most biologically active form (Brigelius-Flohé et al., 2002).

The incorporation of highly hydrophobic bioactive compounds in foods is a challenge not only due to their poor water solubility, fast oxidation and low sensorial detection thresholds (McClements & Decker, 2000), but also because of their low bioaccessibility after the digestion in the gastrointestinal tract (GIT). Since these compounds are typically chemically synthesized or purified, they are commercially available as powders. Therefore, due to their poor water solubility they need to be solubilized into a lipid prior to the emulsification procedure. Moreover, it is important to consider the best lipid carrier for each bioactive compound according to their affinity or the final product to be formulated and its special requirements. It is known that not all lipids and fats present the same behavior through the gastrointestinal tract, thus the type of oil used in the formulation of nanoemulsions will directly influence their biological activity. For instance, it is known that the presence of lipids during the digestion increases the carotenoids bioaccessibility due to the micellarization in the aqueous phase (Maiani et al., 2009; Yeum & Russell, 2002). In this sense, even though little is known about the behavior of vitamin E in the gastrointestinal tract, the lipolysis of the lipid core in which it is included seems crucial for its release (Brigelius-Flohé & Galli, 2010). Therefore,

besides the physicochemical characteristics of nanoemulsions, the selection of the lipid carrier and its digestibility significantly affects the functionality of the lipophilic compounds during the GIT (Singh, Ye, & Horne, 2009). The behavior of nanoemulsions during the GIT and their real benefits over conventional emulsions will be discussed further.

4 Fabrication methods

Typically, the approaches to form nanoemulsions are classified between high-energy or low-energy (McClements & Rao, 2011; Tadros et al., 2004). High-energy methods consist in applying high disruptive forces with mechanical devices, capable of causing the breakup of oil droplets and disperse them into the water phase. On the other hand, low-energy approaches rely on the spontaneous formation of tiny oil droplets within mixed oil–water–emulsifier systems when the solution or environmental conditions are altered, such as composition or temperature (Anton & Vandamme, 2009; Solans, Izquierdo, Nolla, Azemar, & Garcia-Celma, 2005). Even though both approaches are able to form tiny droplets, the high-energy methods are generally more likely to be used in the food sector due to their scale up and equipment availability, in addition to their ability to produce nanoemulsified systems without the addition of organic solvents. Therefore, in this review we will focus on the high-energy approaches to fabricate nanoemulsions.

When two immiscible liquids are placed together, they tend to adopt their thermodinamically most stable state, which is a layer of oil on the top of the aqueous phase (McClements, 2005). To form an emulsion, applying energy via mechanical stress is needed in order to disrupt and mix the lipid and water phases. However, droplet formation in the nano range requires a higher energy input. The presence of surfactant molecules that are adsorbed at the surface of oil droplets prevents them from further re-coalescence. In this sense, the most commonly used devices to form nanoemulsions are high-pressure homogenization and ultrasonication. **Table 2** presents an overview of the articles reporting nanoemulsions by high-energy emulsification.

Table 2. Review of the published works reporting nanoemulsions produced by high-energy methods (high-pressure homogenization or ultrasonication)

Fabrication method	Processing conditions	Lipid carrier	Functional compound	Emulsifier	Droplet size (nm)	Reference
High Pressure Homogenization	50-150 MPa; 1-10 cycles	-	Lemongrass essential oil	Tween80 and sodium alginate	53-7 nm	(Salvia-Trujillo, 2013)
	50-150 MPa; 1-5 cycles	Silicon oil	-	Tween20, sodium caseinate or sodium dodecyl sulfate (SDS)	140-110 nm	(Lee,L. et al. 2013)
	150 MPa; 10 cycles	Medium chain triacylglycerol (MCT)	β-carotene	Octenylsuccinic anhydride (OSA)-modified starch	150 nm	(Liang,R. 2013)
	10 KPa; 10 cycles	MCT and corn oil	Thyme oil	Tween80	diameter < 200 nm	(Chang,Y. 2012)
	15,000 psi; 5 cycles	Corn oil:hexane	-	β-lactoglobulin	radius < 100 nm	(Troncoso 2012)
	70-280 MPa; 1-10 cycles	Sunflower oil	-	Tween80, sodium dodecyl sulfate (SDS), sucrose palmitate, soy lechitin or modified starch	90-190 nm	(Donsì,F. 2012)
	9000 psi; 3 cycles	Orange oil	β-carotene	β-lactoglobulin	79 nm (radius)	(Qian,C. 2012)
	12K psi; 5 cycles	Corn oil, MCT, orange oil	Polymethoxyflavones	β-lactoglobulin, lyso-lecithin, Tween, and dodecyl trimethyl ammonium bromide (DTAB)	diameter < 100 nm	(Li,Y. 2012)
	200–500 bar; 3 cycles	Palm oil	Vitamin E	Tween 40	200 nm	(El Kinawy,O.S. 2012)
	300, 800 or 1200 bar; 12 cycles	Palm oil	α-tocopherol	Whey proteins and/or lechitin	431-201 nm	(Shukat,R. 2011)
	9000 psi; 3 cycles	-	Lemon oil	Sucrose monopalmitate (SP90)	radius < 100 nm	(Rao 2011)
	4-14 Kbar; 1-8 cycles	Corn oil	-	β-Lactoglobulin, sodium caseinate, Tween20 or sodium dodecyl sulfate (SDS)	231-146	(Qian 2011)
	300 MPa; 5 cycles	Sunflower oil	Carvacrol, limonene and cinnamaldehyde	Lecithin, pea proteins, sugar ester and a combination of tween 20	123-293 nm	(Donsì,F. 2011)

				and glycerol monooleate		
	1000 bar; 5 cycles	Hexadecane or peanut oil	-	Sodium dodecyl sulfate (SDS), Tween 80,	120-80 nm	(Wooster 2008)
	20 MPa; 1 cycle	-	d-limonene	Maltodextrin and modified starch	570 nm	(Jafari 2007)
Ultrasounds	750 W; 15 min	-	Basil essential oil	Tween80	41-29 nm	(Ghosh,V. 2013)
	750 W; 30 min	-	Cinnamon oil	Tween80	65 nm	(Ghosh,V. 2013)
	12-28.1 W; 70-170 s	-	d-limonene	Sorbitan trioleate, polyoxyethylene (20) oleyl ether	444-24 nm	(Li,P.-H. 2012)
	30-100 μm; 30-180 s	-	Lemongrass essential oil	Tween80 and sodium alginate	35-5 nm	(Salvia-Trujillo 2012)
	20-40% of the acoustic power (W); 20 min (pulses of 10 s sonication/30 s without sonication)	Soybean oil and wax	-	Lipoid s75 and PEG-surfactants	180-50 nm	(Delmas 2011)
	30 μm; 5-20 min	Sunflower oil and canola oil	-	Tween 80, Span 80 and sodium dodecyl sulfate (SDS), polyethylene glycol (PEG)	180-40 nm	(Leong 2009)
	40 W; 4 pulses (15 s sonication/15 s without sonication)	Soybean, sesame and olive oils	-	Pluronic F68	368-380 nm	(Wulff-Pérez,M. 2009)
	340 W; 17-119 s	Flaxseed oil	-	Tween40	140-130 nm	(Kentish 2008)
	100 μm; 20 s	-	d-limonene	Maltodextrin and modified starch	680 nm	(Jafari 2007)

4.1 High Pressure Homogenization

Conventional emulsification through high pressure homogenization is the most widely spread method among industrial processes due to their versatility and possibility of scaling up. Nevertheless, specific types of high pressure homogenizers are able to produce emulsions with droplet sizes in the nanometric range. There are several types of high pressure homogenizers. The most commonly used are high pressure valve homogenizers and microfluidizers. In the case of high pressure valve homogenizers a coarse emulsion is pumped towards a chamber where the liquid is forced through a narrow valve at the end of the chamber (McClements & Rao, 2011). The intense disruptive forces that the liquid experience as passing through the valve cause the breakdown of larger particles into smaller ones. Donsì and co-workers (Donsì, Sessa, & Ferrari, 2012) stated that the geometry of the high pressure valve homogenizers has a significant effect on the droplet size distribution of nanoemulsions. However, the treatment pressure and the number of passes through the chamber are the most determining factors of the droplet size of nanoemulsions. In this regard, the higher the pressure and the number of cycles, the smaller the lipid particle size (Donsì et al., 2012). Microfluidizers are similar to high pressure valve homogenizers but the design of the chamber where the treatment is conducted is substantially different. Microfluidization processing implies that a coarse emulsion is passed through and interaction chamber using a high-pressure pumping device (Jafari, He, & Bhandari, 2007). The interaction chamber consists of two flow channels, which are designed so that they cause two streams of the coarse emulsion to impinge on each other at high velocity, thus creating a very high shearing action that provides an exceptionally fine emulsion (Mahdi Jafari, He, & Bhandari, 2006). There are several shapes of the microfluidization channels to create the nanoemulsion, but the most commonly used are the “Y” shapes. So far, consistent results have been reported by several authors (Qian & McClements, 2011; Laura Salvia-Trujillo et al., 2013), who describe a decrease in the droplet size of nanoemulsions by increasing the microfluidization pressure and number of cycles. However, a re-coalescence of lipid droplets was observed in nanoemulsions due to an “over-processing” during microfluidization (Jafari et al., 2008). This phenomenon might be related to a too little surfactant concentration or an inefficient adsorption rate, which is also a crucial factor when forming nanoemulsions. Recently, Lee and co-workers (Lee & Norton, 2013) have reported a more effective droplet disruption in

microfluidized nanoemulsions processed with a single pass in comparison with high pressure valve homogenization working at the same pressure. Besides the processing conditions and the surfactant chemistry, the viscosity of both the continuous and lipid phases is also shown to be crucial during the formation of nanoemulsions. It has been observed that low viscosity oils render nanoemulsions with smaller droplet sizes (Wooster et al., 2008). Also Qian and co-workers (Qian & McClements, 2011) observed that the minimum droplet size achievable decreased as the ratio of disperse phase to continuous phase viscosities decreased.

During emulsification by high-pressure homogenization there is an intrinsic increase in the temperature of the sample, which must be taken into account especially when it comes to the production of nanoemulsions incorporating heat-sensitive active compounds. High pressure valve homogenizers and microfluidizers are usually equipped with a cooling coil at the outlet of the treatment chamber, since the product can otherwise reach temperatures as high as 70 ºC. For instance, Shukat and co-workers (Shukat & Relkin, 2011) observed a substantial degradation of α-tocopherol in palm oil nanoemulsions produced with high pressure homogenization. Similarly, Donsì and co-workers (Donsì, Annunziata, Vincensi, & Ferrari, 2011) reported a degradation of certain volatile compounds after high-pressure homogenization of nanoemulsions containing essential oils. Therefore, the temperature of the process is a critical factor to consider when producing nanoemulsions as carriers of active ingredients.

4.2 Ultrasounds

Ultrasonication consists on applying ultrasonic waves of high frequency (typically higher than 20 kHz) by immersing a sonotrode in a liquid. Sonication of liquid food products has been described as a method for microbial inactivation (Knorr, Zenker, Heinz, & Lee, 2004; Piyasena, Mohareb, & McKellar, 2003), food extraction (Vilkhu, Mawson, Simons, & Bates, 2008) and also for emulsification of oil-in-water emulsions (McClements, 1995). Recently, the production of nanoemulsions by sonication has been described in several research papers, as can be observed in Table 2. Emulsification with ultrasonication is mainly caused by cavitation phenomena. During ultrasonication treatment there are pressure fluctuations in the fluid, which cause the cyclical growth and compression of air bubbles that are within the fluid. Eventually, the air bubbles reach a critical size, becoming unstable and violently collapse (Barbosa-Cánovas &

Rodríguez, 2002; Soria & Villamiel, 2010). The implosion of cavitation bubbles leads to the generation of high shear forces, hot spots and turbulence in the cavitation zone (Soria & Villamiel, 2010). This effect causes the breakup of oil droplets of coarse emulsions leading to particle sizes in the nanometric range.

The main factors affecting the final droplet size in ultrasonically generated nanoemulsions are the treatment time and intensity, which is directly related with the amplitude of the sonication wave. In this sense, several works report a reduction in the droplet size of nanoemulsions after increasing the sonication amplitude and treatment time (Leong et al., 2009; P.-H. Li & Chiang, 2012; L Salvia-Trujillo et al., 2012). Also, the ultrasonication treatment can be conducted in both, batch or continuous mode by adapting a continuous flow cell assembled to the sonotrode. Kentish and co-workers (Kentish et al., 2008) determined that ultrasonication with a continuous flow cell may produce nanoemulsions with a broader size distribution in comparison with the ones produced by batch mode due to non-homogenous treatment of the liquid in the flow chamber. The minimum droplet size of nanoemulsions is independent of the design of the flow cell, whereas using over-pressure in the flow cell leads to a more efficient process (Leong et al., 2009).

Ultrasounds are a feasible technology to form nanoemulsions, but they might present several drawbacks when incorporating sensitive active compounds. First, the hot spots generated due to bubble implosion and high shear rates may cause the temperature increase of the emulsion and this can cause a detrimental effect of heat sensitive compounds. Moreover, it has been observed that cavitation phenomena during ultrasound treatment can lead to the hydrolysis or oxidation of triglycerides and therefore the degradation of lipids (Chemat et al., 2004), which is caused by the appearance of reactive species in the sonicated fluid (Riesz, Berdahl, & Christman, 1985). Another limitation for the commercial application of ultrasounds is that since a titanium ultrasonic horn tip is used, titanium ions or particles can be emitted into the product by cavitational abrasion of the sonotrode (Freitas, Hielscher, Merkle, & Gander, 2006).

5 Nanoemulsion characterization

It is important to determine the lipid nanoparticle characteristics and those of the whole nanoemulsion system. Due to their small particle size and also unknown behavior of matter at the nanoscale, it is difficult to measure the nanoemulsions physicochemical characteristics. In this section we will present the most commonly used techniques to measure the nanoemulsion properties.

5.1 Droplet size

The size of lipid nanoparticles in nanoemulsions is probably the most important parameter to be measured, since it directly influences the optical, rheological and release characteristics, as well as potentially altering its biological fate (McClements & Rao, 2011). Typically, the size of lipid particles in the sub micron range is measured by Dynamic Light Scattering (DLS) technique, which is also sometimes referred Photon Correlation Spectroscopy or Quasi-Elastic Light Scattering). DLS techniques are based on measurements of the translational diffusion coefficient of particles determined by analyzing the interaction between a laser beam and an emulsion (Hunter, 2001). When a laser beam is directed through an ensemble of particles, the light is scattered depending on their relative position, which is due to the Brownian motion of the particles (Xu, 2002). As a general rule, the larger the particle, the slower the Brownian motion will be, and vice versa. The change in the scattering pattern is monitored using a detector that measures the intensity of the photons at constant time intervals and a determined scattering angle (McClements, 2005). The intensity and the scattering patterns can be correlated by means of an autocorrelation function (Horne, 1995). Therefore, the Brownian motion of particles dispersed in a liquid is related with their hydrodynamic diameter by the Stokes-Einstein equation:

$$d(H) = \frac{kT}{3\pi\eta D}$$

where:

d(H) = hydrodynamic diameter

D = translational diffusion coefficient

k = Boltzmann's constant

T = absolute temperature

η = viscosity

The measurement of lipid nanoparticles by DLS is an indirect measure. The mathematical models used by these instruments to calculate the particle size distribution from the intensity received by the detector usually assume that the particles are homogeneous spheres with well-defined properties (such as refractive index and density) (McClements & Rao, 2011). Consequently, there may be some errors in the reported particle size distributions in nanoemulsions where the particles are core-shell structures rather than simple homogeneous spheres (McClements & Rao, 2011). Moreover, this technique is valid to measure particle size and size distribution in dilute emulsions, where there are no particle-particle interactions (McClements, 2005). Therefore, it is often necessary to dilute the sample before measuring its particle size. This might not be a problem for nanoemulsions as their particle size remains constant after dilution, but might change the particle size of microemulsions that are thermodynamically stable. In addition, the mean particle size (*d(H)*) of DLS would only be suitable for the characterization of monodisperse nanoemulsions, since the presence of a few bigger particles scatters the light more intensely, leading to a misinterpretation of the distribution of particle sizes. In this sense, when heterogeneous samples in terms of droplet size with bigger particles are to be measured, the use of Static Light Scattering (SLS) techniques is usually needed, since it can cover a broader distribution of particle sizes (McClements, 2005). It uses the same principle as DLS but it does not consider the Brownian motion of the particle set. The particle size measured by SLS can be shown by several parameters. Typically the $d_{4,3}$ or the $d_{3,2}$, which are the volume mean diameter or the surface and the mean diameter respectively, are the most common parameters used to report the mean particle size. For instance, the $d_{4,3}$ is more sensitive to larger particles since it considers the volume of the lipid droplets, thus being useful when there is a special interest in detecting flocculation or coalescence phenomena in nanoemulsions formulation or in monitoring changes of nanoparticles during digestion conditions.

5.2 Emulsion stability

The stability of emulsions after being produced or during storage is a critical parameter to be measured. Emulsions and nanoemulsions are kinetically stable but thermodynamically unstable because the contact between oil and water is unfavorable, so they will always tend to breakdown over time (Taylor, 1998). As a consequence, the

droplet size might change or the lipid and aqueous phase might tend to separate over time (weeks, days, months or years). Several destabilization phenomena might cause emulsion breakdown (**Figure 1**).

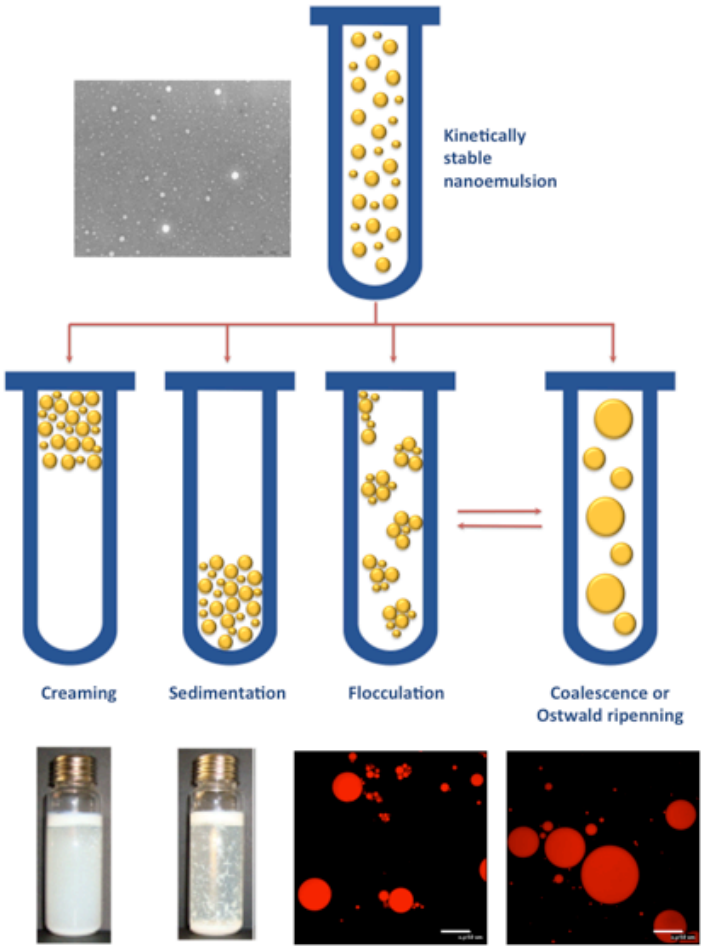

Figure 1. Destabilization phenomena that can occur in nanoemulsions. TEM image of initial nanoemulsions is showed with a scale bar of 500 nm. Creaming and sedimentation phenomena are shown by direct observation. Flocculation, coalescence and Ostwald ripening are shown with confocal micrographs with a scale bar of 50 μm.

Gravitational separation (creaming or sedimentation) of emulsions occurs when oil droplets move upwards (creaming) or downwards (sedimentation) due to density differences between oil and water (McClements, 2007). On the other hand, flocculation happens when lipid droplets form aggregates but individual droplets remain in the same droplet size. Moreover, gravitational separation and flocculation are reversible phenomena, but might lead to irreversible coalescence of oil droplets (McClements, 2007). Coalescence is the process whereby two oil droplets merge together to form a single larger droplet (McClements, 2005). Ostwald ripening is the phenomena whereby there is an oil mass transfer from smaller droplets to bigger ones, thus increasing the size of oil droplets (McClements, 2007; Taylor, 1998). In general, the solubility of a

substance increases as the size of the particle containing it decreases. Consequently, there is a concentration gradient from smaller particles to larger ones (McClements, 2005). Nanoemulsions containing essential oils are particularly sensitive to Ostwald ripening due to the relatively high solubility of flavor oils in water, thus facilitating the transfer of triglycerides through the aqueous phase (McClements & Rao, 2011).

The stability of nanoemulsions will directly influence their bulk physicochemical and functional properties. Therefore, to measure the stability parameters of nanoemulsions is of great importance. Several parameters are reported to indicate the short/medium term stability of emulsified lipid nanoparticles. On one hand, the electrical characteristics of the interface that separates the oil and water phases plays a crucial role in determining the overall nanoemulsion stability, since it governs the electrostatic interactions of droplets (McClements, 2005). The droplet electrical charge is a consequence of the adsorption of emulsifier molecules that are ionized or ionizable, leading to neutral, positively or negatively charged lipid particles. The ζ-potential is the effective surface potential of a droplet suspended in a medium, which takes into account that charged species in the surrounding medium may adsorb to the surface of the droplet and alter its net charge (McClements, 2005). Typically, particles with ζ-potential more positive than +30 mV or more negative than -30 mV are considered to be electrically stable, since electrical charge of droplets is strong enough to assume that repulsive forces between droplets are predominant between oil droplets (Heurtault, Saulnier, Pech, Proust, & Benoit, 2003; Preetz, Hauser, Hause, Kramer, & Mäder, 2010). Therefore, the presence of lipid nanoparticles with a strong electrical charge is a sign of nanoemulsion stability. The most extended method to measure the ζ-potential of nanoemulsions is the electrophoresis technique, which measures the direction and velocity of particles movement under a determined electric field by measuring the light scattering of lipid droplets. In this sense, lipid particles with a large enough ζ-potential are thought to resist flocculation or coalescence in a higher extent, thus being an indicator of long term stability. Besides the type of emulsifier used to stabilize the nanoemulsion, a number of other factors influence the ζ-potential. The pH of the aqueous phase surrounding the oil droplets can change the ionization degree of the emulsifier molecules, whereas the presence of salts in the aqueous media, and thus an increased ionic strength, creates an “screening effect”. Moreover, other species like biopolymers or metallic ions may also adsorb on the droplet surface and therefore change the electrical charge of oil droplets.

On the other hand, specific destabilization phenomena can be measured with a number of analytical techniques. Gravitational separation can be simply measured by placing the nanoemulsion in a transparent test tube for a certain period of time and measure the height of the lower layer (serum) and the upper layer (cream) (McClements, 2005). The results can be expressed as creaming index, using the following expression (Pongsawatmanit et al., 2006):

$$CI = \frac{H_s}{H_t} \times 100$$

Where CR is the creaming index, H_s is the height of the serum layer and H_t is the total height of the sample.

Even though determining the creaming index visually is an easy and fast characterization method, it might not be precise enough when bounds between layers are not well defined. Other techniques are recommended to monitor the gravitational separation of lipid nanoparticles, being light scattering the most frequently used (Chanamai & McClements, 2000). The sample is placed in a transparent test tube where a beam of infrared light is directed to different heights of the sample using a stepper motor, and the percentage of transmitted or scattered light is measured (McClements, 2005). This method provides information about the concentration of droplets versus the height profile of the sample, thus being able to monitor the movement of particles upwards and downwards. Also there are available laboratory devices capable of measuring the droplet size at the same time. Thus this would facilitate to determine flocculation, coalescence or Oswald ripening due to droplet size increase. However, these devices are not commonly used to determine the average droplet in nanoemulsions since they are not specifically designed to measure submicron lipid particles.

5.3 Microstructure

The measurement of the particle size of lipid nanoparticles is crucial, since it has a direct impact on their functional properties. The measurement of nanoparticles by DLS presents several limitations since it may fail to recognize the presence of a small population of large droplets present in nanoemulsions (Benita & Levy, 1993). Moreover, most samples need to be diluted prior to DLS observations and consequently reversible destabilization phenomena might remain unnoticed (Klang & Valenta, 2011). Therefore, it is highly recommended to verify the data of the particle size obtained by

light scattering techniques with additional analysis such as direct observation methodologies. The use of microscopy techniques gives much more precise information about the microstructure of the lipid nanoparticles as well as possible interactions between them. However, the sub micron size of nanoparticles complicates the manipulation and observation of the samples. Recently, several techniques have been proven as useful for characterizing nanoemulsions and detecting nanoparticles in foods, such as Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) or Atomic Force Microscopy (AFM) (Blasco & Picó, 2011; Dudkiewicz et al., 2011; Klang, Matsko, Valenta, & Hofer, 2011; Tiede et al., 2008).

SEM consists on scanning a focused electron beam across the sample and an image of the surface of the sample is generated (Bogner, Jouneau, Thollet, Basset, & Gauthier, 2007). Usually, the sample preparation implies a fixation, drying and coating with gold particles (Klang, Matsko, Valenta, et al., 2011). The main drawback of this analysis technique is that the information revealed about the internal structure of the nanoparticles is limited (Klang, Matsko, Valenta, et al., 2011) so other techniques are preferred. Moreover, the presence of surfactants during nanoemulsion preparation can sometimes inhibit their characterization via SEM due to the formation of a smooth camouflaging coating in the particle surfaces (Luykx, Peters, Van Ruth, & Bouwmeester, 2008). On the other hand, TEM is a technique where an electron beam is transmitted through a sample, detected and focused later on by multiple electromagnetic lenses (Klang, Matsko, Valenta, et al., 2011). TEM is capable of achieving a resolution up to 0.2 nm, which makes it suitable for the analysis of sub micron particles (Silva, Cerqueira, & Vicente, 2012). Recently, TEM has been successfully used to investigate and characterize nanoemulsions (Araújo et al., 2011; Kelmann, Kuminek, Teixeira, & Koester, 2007; Nam, Kim, Shim, Han, & Kim, 2010; Laura Salvia-Trujillo et al., 2013; Seki et al., 2004). However, it presents several drawbacks. On one hand, it requires extensive and time-consuming sample preparation, which may change the structure of the sample. On the other hand, there is a risk of sample damage due to the electron beam going across the sample (Luykx et al., 2008). For this reason, the use of cryo-TEM techniques to characterize nanoemulsions has been highly recommended, since it avoids the staining of the samples, delivering detailed information about the internal structure of the nanoparticles in their native state (Klang, Matsko, Valenta, et al., 2011; Kuntsche, Klaus, & Steiniger, 2009). Freeze-etching or freeze-fracturing methodologies consist on rapid freezing of the sample, fracturing, replication and observation by TEM

(Severs, 2007), whereas cryo electron microscopy consists on the cryo-fixation of a liquid sample to obtain a vitrified thin layer (Klang, Matsko, Valenta, et al., 2011; Kuntsche et al., 2009). Cryo-TEM has been used recently to characterize nanoemulsions (Klang, Matsko, Raupach, El-Hagin, & Valenta, 2011; Klang, Schwarz, et al., 2011; Yilmaz & Borchert, 2005) and so far seems to be the most recommendable technique to analyze the microstructure of soft matter like lipid nanoparticles.

Recently, the use of AFM has been introduced to study the microstructure of nanoemulsions due to its high resolution (± 0.1nm) (Silva et al., 2012). It consists on scanning the surface of a sample by measuring the force after contact with a cantilever, which has been previously immobilized onto a mica or glass surface (Giessibl, 2003). It has been described as a useful tool to characterize nanoemulsions and specially to study interfacial phenomena (Morris, Woodward, & Gunning, 2011; Shimoni, 2008). Several research works used AFM to study the physic-chemical properties of nanoemulsions with good results to measure the lipid particle size (Bazylinska, Skrzela, Szczepanowicz, Warszynski, & Wilk, 2011; Mao et al., 2009; Laura Salvia-Trujillo et al., 2013). **Figure 2** displays an AFM image of a lemongrass oil-alginate nanoemulsion with droplet sizes below 100 nm (Laura Salvia-Trujillo et al., 2013). When measuring lipid nanoparticles, it is usually recommended to work in non-contact or semi-contact mode to avoid excessive adhesion of the cantilever tip with the nanoparticles. In this sense, wider nanoparticles compared to DLS measurements have been observed sometimes due to the tip broadening effect, which takes place when the tip is in contact with soft or sticky biomaterials (Luykx et al., 2008; Yang et al., 2007). For instance, Preetz and co-workers (Preetz et al., 2010) have reported differences between droplet sizes of nanoemulsions depending on the measurement technique used, since they found that the mean droplet size determined by DLS was 150 nm, whereas it ranged between 50 and 500 nm if they were observed by TEM or AFM. Despite the differences with DLS measurements, AFM is a good complementary tool to characterize lipid nanoparticles.

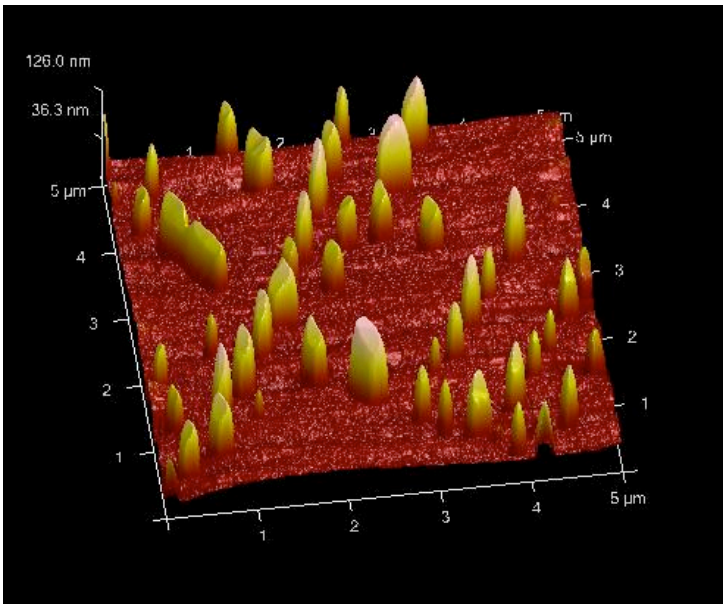

Figure 2. Atomic force microscopy image of microfluidized lemongrass oil-alginate nanoemulsion (3 cycles at 150 MPa). Adapted from Salvia-Trujillo et al. (2013)

Another useful technique that has been recently introduced to investigate the properties of lipid nanoparticles is Laser Scanning Confocal Microscopy (LSCM). In this technique, a laser as point illumination source is focused onto an entrance pinhole, providing a bright focused point source. Light from this point source is collected by a condenser and focused to a focus plane, where the sample is placed. The light emerging from the focused specimen is collected by the objective lens and focused at a second (exit) pinhole and to the detector (Conchello & Lichtman, 2005). The major advantage of using this technique is that it delivers very high-resolution images and avoids the scattering of light from out-of-focus specimens within the sample. The sample has to be previously labeled with a fluorescent dye and the system is equipped with a fluorescent detector. This technique has been typically used to study the microstructure of emulsions or biopolymers in complex systems (Dickinson, 2006). The fluorescent dye for lipids is usually Nile red, whereas for hydrophilic ingredients, e.g. proteins, it is Nile blue or fluorescein for sodium alginate. However, LSCM has a limit of detection above 100 nm, therefore it is not able to measure precisely the droplet size of nanoemulsions. However, it has been recently used to detect aggregation or coalescence of nanoemulsions as well as to study the changes experienced by nanoemulsions during their path in the gastrointestinal tract (Hur, Decker, & McClements, 2009; L Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013a). **Figure 3** shows the behavior of

nanoemulsions containing β-carotene during simulated mouth (A), stomach (B) and small intestine (C) (L Salvia-Trujillo et al., 2013a).

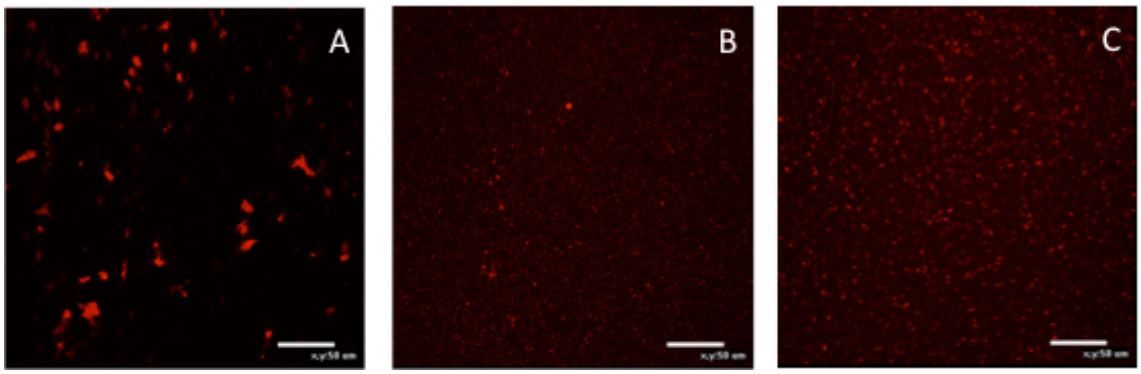

Figure 3. Confocal fluorescence microscopy images of corn oil-in-water emulsions loaded with β-carotene and stabilized by Tween 20 in their path through mouth (A), stomach (B) and small intestine (C) simulated conditions. Adapted from (Salvia-Trujillo et al., 2013a).

6 Potential advantages over conventional emulsions

6.1 Antimicrobial nanoemulsions

The encapsulation of antimicrobials in nanoemulsions arises as an attempt to improve the stability and the delivery of preservatives to food products. Most of the currently approved chemicals that can be used as antimicrobials in foods are water soluble, thus they are not suitable for incorporation in lipid nanodroplets systems. For this reason, the vast majority of research works dealing with nanoemulsions are conducted with natural essential oils as lipid phase. In the case of essential oils, the delivery in nanoemulsions may increase the effectiveness of active compounds in areas of the food system where target microorganisms are preferentially located. Nanoemulsions might be designed in several ways as delivery systems of antimicrobials for food applications, being possible to incorporate the antimicrobial compound in the lipid core, as a surfactant or both (**Figure 4**) (Jochen Weiss, Gaysinsky, Davidson, & McClements, 2009). On one hand, the lipid phase of the nanoemulsion can be loaded with the antimicrobial compound. In this case the migration of the antimicrobial compound via mass transport from the oil core to the microorganism surface is thought to be governed by the solubility of the antimicrobial in the aqueous phase. Because of their small droplet size, nanoemulsions can be expected to have a better antimicrobial activity than conventional emulsions. This fact can be attributed to the Laplace effect, since the mass transport of the

antimicrobial from the oil phase to the bulk phase is much higher (McClements, 2005). Moreover, nanoemulsions exhibit higher droplet size distribution homogeneity and an increased active surface area, which in principle would enhance the interaction of the active compounds through microbial membranes as well as favor the collision of nanoparticles and microorganisms. On the other hand, it is also possible to use antimicrobial substances as surfactants, such as lysozyme, nisin, lauric arginate (LAE) or SDS, or a combination of an antimicrobial core with an antimicrobial surfactant. Despite the potential applications of nanoemulsions as delivery systems of antimicrobials there is scarce research conducted on that topic and the factual benefits over conventional emulsions are still to be elucidated.

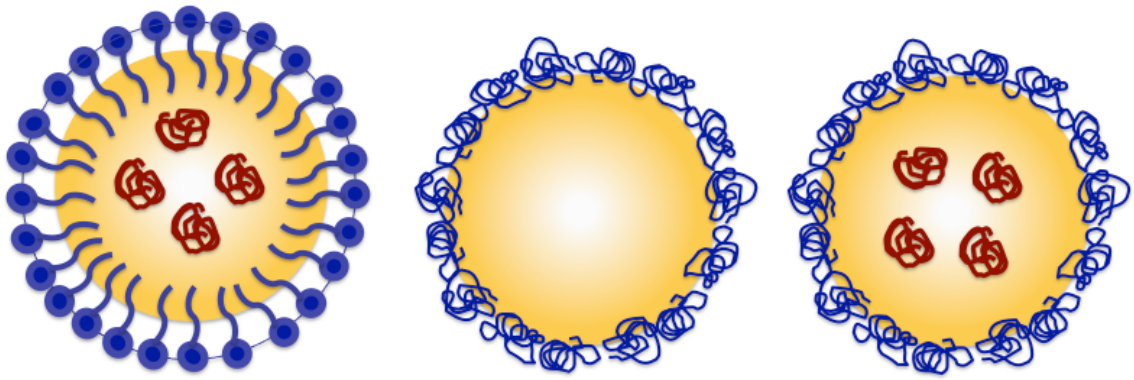

Figure 4. Different structures for antimicrobial nanoemulsions. From left to right: nanoemulsions with antimicrobial lipid and inert surfactant; nanoemulsions with surface-active antimicrobials and inactive lipid; and nanoemulsions with antimicrobial surfactant and lipid. Adapted from Weiss, Gaysinsky, Davidson, & McClements (2009).

Recently, some research works have contributed to establish the mechanism of interaction between essential oil-loaded nanoemulsions and food-borne microorganisms. **Table 3** presents the most relevant research papers concerning the delivery of antimicrobials incorporated in nanomulsions with a potential application in foods. Ziani and co-workers (Ziani et al., 2011) studied the interaction between thyme oil nanodroplets according to their interfacial composition and microbial cells, by using different types of surfactants (cationic, anionic and nonionic). They hypothesized that, since the surfaces of microorganisms are typically negatively charged, oil droplets with a positively charged surface would be attracted and would therefore exhibit an enhanced antimicrobial activity. However, the conclusion of their study was that the partitioning of the cationic or anionic surfactant molecules between the oil droplets and the microbial surface reduces the efficacy of nanoemulsions. Therefore, it would be more suitable to use nonionic surfactants to formulate delivery systems of essential oils to

inactivate microorganisms. At the same time, Donsí and coworkers (Donsì, Annunziata, Sessa, et al., 2011) as well Liang and co-workers (Liang et al., 2012) studied the antimicrobial properties of nanoemulsions containing aromatic compounds loaded in a lipid carrier. In both studies they observed a higher antimicrobial potential of nanoemulsions in comparison with the same concentration of non-emulsified essential oils. Therefore the enhanced penetration of aromatic compounds across microbial membranes seems to be feasible. More recently, Salvia-Trujillo and co-workers (Laura Salvia-Trujillo, Alejandra Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2014) have reported a faster inactivation kinetics of *Escherichia coli* in contact with lemongrass oil nanoemulsions produced by microfluidization, compared with emulsions of a greater droplet size produced by high shear homogenization. On the contrary, they stated that the production of nanoemulsions by ultrasonication depleted the antimicrobial activity of essential oils that could be induced by volatilization of the aromatic compounds during processing. Therefore, it can be stated that nanoencapsulation of essential oils in nanoemulsions may lead to a higher efficacy of the antimicrobial compounds against microorganisms. This could allow reducing the concentration of essential oils used for food preservation, overcoming at the same time the sensorial drawbacks due to their strong flavor. However, besides the nanoemulsion droplet size, other factors, such as a correct release from the oil droplets, might affect the bactericidal activity of emulsified antimicrobials; hence, the selection of the appropriate emulsifier seems crucial as well.

Table 3. Review of the most relevant results concerning the functionality of edible nanoemulsions in comparison with conventional emulsions.

Functional compound	Lipid carrier	Emulsifier	Droplet size (nm)	Fabrication method	Processing conditions	Functionality	Reference
Antimicrobial compounds							
Thyme essential oil	Corn oil (as ripening inhibitor)	Tween 80, lauric arginate (LAE) or sodium dodecyl sulfate (SDS)	≈ 200 nm	High Pressure Homogenization	9 kPa; 5 cycles	Impact of the nanemulsions interfacial properties on microbial membranes. Combination of thyme oil and antimicrobial surfactants (SDS or LAE) decreased the overall antimicrobial efficacy.	(Ziani 2011)
Peppermint essential oil	MCT	Modified starch	550-146 nm	High Pressure Homogenization	50-150 MPa; 1-20 cycles	Nanoemulsions with a prolonged long-term antimicrobial activity in comparison with unformulated oil	(Liang,R. 2012)
A mixture of terpenes and d-limonene	Sunflower oil and palm oil	Soy lecithin, Tween 20, and glycerol monooleate, modified starch	365-74 nm	High Pressure Homogenization	350 MPa; 10 cycles	Nanoemulsions with d-limonene with a lower minimum inhibitory concentration (MIC) compared to the non-encapsulated oil. Delayed microbial growth in orange or pear juices with the use of nanoemulsions.	(Donsì,F. 2011)
Lemongrass essential oil	-	Tween 80	< 10 nm	High Pressure Homogenization	50-150 MPa; 1-10 cycles	Nanoemulsions produced by microfluidization showed a faster inactivation kinetics against *Escherichia coli*	(Salvia-Trujillo,Laura, 2014)
Bioactive compounds							
β-carotene	Mixtures of medium-chain triglyceride (MCT) and corn oil as long-chain triglyceride (LCT)	Tween 20	415-146	High Pressure Homogenization	9000 psi; 3 cycles	The bioaccessibility of β-carotene was enhanced in low-fat nanoemulsions with increasing the percentage of long chain tryglycerides (LCT) in the lipid phase.	(Salvia-Trujillo,L. 2013)

β-carotene	Corn oil	Tween 20	23μm - 200 nm	High Speed Homogenization or High Pressure Homogenization	10,000 rpm for 2 min; 4 kpsi and 3 cycles; or 9 kpsi 5 cycles	Enhanced β-carotene bioaccessibility in nanoemulsions with smaller droplet size in comparison with conventional emulsions.	(Salvia-Trujillo 2013)
-	Medium chain triacylglycerol (MCT)	Modified starch, Tween 20 and whey protein isolate	155-172	Ultrasounds	175 W for 5 min	No cytotoxicity of nanoemulsions on Caco-2 cell monolayers	(Yu,H. 2013)
-	Corn oil:hexane	Tween 20	30–85 nm (radius)	High Pressure Homogenization and solvent displacement	15,000 psi; 5 cycles	Increased oil digestibility at decreasing emulsion droplet size	(Troncoso,E. 2012)
β-carotene	Corn oil as long chain triglycerides (LCT), medium chain triglyc-erides (MCT) or orange oil	Tween 20	< 200 nm	High Pressure Homogenization	9000 psi; 3 cycles	β -carotene bioaccessibility is highly dependent of the lipid carrier: in the order LCT $\gg$ MCT > orange oil	(Qian 2012)
Curcumin	MCT	Modified starch, Tween 20, whey protein isolate	218 nm	Ultrasounds	175 W for 5 min	Oral bioavailability of curcumin loaded in nanoemulsions enhanced 9-fold in comparison with the crystalline form	(Yu,H. 2012)
Curcumin	Long, medium, and short chain triacylglycerols (LCT, MCT and SCT)	β-lactoglobulin	< 100 nm (radius)	High Pressure Homogenization	9000 psi; 5 cycles	Curcumin bioaccessibility was highly dependent of the lipid carrier: the higher the chain length the higher the bioaccessibility. Nanoemulsions presented lower bioaccessibility than conventional emulsions	(Ahmed, 2012)
Resveratrol	Peanut oil	Soy lechitin or tween20	< 180 nm	High Pressure Homogenization	300 MPa; 10 cycles	Resveratrol with higher chemical and cellular antioxidant activity. Nanoencapsulated resveratrol non-metabolized is potentially absorbable in the intestinal wall.	(Sessa, 2011)

6.2 Bioactive nanoemulsions

Many bioactive compounds, such as several types of vitamins used in food fortification are lipophilic, so they are typically emulsified prior to incorporation into foods. Understanding the mechanisms of lipid digestion is a major goal to be achieved due to the importance of lipophilic nutrients in the human diet. It is known that emulsion droplet size and interfacial properties of oil droplets are key factors to modulate the digestibility of oils during the gastrointestinal tract as well as the release of encapsulated bioactive compounds.

Three recently published reviews by McClements & Li (McClements & Li, 2010), by Singh, Ye and Horne (Singh et al., 2009) and by Golding and Wooster (Golding & Wooster, 2010) report in detail the main changes in nanoemulsion structure during their path in the gastrointestinal tract (GIT). The digestion of lipids is a complex physicochemical and enzymatic process that basically consists on three steps in the mouth, stomach and small intestine (**Figure 5**). In the mouth, the presence of salts may alter the ionic strength of the system and induce changes in the emulsion stability. Also the mucin in the saliva can cause aggregation of nanodroplets by bridging or depletion flocculation. However, when the emulsion achieves the stomach, due to the low pH and shear conditions, the droplets can be re-dispersed or coalesce depending on the nanoemulsion characteristics (Salvia-Trujillo et al., 2013a). The hydrolysis of the oil droplets starts at the small intestine of the human gut and is performed by the pancreatic lipase. In some cases, the surfactant used to stabilize nanoemulsions can inhibit the adsorption of the lipase on the oil droplet surface. In this case, the bile salts present in the gut can displace the surfactant molecules of the oil-water interface, allowing the binding of the enzyme on the oil-water interface (Reis, Holmberg, Watzke, Leser, & Miller, 2009). As the lipolysis takes place, free fatty acids and monoglycerols are generated and accumulated on the oil surface. Due to their high surface activity, they can displace the lipase and interrupt the lipolysis reaction. Bile salts remove these digestion products from the oil droplet surface by solubilizing them in mixed micelles, thus facilitating the complete digestion. Finally, products from lipolysis as well as the encapsulated lipophilic compounds, if any, are solubilized in mixed micelles or unilamellar phospholipid vesicles, enabling them to adsorb across the intestinal lumen.

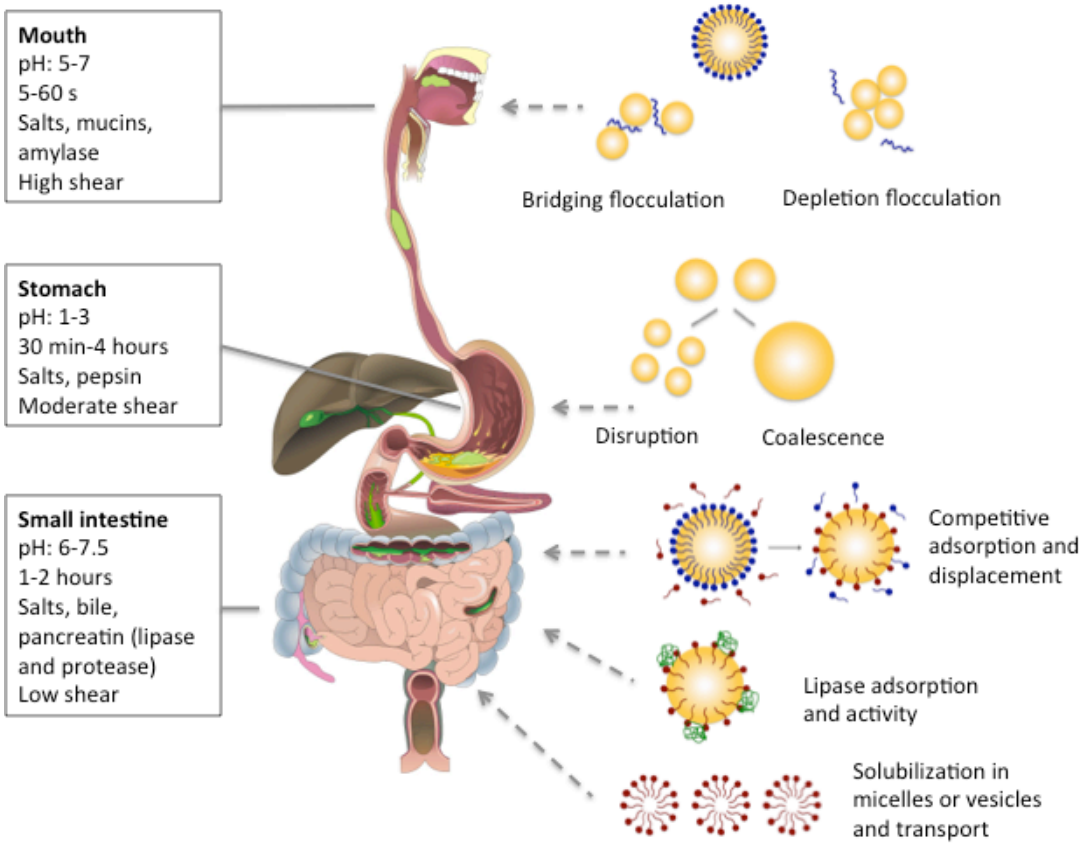

Figure 5. Representation of the possible changes in nanoemulsion structure during their path through the gastrointestinal tract (GIT). Adapted from Singh et al., (2009) and (McClements & Li, 2010). The schematic representation of the human digestive apparatus was obtained from the database of the Spanish Ministry of Education (http://recursostic.educacion.es/bancoimagenes/web/).

The comprehension of the lipid digestion in the GIT has been crucial to carry out the investigations dealing with the bioaccessibility and bioavailability of bioactive compounds loaded in nanoemulsions. The most significant research studies that contributed to describe the benefits of incorporating active lipophilic compounds in nanoemulsions are summarized in **Table 3**. On one hand, seems clear that the co-administration of hydrophobic bioactive compounds with a lipid phase facilitates their absorption after digestion (Pouton & Porter, 2008). For instance, Yu and co-workers (Yu & Huang, 2012) found that the bioavailability of curcumin-loaded nanoemulsions was increased 9-fold compared with the crystalline form. On the other hand, two other main factors might determine the functionality of active compounds in nanoemulsions being the droplet size and type of lipid carrier. Firstly, several authors (Salvia-Trujillo et al., 2013a; Troncoso, Aguilera, & McClements, 2012) have reported that the smaller the

droplet size of nanoemulsions, the higher the bioaccessibility of bioactive compounds encapsulated, which could attributed to the higher digestibility of the lipid phase. Namely, Salvia-Trujillo and co-workers (Salvia-Trujillo et al., 2013a) reported a β-carotene bioaccessibility of 60 % after *in vitro* digestion nanoemulsions with an initial droplet size of 120 nm, whereas in conventional emulsions was 34 %. The lower bioaccessibility of carotenoids in emulsions with a larger droplet size could be attributed to both a higher amount of non-digested oil that could have retained the β-carotene, and also a lower amount of mixed micelles to solubilize the bioactive compound. It can be stated that, in general, a reduction of the droplet size up to the nanometric scale would in principle favor the contact between the oil droplets and the lipase, facilitating the digestibility of the oil and, in consequence, the functionality of the active compound. However, a research conducted by Ahmed and co-workers (Ahmed, Li, McClements, & Xiao, 2012) evidenced a lower bioaccessibility of curcumin-loaded nanoemulsions compared with the conventional emulsions. Therefore, besides the droplet size, the role of the type of bioactive compound encapsulated is still due to be elucidated. Secondly, the composition of the lipid carrier, in terms of the length of the fatty acids of the oil phase, also shows to determine the bioaccessibility of the active compounds in the nanoemulsion system. In this sense, it has been reported that the *in vitro* digestion of nanoemulsions containing lipid carriers with a longer chain of fatty acids led to a higher bioaccessibility of lipophilic active compounds (Ahmed et al., 2012; Qian et al., 2012b; L Salvia-Trujillo et al., 2013b). For instance, Qian and co-workers (Qian et al., 2012b) observed that the lipid digestibility of nanoemulsions decreased in the order of long chain triglycerides (LCT; corn oil) > medium chain triglycerides (MCT; miglyol) > short chain triglycerides (SCT; orange oil). This could be correlated with the bioaccessibility of β-carotene, as it was higher in nanoemulsions with a greater lipid digestibility (LCT>MCT>SCT). This behavior might be explained by three reasons: (i) the higher digestibility of LCT favors the formation of more mixed micelles; (ii) there is less non-digested lipid phase that can bind the active compound, and; (iii) the longer free fatty acids released from the digestion of LCT nanoemulsions are able to form bigger mixed micelles to solubilize the lipophilic active compound. These results were further confirmed by Salvia-Trujillo and co-workers (Salvia-Trujillo et al., 2013b), observing a higher β-carotene bioaccessibility in noemulsions at increasing ratios of LCT in the lipid phase.

Based on the recently published research, it might be stated that incorporating lipophilic active ingredients in nanoemulsions seems to enhance the lipid digestibility and therefore the bioactive bioaccessibility. However, the biological fate of the non-digested oil fraction should be considered, as it could be adsorbed in the small intestine epithelium cells due to their sub-micron size and subsequently bioaccumulated in the liver instead of being excreted (Carey, Small, & Bliss, 1983; Mu & Høy, 2004). Up to our knowledge, there is only one recently published work dealing with in vivo toxicity testing of nanoemulsions conducted by Yo and co-workers (Yu & Huang, 2013). The authors reported no toxicity of nanoemulsions with droplet diameter below 200 nm in Caco-2 cells (small intestine cells). However, they detected a greater loss of viability in HepG2 cells (liver cells) after exposure to nanoemulsions compared with conventional emulsions. These results indicate that, despite the potential use of nanoemulsions to enhance the bioavailability of certain food ingredients, there is a need of information regarding the biological fate and potential toxicity of ingested nanoemulsions.

7 Applications in food products

The potential benefits of using nanoemulsions as delivery systems of food ingredients has launched to the market a number of upcoming products containing lipid nanoparticles. When it comes to scientifically based approaches, there are few research works evidencing the advantages of using nanoemulsions in foods. As far as we are concerned, only two research works are published with the application of nanoemulsions in complex foods. Donsì and co-workers (Donsì, Annunziata, Sessa, et al., 2011) incorporated nanoemulsions (d<200 nm) of terpenes as antimicrobials in orange and pear juices and evaluated the inactivation of inoculated *Lactobacillus delbrueckii* as well as their physicochemical characteristics during 16 days of storage time at 32 ºC. The addition of low concentrations of the nanoencapsulated terpenes was able to delay the microbial growth (1.0 g/l terpenes) or completely inactivate the microorganisms (5.0 g/l terpenes) while minimally altering the organoleptic properties of the fruit juices. Also in a recent work, Joe and co-workers (Joe et al., 2012) applied a sunflower-oil based nanoemulsion to extend the shelf life of fish steaks. They observed a significant reduction in the heterotrophic, H_2S and lactic acid bacteria populations as well as a significant shelf life extension during storage compared with the non-treated

samples. From the industrial point of view, despite the lack of scientific evidence of the efficiency of such kind of antimicrobial and bioactive delivery systems a number of food products are already available in the food market containing functional lipid nanoparticles. **Table 4** presents a list of commercialized products that incorporate nanosized delivery systems. In most of these products, the manufacturers provide the nanoemulsions or nanocapsules that are loaded with active compounds, such as coenzyme Q10, lycopene, lutein, β-carotene, omega-3, vitamins A, D3 and E, phytosterols or isoflavones ready to be used and incorporated in complex food formulations. In all cases they claim a protection of the active compound during food processing operations as well as enhanced bioavailability of the nutrients after digestion. However, the lack of standardized analytical techniques to proof such health claims opens a gap in the information to consumers.

Even though the research and the commercialization of food products incorporating nanoparticles as carriers of lipophilic active ingredients is boosted due to their appealing functional properties, their real benefits after being incorporated in food matrices and after food handling operations has yet to be confirmed. Therefore, the future research trends in this topic need to be directed towards the elucidation of the actual advantages of foods containing lipid nanoparticles in a more comprehensive approach, considering potential loss of functionality due to food matrix effect, food processing or storage.

Table 4. Products available in the market consisting or containing active nanemulsions.

Company	Product	Functionality
Tip Top®	Tip Top UP® Omega-3 DHA	Fortified with nanocapsules containing Omega-3 DHA rich tuna fish oil
Shemen industries	Canola Active oil	Fortified with nonesterified phytosterols encapsulated via a new nanoencapsulation technology (NSSL: Nano-sized self assembled liquid structures, developed by NutraLease (Israel) for optimising the absorption and bioavailability of target nutrients
RBC Life Sciences®, Inc.	NanoceuticalsTM Slim Shake Chocolate	Nanoscale ingredients that scavenge more free radicals, increase hydration, balance the body's pH, reduce lactic acid during exercise, reduce the surface tension of foods and supplements to increase wetness and absorption of nutrients
Shenzhen Become Industry & Trade Co., Ltd.	Nanotea	Nano-fine powder produced using nanotechnologies
Aquanova	NovaSOL sustain	Nano-carrier that introduces CoQ1O to address fat reduction and alpha-lipoic acid for satiety
Nano-Sized Self assembled Liquid Structures (NSSL) Supplements	NutraLease	Beverages containing encapsulated functional compounds such as coenzyme Q10, lycopene, lutein, β-carotene, omega-3, vitamins A, D3 and E, phytosterols and isoflavones are available. It is claimed that nanoemulsions can capture the flavor and protect it from temperature, oxidation, enzymatic reactions and hydrolysis and are thermodynamically stable at a wide range of pH values
Lypo-Spheric Vitamin C	LivOn Labs	The "smart" liposomal Nano-spheres® that encapsulate Lypo-Spheric™ Vitamin C gently slip across the intestinal wall and into the blood. That's because they are able to completely bypass a very restrictive nutrient transport system that radically limits the bioavailability of all non-liposome encapsulated forms of vitamin C.

Adapted from the Project on Emerging Technologies, 2009 (http://www.nanotechproject.org)

8 Regulation

Even though the potential benefits of the use of engineered nanoparticles in foods are arising with high expectations for the food industry, the associated risks that they may lead are still unknown. Nanomaterials may have a wide range of potential toxic effects, depending on their chemical nature (organic or inorganic), particle size distribution, particle shape, surface state (e.g. surface area, surface functionalisation, surface treatment), state of aggregation/agglomeration etc. There is a real need to elucidate the toxicology and biological fate of nanoparticles after digestion, to determine their behavior during the gastrointestinal tract and further possible bioaccumulation in human cells and tissues. The efforts of the scientific community are oriented towards this purpose, but the analytical techniques need validation and experiments with mice or human are expensive and tedious. Despite the lack of knowledge in this respect, the presence of food products containing nanoparticles in the market is growing. This has driven the authorities worldwide to release guidelines and regulations dealing with the use of nanomaterials in foods. Recently, some review papers have been published to describe the current situation according to the regulation of nanoparticles used in foods (Card, Jonaitis, Tafazoli, & Magnuson, 2011; Chau, Wu, & Yen, 2007; Cushen, Kerry, Morris, Cruz-Romero, & Cummins, 2012; Magnuson et al., 2013; Sandoval, 2009).

Concerning the regulation in the European Union, efforts have been made to control the use of nanomaterials in foods and there are several effective documents that are valid at the moment. As a general basis, the incorporation of engineered nanomaterials in foods must fulfill the current regulation for food additives (EU 1333/2008), being considered as new food ingredients. Already on February 2009, the European Food Safety Authority (EFSA) adopted a scientific opinion on the potential risks arising from nanoscience and nanotechnologies on food and feed safety. Recently, the Commission of the European Parliament, the Council and the European Economic and Social Committee, on the Second Regulatory Review on Nanomaterials (Brussels, 3.10.2012), compiled an inventory of nanomaterial types and uses, including safety aspects. As a conclusion of the communication, it is suggested that there is limited scientific evidence whether nanoparticles pose a risk for human health or not. The available toxicological knowledge on nanomaterials suggests that, despite the particle size, their toxicity relies on their nature and also reactivity with biological tissues. As a consequence, some

nanomaterials might be hazardous and some of them might be not, so there is a need for a more focused risk assessment of nanomaterials on a case-by-case basis. Moreover, due to the need of specific guidelines for nanoparticles, the EFSA published on May 2011 a guide for the risk assessment of engineered nanomaterials in foods as well as consumer exposure, considering their characterization, potential toxicity and biological fate. In this document, guidance on the characterization as well as the testing approaches to identify hazards arising from nanoparticles in foods is provided. In this report, it is stated that if the full digestion of a nanoparticle in the gastrointestinal tract can be proven, so there is no risk of potentially absorbed species and bioaccumulation in human gut, the toxicology evidence and regulation of the non-nano form can be used for commercial purposes. This statement is specifically relevant in the case of nanoemulsions, which are typically formulated with digestible oils and therefore would be exempt of special toxicological studies to be placed in the food market.

On October 2011, the European Parliament and the Council of the European Union published the Regulation EU nº 1169/2011 on the provision of food information to consumers, where it is stated that the presence of engineered nanomaterials in foods must be properly labeled. In September 2013, an amendment to the EU 1169/2011 has been submitted in order to distinguish between naturally occurring and manufactured nanoparticles. This new regulation will apply from December 2014. Therefore, the consumer acceptance for the successful commercialization of foods containing nanosized particles is crucial. For this achievement, the scientific community is due to provide reliable information dealing with the real associated risks of consuming engineered nanoparticles.

In the United States of America, there is a lack of specific regulation with respect to nanomaterials in food products. So far, the Food and Drug Administration (FDA) has released two guides regarding the application of nanomaterials in foods. On June 2011, they published a guide for food manufacturers to take into account possible considerations when incorporating nanoparticles in foods. Later on, on April 2012, the FDA also released a guide to assess the effect of manufacturing processes, including emerging technologies, which may cause significant changes on food ingredients already placed in the market and therefore may have an impact on their safety.

9 Future prospects

The results of research conducted so far indicate the potential benefits of nanoemulsions as delivery systems of active food ingredients, such as antimicrobials or bioactive compounds. In general, a higher functionality is observed due to their active surface area, leading to a greater interaction with microbial cells in the case of antimicrobial nanemulsions or a higher digestibility and resulting bioavailability in the case of nanoemulsions containing bioactive compounds. Nevertheless, the promising expectations arising from the recently published data are based on few research papers with concluding results that merit scrutiny and verification. On one hand, there is a need of further studies including a wide range of bioactive compounds or other food ingredients loaded in nanoemulsions to give a more comprehensive overview of the behavior and functionality of such kind of lipid nanoparticles. On the other hand, most of the research studying the functionality of lipid nanoparticles is conducted in model solutions. Therefore, the possible interactions between lipid nanoparticles and macromolecules present in food formulations, such as proteins, carbohydrates or fibers, are to be elucidated in order to present reliable data to the producers and consumers about the real benefits of nanoemulsions. In fact, it could be expected that the behavior of lipid nanoparticles in the GIT may change due to binding to other food components, thus keeping the lipases from adsorbing to the lipid droplets surface. Therefore the next step towards evidencing the real advantages of using nanoemulsions in foods would be carrying out experiments incorporating lipid nanoparticles in food matrices to study their functionality and biological fate. Moreover, despite lipid nanoparticles are usually formulated with edible ingredients and show a similar pattern compared to the digestibility of conventionally emulsified lipids, their toxicological safety cannot be certainly assured. Therefore, it should be noted that the biological path of lipid nanoparticles once they enter the human gut should be described in order to assess their biological fate, tissue location and possible bioaccumulation. For this purpose, complementary *in vivo* studies to the already established *in vitro* methodologies should be carried out to compare and verify the already existing information.

10 Concluding remarks

In the last decade there has been a rise in the interest for nanoemulsions among the food sector, previously introduced in the pharmaceutical and cosmetic sector, as delivery

systems of poor water-soluble ingredients. Their claimed potential advantages over conventional emulsions, such as improved stability and increase active surface area, have pointed out their direct application in the food ingredient sector as carriers of antimicrobials or vitamins. There is a strong research activity among food scientists to provide a comprehensive understanding of their formation, characterization and assessment of their biological activity. However, there is still scarce scientific evidence about their biological fate in the human gut as well as their possible toxicological implications of their consumption. In this sense, the authorities and government organizations are also working towards the regulation of the use of edible lipid nanoparticles. Despite the efforts to elucidate the real implications of incorporating nanoemulsions in foods, there is still a gap on the knowledge of their behavior compared with conventional emulsions. Therefore, further investigations regarding their behavior after incorporation to foods and during the food production chain, as well as their biological fate in the gastrointestinal tract are required in order to provide a more comprehensive understanding of their real benefits.

11 Aknowledgements

This study was supported by the Ministerio de Ciencia e Innovación (Spain) throughout the project AGL2009-11475. Laura Salvia-Trujillo thanks the Ministry of Science and Education (Spain) for the predoctoral grant. Prof. Olga Martín-Belloso thanks the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia 2008 Award.

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OBJECTIVES

The main objective of the current research was to design edible nanoemulsion-based delivery systems containing antimicrobials or bioactive compounds, being essential oils or β-carotene respectively. The initial hypothesis was that the emulsified active lipids with droplets in the nano-range can interact faster with the surrounding biological membranes or biomacromolecules compared with conventional emulsions, thus potentially showing an enhanced functional activity. Therefore, the purpose of the present thesis was to study the factors affecting the physicochemical characteristics and functionality of nanoemulsions containing essential oils or β-carotene. In order to achieve the above-mentioned general goal, the following specific objectives were proposed:

- To evaluate the influence of ultrasounds or microfluidization processing parameters on the physicochemical properties and antimicrobial activity of nanoemulsions containing lemongrass essential oil.
- To study the effect of the concentration of the components on the formulation of lemongrass essential oil nanoemulsions.
- To assess the ability of several essential oils to form nanoemulsions and their respective antimicrobial activity.
- To determine the feasibility of using nanoemulsion-based edible coatings containing essential oils to preserve the quality fresh-cut Fuji apples during refrigerated storage.
- To elucidate the influence of the emulsion droplet size on the *in vitro* lipid digestibility and β-carotene bio-accessibility.
- To study the effect of the lipid carrier composition and concentration on the *in vitro* lipid digestibility and β-carotene bio-accessibility.

MATERIAL AND METHODS

Material and Methods

Table of contents

In this section the logical sequence of actions carried out to fulfill the research objectives is depicted (Figure 1), and the experimental design as well as material and methods are described.

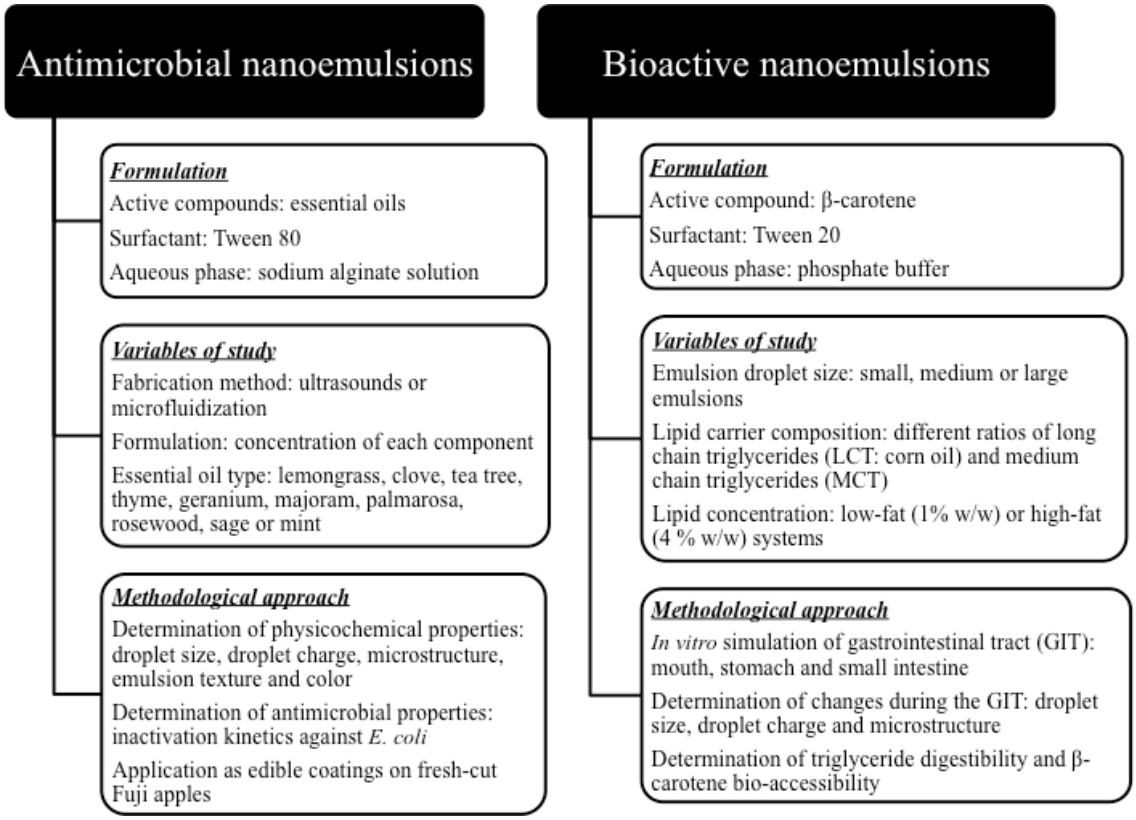

Figure 1. Project description to fulfill the proposed objectives and the corresponding methodological approach.

1 Nanoemulsion formation

The ingredients used in the formulation were previously mixed to obtain a coarse emulsion prior to the nanoemulsion formation. In those emulsions or naoemulsions formulated with sodium alginate as aqueous phase, it was previously dissolved in hot Mili-Q water at 70 ºC and continuous stirring until it was completely dissolved. Several antimicrobial essential oils were tested depending on the set of experiments, and were used directly with any further preparation. In the case of bioactive nanoemulsions, the oil carrier was enriched with β -carotene at 0.5% (w/w) by sonication (1 min) and mild heating (< 50ºC for 5 min) until complete dissolution, and the aqueous phase consisted on a phosphate buffer. Coarse emulsions were made by mixing the aqueous phase and the lipid phase with the surfactant by means of a laboratory scale digital mixer for 2

min. Nanoemulsions were produced by ultrasounds or microfluidization depending on the set of experiments.

Ultrasound treatment

A UP400S Hielscher sonifier (Hielscher Ultrasound Technology, Teltow, Germany), of 400 W nominal power and a frequency of 24 kHz equipped with a 22 mm sonotrode, was used to perform the ultrasonic treatment. The coarse emulsion was pumped into a stainless steel ultrasonic flow cell by means of a peristaltic pump (model Selecta- PR 2003) set at 100 mL/min giving a residence time of 16.5 s. The ultrasonic flow cell was equipped with a stainless steel double layer with flowing cold water to avoid excessive heating of nanoemulsions. The coarse emulsion was passed through the ultrasonic flow cell varying the sonication time (0, 30, 60, 120, 180 s) and at different ultrasound amplitudes (30, 60 and 100 μm).

Microfluidization treatment

Microfluidization processing implies that a coarse emulsion is passed through and interaction chamber using a high-pressure pumping device to obtain nanoemulsions (M110P, Microfluidics, Massachusetts, USA). The interaction chamber consists of two flow channels, which are designed so that they cause two streams of the coarse emulsion to impinge on each other at high velocity, thus creating a very high shearing action that provides emulsions with sub-micron droplet sizes.

At the outlet of the interaction chamber the product was refrigerated through an external cooling coil immersed in an ice-water bath so that the temperature of the product was always kept below 20 ºC. The number of processing cycles (from 1 to 10) and pressure (from 50 to 150 MPa) used varied depending on the set of experiments.

2 Physicochemical characterization

2.1 Droplet size

Droplet size and droplet size distribution of nanoemulsions were measured using dynamic light scattering (DLS) or static light scattering (SLS) techniques depending on the range of droplet sizes. DLS is more suitable for nanoemulsions with submicron lipid

nanoparticles (1-6000 nm) and narrow size distribution, while SLS is suitable for a wide range of lipid nanoparticles (0.2-2000 μm) with a polydisperse droplet size distribution.

2.1.1 Dynamic light scattering

The oil droplet size was measured using DLS technique with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK) working at 633 nm at 25 ºC and equipped with a backscatter detector (173 º). DLS measures the Brownian motion of nanosized droplets and relates this movement to an equivalent hydrodynamic diameter (Brar & Verma, 2011). The oil droplet size (nm) was characterized by distribution curves in intensity (%), average droplet size and polydispersity index. A polydispersity index near to 1 indicates a heterogeneous distribution between sizes of droplets. The oil absorbance at 633 nm, the oil refractive index and the continuous phase viscosity were determined with a spectrophotometer, a refractometer and a vibro-viscometer, respectively. These parameters were taken into account for the droplet size measurements by DLS.

2.1.2 Static light scattering

The particle size distribution was measured using SLS with a Mastersizer 2000 device (Malvern Instruments Ltd, Worcestershire, UK). Samples were diluted in phosphate buffer and stirred in the small-volume dispersion unit with a speed of 1250 rpm. The particle size was reported as either the surface-weighted mean diameter (d_{43}) or the volume-weighted mean diameter (d_{32}) (McClements, 2005).

2.2 Microstructure

The microstructure of nanoemulsions was assessed by transmission electron microscopy (TEM) and atomic force microscopy (AFM). Changes on the structure of β-carotene-loaded nanoemulsions during the simulated gastrointestinal tract were measured by confocal microscopy.

2.2.1 Transmission electron microscopy (TEM)

Nanoemulsions were observed by negative-staining electron microscopy as a direct measure of their droplet size and shape. The sample was adsorbed onto carbon-coated

copper/palladium grids for 1 min. Then, the grids were washed three times by floating it face-down on drops of sterilized, deionised water for 1 min. Finally, the sample was negatively stained by floating the grids face-down on a drop of 2% (w/v) ammonium molybdate at pH 6.5 for 1 min. The grids were observed in a Tecnai Spirit transmission electron microscope (FEI Company, Netherlands) at an acceleration voltage of 100 kV.

2.2.2 Atomic force microscopy (AFM)

Atomic force microscopy (AFM) experiments were carried out as a complementary tool for droplet characterization. Nanoemulsions were diluted 1:10 with ultra pure mili-Q water and a 20-μL drop was placed onto freshly cleaved mica for 10 minutes. After adsorption to mica surface at room temperature, samples were rinsed with ultra pure mili-Q water and then blow-dried with nitrogen compressed air. Samples were then observed using an extended multimode® 8 AFM head with a Nanoscope V controller (Bruker, Germany). AFM images were recorded in peak force tapping mode with triangular silicon cantilevers (nominal radius of 8 nm and spring constant of 0.5 N/m) at a scan rate of 1 Hz.

2.2.3 Confocal microscopy

Confocal fluorescence microscopy images were taken to determine structural changes that occurred in nanoemulsions within different phases of the gastrointestinal tract (GIT) model. Emulsions and samples taken from the GIT model were dyed with Nile red, a fat-soluble fluorescent dye that was previously dissolved at 0.1% (w/v) in ethanol. An air-cooled argon ion laser Model IMA1010 BOS (Melles Griot, Carlsbad, CA) was used to excite Nile red at 488 nm. A Nikon Confocal Microscope (Nikon D-Eclipse C1 80i, Nikon, Melville, NY) with a 60× oil immersion objective lens was used to capture the confocal images. The resulting fluorescent spectra of Nile red were detected in the 515 nm channel equipped with a narrow pass filter (HQ 515/30 m) with a pinhole size of 150 μm. The images generated had a size of 512 × 512 pixels, with a pixel size of 414 nm, and a pixel dwell time of 61.44 μs. All images were taken and processed using the instrument software program (EZ- CS1 version 3.8, Nikon, Melville, NY).

2.3 ζ-potential

The ζ-potential of lipid nanoparticles was measured by phase-analysis light scattering (PALS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK) to determine the surface charge at the interface of the droplets dispersed in the aqueous phase. Samples placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles.

2.4 Viscosity

A SV-10 vibro-viscometer (A&D Company, Tokyo, Japan) vibrating at 30 Hz was used to measure the viscosity of 10 mL-aliquots of the emulsions and nanoemulsions at room temperature.

2.5 Whiteness index

The color of emulsions and nanoemulsions was measured with a Minolta CR-400 colorimeter (Konica Minolta Sensing, Inc., Osaka, Japan) at room temperature set up for illuminant D65 and 10º observer angle and calibrated with a standard white plate. CIE L*, a* and b* values were determined and the whiteness index (WI) was calculated with the following equation (Eq. 1) (Vargas, Cháfer, Albors, Chiralt, & González-Martínez, 2008):

$$WI = 100 - \left(\left(100 - L \right)^2 + \left(a^2 + b^2 \right) \right)^{0.5} \quad \text{(Eq. 1)}$$

3 Antimicrobial activity determination

The antimicrobial activity of nanoemulsions containing essential oils was assessed by evaluating the *in vitro* inhibition of *Escherichia coli*. The method used was a modification of that previously described by other authors (Teixeira et al., 2007). *E. coli* 1.107 (Laboratoire de Répression des Fraudes, Montpellier, France) was cultured in tryptone soy broth (Bioakar Diagnostic; Beauvais, France) and incubated at 37 ºC with continuous agitation at 120 rpm for 11 h to obtain cells in stationary growth phase. The final concentration reached in the culture was 10^8-10^9 colony forming units/millilitre (CFU/mL). A 0.5 mL-aliquot of overnight bacterial culture was mixed with 0.5 mL of the lemongrass oil-alginate nanoemulsion and 4.5 mL of sterile Mili-Q water. To

determine the inactivation kinetics, an aliquot was taken after 5, 15 and 30 minutes of contact time to be serially diluted and spread on McConkey agar (Biokar Diagnostics, Beauvais, France) plates. A control was performed with the same method, replacing the nanoemulsion by sterile Mili-Q water. Colony counts were determined after incubation of agar plates at 37 ºC for 24 hours.

3.1 Modelling *E. coli* inactivation

The inactivation kinetics of nanoemulsions against *E. coli* were adjusted to a Weibull distribution model, by the following equation (Eq. 2) (Peleg, 2006):

$$\ln S(t) = -\left(\frac{t}{\beta}\right)^{\alpha} \qquad \text{Eq. (2)}$$

where S is the survival fraction (N/N_0) of *E. coli*, t is the contact time (min), β is the scale factor, and α is the shape factor. β represents the contact time (min) necessary to inactivate the first log-cycle of the microbial population. α describes the shape of the inactivation curve. A shape factor < 1 indicates a concavity upwards, and > 1 a concavity downwards.

The model was fitted to experimental data by non-linear regression using the Statgraphics Plus v.5.1 Windows package. The adjusted regression coefficients (R^2 adj) and accuracy factor (A_f, Eq. 3) (Ross, 1996) were calculated to evaluate the fitting of the model to experimental data.

$$A_f = 10^{\frac{\sum\left|\log\left(\frac{predicted}{observed}\right)\right|}{n}} \qquad \text{Eq. (3)}$$

where log (predicted/observed) is the logarithmic relation between the predicted and the experimental values, and n is the number of observations.

The highest the R^2 adj values and the nearer the A_f values to 1, the better the fitting of the model to experimental data.

4 Pseudo-ternary phase experimental design

An experimental mixture design was used to determine the effect of the concentration of sodium alginate, lemongrass essential oil (LEO) and surfactant on the formation of oil-in-water nanoemulsions. A D-optimal design was used to build up the pseudo-ternary phase diagrams and to define the optimum number of experiments. It was set up using

the software Design Expert 8.0.4 (Stat Ease Inc., Minneapolis, MN). The concentration of each component was limited according to the following constraints expressed in volume basis (v/v): 0.001 < LEO < 0.02; 0 < tween 80 < 0.06; 0 < sodium alginate < 0.02; 0.9 < water < 0.999; EO + tween 80 + sodium alginate + water = 1. The minimum concentration of LEO was set below its minimum inhibitory concentration (Hammer, Carson, & Riley, 1999) to see if there is an enhancement of the antimicrobial activity after emulsification, whereas the maximum concentration was set to limit its impact on sensory attributes. Tween 80 concentration was bound to an oil:surfactant ratio of 1:3, assuring a complete coating of lipid droplets surface with adsorbed surfactant molecules (Qian & McClements, 2011). Sodium alginate maximum concentration was set according to its viscosity, to avoid the subsequent block of the high-pressure homogenizer micro channels. The droplet size (z-average), ζ-potential, viscosity, color and antimicrobial activity of the mixtures were the responses measured for the different formulations. All samples were randomly prepared and analyzed during the same day without further storage. Two replicate analyses of each sample and parameter were performed and the mean value was calculated. Afterwards, experimental data was represented graphically with pseudo-ternary phase diagrams and modeled with a Scheffe polynomial equation (Eq. 4) for the 4 components to predict their effect in the formulation. In those responses where the differences between experimental values were very high, a Box-Cox transformation was applied to data to normalize the data and stabilize the variance. The statistical significance of the models for each response was considered regarding their adjusted R^2 and *p*-values.

$$Y = \sum \beta_i x_i + \sum\sum \beta_{ij} x_i x_j + \sum\sum\sum \beta_{ijk} x_i x_j x_k \qquad \text{Eq. (4)}$$

Where:

Y is the response variable; β_i is the first-order coefficient, β_{ij} is the second-order coefficient; β_{ijk} is the third-order coefficient; x_1 stands for LEO; x_2 stands for Tween 80; x_3 stands for water; x_4 stands for sodium alginate.

5 Application of antimicrobial nanoemulsions as edible coatings on fresh-cut Fuji apples

Coarse emulsions were prepared by mixing LEO at different concentrations (0.1%, 0.5% or 1% v/v) with the sodium alginate (2% w/v) solution and tween 80 (1% v/v). Then, coarse emulsions were treated by microfluidization (3 cycles at 150 MPa) to obtain nanoemulsions. Emulsions and nanoemulsions were degassed under vacuum and further used as film-forming solutions to form the edible coating onto fruit surface. Coarse emulsions and nanoemulsions containing different concentrations of lemongrass essential oil to further form the edible coatings were characterized in terms of emulsion droplet size, ζ-potential, viscosity and whiteness index were determined as described above.

5.1 Fresh-cut fruit coating

Apples were washed with a sodium hypochlorite solution and rinsed with tap water. The excess of water was removed from the surface with absorbent paper prior to processing. Both poles of sanitized fruits were cut off with a sharp knife and then six pieces (1.4 cm diameter per 2.0 cm height) were taken out from each apple with a hollow stainless steel sampling tube. A maximum of four fruits were processed at the same time to minimize excessive exposure to aggressive conditions. Apple pieces were dipped for 2 min into water (control), into the alginate solution (with Tween 80 but without LEO) or into the emulsions or nanoemulsions containing different concentrations of LEO. Residual solutions were allowed to drip off for 1 min, before submerging the coated fruits for 2 min in a solution of calcium chloride solution (2% w/v) containing citric acid (1% w/v). Ten pieces of uncoated or coated apple were placed in polypropylene trays of 500 cm^3 (Mcp Performance Plastic LTD, Kibbutz Hamaapil, Israel) and wrap-sealed using a 64 μm thickness polypropylene film with a permeability to oxygen of 110 cm^3 O_2 m^{-2} bar^{-1} day^{-1} at 23 ºC and 0% RH (Tecnopack SRL, Mortara, Italy) using a packaging machine (Ilpra Foodpack Basic V/G, Ilpra, Vigenovo, Italy). Trays were heat-sealed and stored in darkness at 4 ºC. Analyses were carried out periodically during 14 days of storage for randomly sampled pairs of trays.

5.2 Fresh-cut fruit microbial evaluation

5.2.1 *Escherichia coli* inactivation

To assess the bactericidal effect of edible coatings containing LEO, *E. coli* was inoculated on the coated apple slices. *E. coli* 1.107 (Laboratoire de Répression des Fraudes, Montpellier, France) was cultured in tryptone soy broth (Bioakar Diagnostic; Beauvais, France) and incubated at 37 ºC with continuous agitation at 120 rpm for 11 h to obtain cells in stationary growth phase. The final concentration reached in the culture was 10^8-10^9 colony forming units/milliliter (CFU/mL). Afterwards, a 500µL aliquot was spread homogeneously over 10 pieces of fruit. Then, fruit was coated as described previously without being used for any analysis other than *E. coli* determination. For *E. coli* counts evaluation, 10 g of fruit were taken from each tray, placed in a sterile plastic bag and mixed with 90 mL of saline peptone water (0.1 g peptone/100 ml water + 0.85g NaCl/100 ml water) for 1 min with a stomacher blender (IUL Instruments, Barcelona, Spain). Serial dilutions were prepared and spread on McConkey agar plates, which in turn were incubated at 37 ºC for 24 hours. Counts were expressed as $\log_{10}$ CFU/mL.

5.2.2 Microbial stability

Counts of psychrophilic bacteria or molds and yeasts on fresh-cut *Fuji* apples were determined during cold storage. A representative apple sample of 10 g was taken of each tray in aseptic conditions and placed in a sterile plastic bag. Then, 90 mL of sterile saline peptone water (0.1 g peptone/100 ml water + 0.85g NaCl/100 ml water) were added and homogenized for 1 min in a stomacher blender (IUL Instruments, Barcelona, Spain). Serial dilutions were made and 0.1 mL was spread over previously prepared plate count agar (PCA) or chloramphenicol glucose agar (CGA) and incubated at 5 ± 1 ºC for 10 days or for at 25 ± 1 ºC for 3 days for determination of psychrophilic bacteria or molds and yeasts, respectively. Plate counts were expressed as $\log_{10}$ CFU/mL.

5.2.3 Microbial growth modeling

The growth of molds, yeasts and psychrophilic bacteria were adjusted to a Gompertz model with the following equation (Eq. 5) (McKellar & Lu, 2004):

$$\log_{10} X(d) = A + C\exp(-\exp(-B(d-\mu))) \qquad \text{Eq. (5)}$$

where X is the number of cells (CFU/mL), d is the time of storage expressed in days, A is the asymptotic count as d decreases to 0, C is the difference in value of upper and

lower asymptote, *B* is the relative growth rate at μ, and μ is the day at which the absolute growth rate is maximum.

5.3 Fresh-cut fruit quality evaluation

5.3.1 Headspace gas analysis

The composition of the headspace of each tray was analyzed with a gas chromatograph equipped with a thermal conductivity detector (Micro-GC CP 2002 gas analyzer, Chrompack International, Middelburg, The Netherlands). A 1.7ml sample was automatically withdrawn from the headspace atmosphere. Portions of 0.25 and 0.33 mL were injected for O_2 and CO_2 determination, respectively. The O_2 content was analyzed with a CP-Molsieve 5 Å packed column (Chrompack International, Middelburg, The Netherlands) (4 m×0.32 mm, df=10 mm) at 60 ºC and 100kPa. For quantification of CO_2, ethylene (C_2H_4) and ethanol (C_2H_5OH), a Pora- PLOT Q column (Chrompack International, Middelburg, The Netherlands) (10 m×0.32 mm, df=10mm), held at 70 ºC and 200 kPa, was used. Two trays were taken at each sampling time to perform the gases analysis and two replicates were carried out for each one.

5.3.2 Color measurements

Cut apples surface color was directly measured with a Minolta chroma meter (Model CR-400, Minolta, Tokyo, Japan). The equipment was set up for illuminant D65 and 10º observer angle and calibrated using a standard white reflector plate. Twenty apple cylinders were analyzed for each pair of trays. Three readings were made in each replicate by changing the position of the apple pieces. Color was measured through changes in L and h* values. Numerical values of a* and b* parameters were employed to calculate the hue angle (h*) according to the following equation (Eq. 6):

$$h^* = \arctan\frac{b^*}{a^*} \qquad \text{Eq. (6)}$$

5.3.3 Firmness determination

The texture of apple slices was measured using a TA-XT2 Texture Analyzer (Stable Micro Systems Ltd, England, UK) by measuring the maximum penetration force required for a 4-mm-diameter probe to penetrate into apple cubes (20 mm high) to a

depth of 10 mm at a speed rate of 5 mm s^{-1}. Apple cubes were placed perpendicular to the probe to allow the penetration in their geometric center.

6 *In vitro* digestion of nanoemulsions

6.1 Simulated gastrointestinal tract (GIT) model

An *in vitro* GIT model consisting of mouth, gastric and intestinal phases was used to simulate the biological fate of nanoemulsions loaded with β-carotene:

Mouth phase: Simulated saliva fluid (SSF), containing mucin and various salts, was prepared according to a previous study (Sarkar, Goh & Singh, 2009). A 5 mL-aliquot of the emulsions were mixed with 5 mL of SSF, so that the final mixture contained 2% (w/w) oil. The pH of the mixture was adjusted to 6.8 and it was incubated at 37 ºC for 10 min with continuous agitation at 100 rpm.

Gastric phase: Simulated gastric fluid (SGF) was prepared using a method reported previously (Sarkar, Goh, Singh & Singh, 2009) by dissolving 2 g of NaCl and 7 mL of HCl (37%) in 1 L of water and adjusting the pH to 1.2 using 1.0 M HCl. The "bolus" sample from the mouth phase was mixed with SGF at a 50:50 volume ratio so that the final mixture contained 1% (w/w) oil. The pH of the sample was adjusted to 2.5 using NaOH (1M) and incubated at 37 ºC for 2 hours with continuous agitation at 100 rpm.

Small intestinal phase: A pH-stat (Metrohm, 147 USA Inc.) device was used to simulate the conditions in the small intestinal phase of the gastrointestinal tract (McClements & Li, 2010). An aliquot of 30 mL of the sample was placed in a temperature-controlled (37 ºC) chamber and the pH was set at 7.0 using NaOH solution. Then, 4 mL of bile extract (46.87 mg/mL) and 1 mL of calcium chloride (110 mg/mL) solutions dissolved in phosphate buffer were added to the sample and the pH was adjusted to 7.0 if necessary. Afterwards, 2.5 mL of freshly prepared lipase suspension (24 mg/mL) dissolved in phosphate buffer was incorporated into the mixture. The pH of the mixture was monitored and the volume of 0.25 M NaOH (mL) necessary to neutralize the free fatty acids (FFA) released from the lipid digestion (*i.e.,* to keep pH at 7.0) was recorded during 2 h. The amount of free fatty acids released was calculated from the titration curves with the following equation (Eq. 7) (Li & Mcclements, 2010).

It was assumed that each molecule of triglyceride (TAG) generates two molecules of FFA when completely digested (Mu & Høy, 2004):

$$FFA(\%)=\left(\frac{(V_{NaOH}\ (t).M_{NaOH}.M_{oil})}{(m_{oil}.2)}\right).100 \qquad \text{Eq. (7)}$$

where $V_{NaOH}(t)$ is the volume of NaOH solution required to neutralize the FFAs produced at digestion time t (L), M_{NaOH} is the molarity of the NaOH solution used to titrate the sample (mol L^{-1}), M_{oil} is the molecular weight of the oil (g mol^{-1}), and m_{oil} is the total weight of oil initially present in the incubation cell (g). The molecular weight of the corn and MCT oil was considered 800 g mol^{-1} and 400 g mol^{-1}, respectively. The molecular weight of the oil phase in the mixed emulsions was calculated proportionally to the ratio of both oils in the blend. Blanks were carried out in the absence of oil in the samples and subtracted from the reported values.

6.2 β-carotene bioaccessibility

The bioaccessibility of β-carotene was determined after the samples had been subjected to the simulated GIT model using the method described previously (Qian, Decker, Xiao, & McClements, 2012). An aliquot of raw digesta was centrifuged at 4,000 rpm for 40 min at 25 ºC (CL10 centrifuge, Thermo Scientific). The supernatant was collected and was assumed to be the "micelle" fraction in which the bioactive compound is solubilized. In some samples, a top layer of tiny droplets of non-digested oil was observed and it was removed from the micelle fraction. Aliquots of 5 mL of the raw digesta or the micelle fraction were mixed with 5 mL of chloroform, vortexed and centrifuged at 1750 rpm for 10 min at 25 ºC. The bottom layer containing the solubilized β-carotene was collected, while the top layer was mixed with an additional 5 mL of chloroform and the same procedure was repeated. The bottom chloroform layer was added to the previous one and analyzed spectrophotometrically (Ultraspec 3000 pro, GE Health Sciences, USA) at 450 nm. A cuvette containing pure chloroform was used as a reference cell.

The concentration of β-carotene extracted from a sample was determined from a calibration curve of absorbance *versus* β-carotene concentration in chloroform. The bioaccessibility was then calculated using the following equation (Eq. 8):

$$Bioaccessibility = 100 \times \left(\frac{C_{Micelle}}{C_{RawDigesta}} \right) \quad \text{Eq. (8)}$$

where $C_{micelle}$ and $C_{RawDigesta}$ are the concentration of β-carotene in the micelle fraction and in the overall sample (raw digesta) after the pH-stat experiment, respectively.

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PUBLICATIONS

PUBLICATIONS

Chapter I

Physicochemical characterization of lemongrass essential oil-alginate nanoemulsions: effect of ultrasound processing parameters

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Food and Bioprocess Technology 6 (9), 2439-2446

Abstract

The formation of lemongrass oil (1% v/v) nanoemulsions in aqueous sodium alginate solution (1% w/v) containing tween 80 (1% v/v) as non ionic surfactant was studied in terms of droplet size, electrical charge, viscosity and whiteness index considering different ultrasonication times (0, 30, 60, 120 and 180 s) and amplitudes (30, 60, 100 μm). The droplet size and size distribution of the emulsions decreased at increasing treatment time and amplitude. The minimum average droplet size observed in nanoemulsions was 4.31 ± 0.18 nm with a narrow size distribution. The interface electrical charge of the coarse emulsion was -18.0 ± 2.9 mV whereas in ultrasonicated nanoemulsions it diminished up to -55.8 ± 6.4 mV when the sonication time was extended for 180 s. The viscosity of nanoemulsions also decreased at increasing treatment time and amplitude. Moreover, nanoemulsions became translucent after sonicating for 180 s at 30, 60 or 100 μm with whiteness indices of 28.61 ± 0.17, 27.93 ± 0.21 and 27.86 ± 0.33, respectively. Therefore, it can be stated that ultrasound processing might be a feasible technology to produce highly translucent lemongrass oil-alginate nanoemulsions, with extremely small droplet sizes and high stability to be used as delivery systems of essential oils in food products. However, it is necessary to investigate the effect of ultrasound processing parameters on the antimicrobial potential of essential oils incorporated to nanoemulsions.

Keywords

Nanoemulsions, sodium alginate, essential oils, ultrasounds, droplet size, ζ-potential

Table of contents

1 Introduction

There is a growing awareness and concern among consumers regarding the use of synthetic chemical additives in foods. Therefore, foods preserved with natural additives have become more and more popular (Holley & Patel, 2005). In this sense, plant essential oils (EO) could be an alternative to chemical preservatives for extending the shelf life of food products. In fact, EO not only have shown *in vitro* antimicrobial activity against several food borne pathogens (Hammer, et al., 1999) but also in food systems (Tajkarimi, et al., 2010). Moreover, they are often used as flavouring agents in several food products (Fernaroli, 1995). In particular, lemongrass (*Cymbopogon citratus*) essential oil, apart from its appealing citric flavour, has shown to have a strong antimicrobial potential (Raybaudi-Massilia, et al., 2006), thus being susceptible for incorporation to food products. However, the use of EO in food products still presents limitations. On the one hand, their intense and persistent aroma significantly affects the organoleptical properties and consumer acceptance of foods (Raybaudi-Massilia, et al., 2008). On the other hand, they may present toxicological effects at high doses (Burt, 2004). Therefore, there is a need to reduce the concentration of EO incorporated to food products to avoid consumer rejection, toxicological effects, and to take in to account economical aspects (Sánchez-González, et al., 2011). In addition, their poor water solubility represents technological limitations for the industrial scaling up.

Oil in water nanoemulsions have been described as delivery systems to solubilize lipophilic ingredients, being a method to encapsulate ingredients into droplets, with smaller size in comparison with conventional emulsions (McClements & Rao, 2011; McClements, 2011; Sagalowicz & Leser, 2010; Solans, et al., 2005; Mason, et al., 2006). In this sense, nanoemulsions would be a good alternative to solubilize and incorporate essential oils to food products. The emulsion droplet size and size distribution determines emulsion properties, such as stability, rheology, appearance, color, texture and shelf life (McClements, 2005; Becher, 2001). Reducing the size of the active compounds incorporated within a solution would increase the surface area per mass unit of nano-sized biomaterials thus enhancing their biological or functional activity (Flanagan & Singh, 2006; Imran, et al., 2010). Therefore, the control, prediction, measurement and report of the droplet size in nanoemulsions are determining parameters (Jafari, et al., 2008). Moreover, nanoemulsions seem to present

optical transparency, enhanced functionality and physical stability, which would make them very attractive as delivery systems for food products (Gutiérrez et al., 2008). For this purpose, several stabilizers and biopolymers, such as polysaccharides, are being used to formulate nanoemulsions (McClements, 2011). Recently, the generation of nanoemulsion-based delivery systems by means of ultrasonication has been reported. For instance, Spinelli et al. (2010) obtained synthetic oil nanoemulsions with droplet sizes lower than 100 nm by ultrasounds. In other research works presented by Leong et al. (2009) or Kentish et al. (2008) edible oil nanoemulsions are produced by power ultrasounds. However, there is a lack of published data about the effect of ultrasound processing on the formation of nanoemulsions containing essential oils. Moreover, up to our knowledge, there is no reported data about the effect of alginate as a biopolymeric aqueous phase on nanoemulsion formation and stabilization.

Thus, the aim of the present work was to study the influence of ultrasound processing parameters (amplitude-μs and treatment time-s) regarding the droplet size, stability, whiteness index and viscosity of O/W nanoemulsions containing lemongrass oil as lipid phase and sodium alginate in aqueous phase.

2 Material and methods

2.1 Primary emulsion formation

Sodium alginate (1% w/v) (FMC Biopolymers, UK) was dissolved in hot water at 70 ºC and continuously stirred until it was completely dissolved. A coarse or primary emulsion was made by mixing lemongrass essential oil (1% v/v) (Laboratoris Dicana, Spain) as lipidic phase and Tween 80 (1% v/v) (Scharlau, Spain) as surfactant with a laboratory T25 digital Ultra-Turrax mixer (IKA, Staufen, Germany) working at 3400 rpm for 2 min. All samples were prepared using ultra pure water obtained from a Mili-Q filtration system. Physico-chemical characterization of primary emulsion is shown in Table 1.

Table 1 Physicochemical characterization of the coarse lemongrass oil-alginate emulsion obtained with a high speed blender at 3400 rpm for 2 min.

Average droplet size (nm)	1410 ± 366
ζ-potential (mV)	-18 ± 3
Whiteness index	46.8 ± 0.1
Viscosity (mPa.s)	30.8 ± 0.9
Density (g/mL)	1.005 ± 0.001
pH	6.026 ± 0.011

2.2 Nanoemulsion formation

A UP400S Hielscher sonifier (Hielscher Ultrasound Technology, Teltow, Germany), of 400 W nominal power and a frequency of 24 kHz equipped with a 22 mm sonotrode, was used to perform the ultrasonic treatment. The coarse emulsion was pumped into a stainless steel ultrasonic flow cell by means of a peristaltic pump (model Selecta- PR 2003) set at 100 mL/min giving a residence time of 16.5 s. The ultrasonic flow cell was equipped with a stainless steel double layer with flowing cold water to avoid excessive heating of nanoemulsions. The temperature of emulsions was monitored at the outlet of the ultrasonic flow cell (Table 2). For the ultrasound treatment, the coarse emulsion was passed through the ultrasonic flow cell varying the sonication time (0, 30, 60, 120, 180 s) and at different ultrasound amplitudes (30, 60 and 100 μm). Ultrasonicated nanoemulsions were analyzed after processing without further storage.

Table 2 Temperature (ºC) of coarse emulsion and nanoemulsions after ultrasound processing at different sonication times (s) and amplitudes (μm)

	Amplitude		
Sonication time (s)	30 μm	60 μm	100 μm
0	20	20	20
30	21	32	35
60	22	36	41
120	25	39	46
180	27	39	47

2.3 Particle size and electrophoretic mobility

The oil droplet size was measured by dynamic light scattering (DLS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK) working at

633 nm at 25 ºC and equipped with a backscatter detector (173 º), which is appropriate to measure sub-micron particles (Brar & Verma, 2011). DLS measures the Brownian motion of nanosized droplets and relates this movement to an equivalent hydrodynamic diameter (nm). Average droplet size, size distribution curves in intensity (%) and polydispersity index were used to characterize oil droplets dispersion in nanoemulsions. The lemongrass oil absorbance at 633 nm was 0.001 and its refractive index was 1.487. The electrophoretic mobility of oil droplets, also reported as ζ-potential, was measured by phase-analysis light scattering (PALS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK). It determines the surface charge at the interface of the droplets dispersed in the biopolymeric solution.

2.4 Viscosity

Viscosity of emulsions was measured from 10 mL of the nanoemulsions at ambient temperature using a SV-10 vibro-viscometer (A&D Company, Tokyo, Japan) vibrating at 30 Hz. The actual temperature of each sample was recorded with a thermometer. The viscosity of the sodium alginate solution (1% w/v) was also measured, which was 30.00 MPa.s. Therefore, it was considered the viscosity of the dispersant material in lemongrass oil-alginate nanoemulsions.

2.5 Whiteness index

The color of nanoemulsions was measured with a Minolta CR-400 colorimeter (Konica Minolta Sensing, Inc., Osaka, Japan) at room temperature set up for illuminant D65 and 10º observer angle and calibrated with a standard white plate. CIE L*, a* and b* values were determined and the Whiteness Index (WI) was calculated with equation 1 (Vargas et al., 2008):

$$WI = 100 - ((100 - L)^2 + (a^2 + b^2))^{0.5} \quad \text{Eq. (1)}$$

2.6 Statistical analysis

All the experiments were assayed in duplicate, and two replicate analyses were carried out on each parameter in order to obtain mean values. Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, MD) was used to perform the analysis of variance. The least significant difference (LSD) test was run to determine significant differences among treatment conditions at a 5% significance level.

3 Results and discussion

3.1 Droplet size and size distribution

Ultrasound amplitude (μm) and treatment time (s) significantly affected the droplet size and size distribution of lemongrass oil-alginate nanoemulsions, as can be observed in Fig 1 and Fig 2. The average droplet size of lemongrass oil-alginate nanoemulsions decreased at increasing sonication amplitude and time. An ultrasonic treatment at 30 μm for 30, 60, 120 or 180 s reduced the average droplet size of nanoemulsions to 34.95 ± 8.55, 15.43 ± 2.90, 6.88 ± 1.34 or 5.12 ± 0.23 nm, respectively, but the size distribution was always multimodal. This fact indicates that dispersed droplets remain heterogeneous regardless the ultrasonication time. The same behaviour was observed at ultrasonic amplitude of 60 μm. When the treatment was conducted at 100 μm amplitude for 30 s the average droplet size was 10.67 ± 1.80 nm but with large particles that were not ruptured. Nevertheless, increasing the treatment time up to 180 s at 100 μm larger particles were disrupted, achieving a monomodal size distribution with an average droplet size of 4.31 ± 0.18 nm. Thus, the high acoustic power density delivered at 100 μm amplitude would be able to cause the rupture of larger particles. In fact, several authors have reported that high sonication times and power are required for droplet size reduction during emulsification processes in high-viscous media (Canselier et al., 2002), such as the sodium alginate solution used in the present work. Although it is known that increasing the temperature, typically above 80ºC, enhances the formation of nanoemulsions (Rao & McClements, 2011), the temperature increase observed after sonication treatment was not considered to have an effect on nanoemulsion formation.

The droplet radius is the sum of the radius of the oil core and the thickness of the interface, which is determined by the size of the surface active molecules (McClements, 2011). Emulsions are commonly stabilized with non-ionic surfactants, which are small surface active molecules adsorbed at the O/W interface. However, when larger molecules (e.g. biopolymers) are used in an emulsion an increase in the droplet size would be expected. Unfortunately, there is no information about their behavior when non-ionic surfactants and biopolymers are added together in a nanoemulsion system, as in this study. Nevertheless, Jafari et al. (2007) reported sub-micron emulsions of d-limonene oil stabilized by modified starch with an average droplet size of 500 nm approximately after applying an ultrasound treatment at 100 μm for 100 s. Pongsawatmanit et al. (2006) stated that alginate molecules were adsorbed at the

interface of palm oil emulsions containing β-lactoglobulin, forming a multilayered interface. In the present work, it is believed that alginate and Tween 80 molecules are both adsorbed at the oil droplet surface leading to a complex interface.

Previously, other authors have used ultrasounds to obtain nanoemulsions. Namely, Gaikwad & Pandit (2008) studied the effect of ultrasound processing on the droplet size of paraffin, groundnut or soybean oil in water emulsions. They stated that smaller droplet sizes and narrower size distributions were observed at longer sonication times and higher power applied, which is proportional to the amplitude. Spinelli et al. (2010) reported the same behavior in synthetic oil (decane: toluene: cyclohexane; 50: 30: 20) in water emulsions stabilized with non-ionic surfactants, reaching a minimum average droplet size of 30 nm by increasing the sonication time up to 8 minutes. Similarly, Kentish et al. (2008) obtained nanoemulsions of flaxseed oil stabilized with Tween 40 with a minimum average droplet size of 130 nm with an ultrasound treatment for 51 s at 330 W of nominal power. Likewise, Leong et al. (2009) attained sunflower oil nanoemulsions of up to 40 nm of average droplet size by applying 30 μm amplitude ultrasounds for 20 min in a batch mode cell.

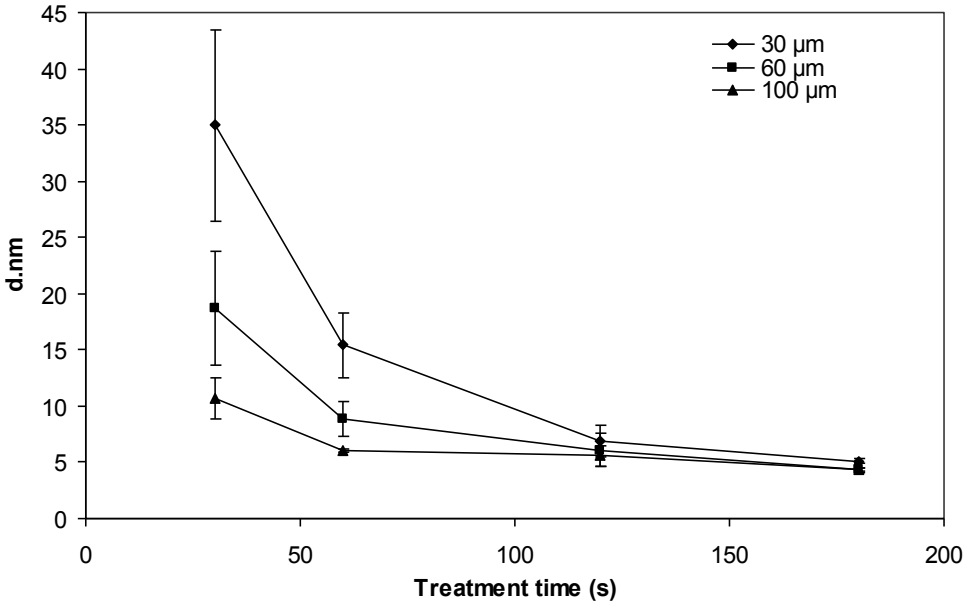

Fig. 1 Effect of ultrasound treatment time at different amplitudes (30, 60 and 100 μm) on the average droplet size of lemongrass oil-alginate nanoemulsions. Data shown are a mean ± standard deviation.

The use of nanoemulsions to incorporate active compounds in foods is emerging with several potential advantages, as the solubilization of essential oils in nano-sized droplets

might lead to an enhanced antimicrobial activity (Rojas-Graü, et al., 2009). However, a result of such increase in the solubility and permeability of active substances may cause unexpected toxicity reactions, being a relevant issue when dealing with food, consumers and health (Sandoval, 2009; Chau, et al. 2007; Chaudhry, et al. 2008). Therefore, it is needed to continue research in order to completely understand the mechanisms of interaction of lipid nanoparticles with food and human body.

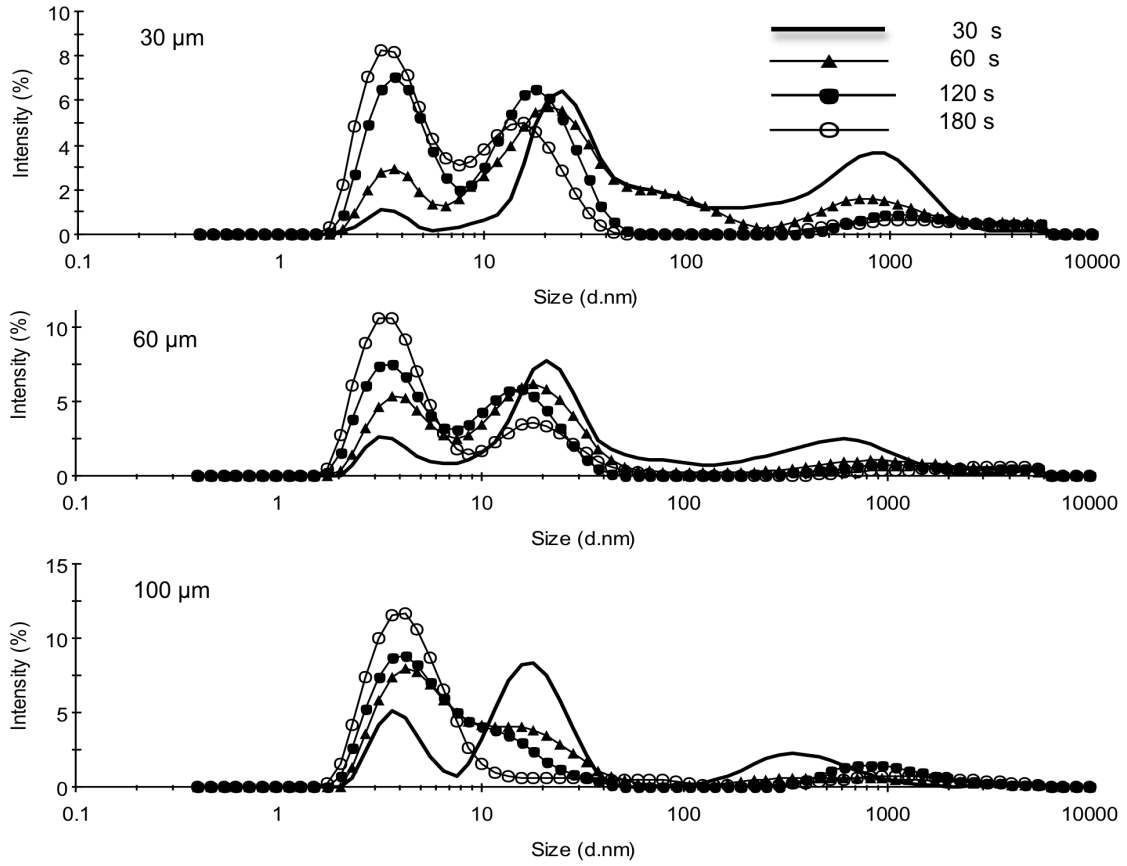

Fig. 2 Effect of ultrasound treatment time at different amplitudes (30, 60 and 100 µm) on the droplet size distribution of lemongrass oil-alginate nanoemulsions.

3.2 Zeta potential

The effect of ultrasound homogenization parameters on the electrical charge of lipid droplets surface is shown in Fig 3. Regarding the coarse emulsion, the electrical charge at interface of lipid drops was -18.0 ± 2.9 mV. When the sonication time increased, the zeta potential became significantly more negative, regardless the ultrasonic wave amplitude. The ζ-potential of emulsions rapidly decreased up to -38.0 ± 10.4 mV after

applying an ultrasound treatment for 30 s. Generally, particles with ζ-potential more positive than +30 mV or more negative than -30 mV are considered to be stable, since electrical charge of droplets is strong enough to assume that repulsive forces between droplets are predominant in the nanoemulsion system (Heurtault et al., 2003). When the sonication time was extended up to 180 s the ζ-potential of oil drops ranged between -46.0 ± 3.3 and -55.8 ± 6.4 mV, thus leading to highly stable nanoemulsions.

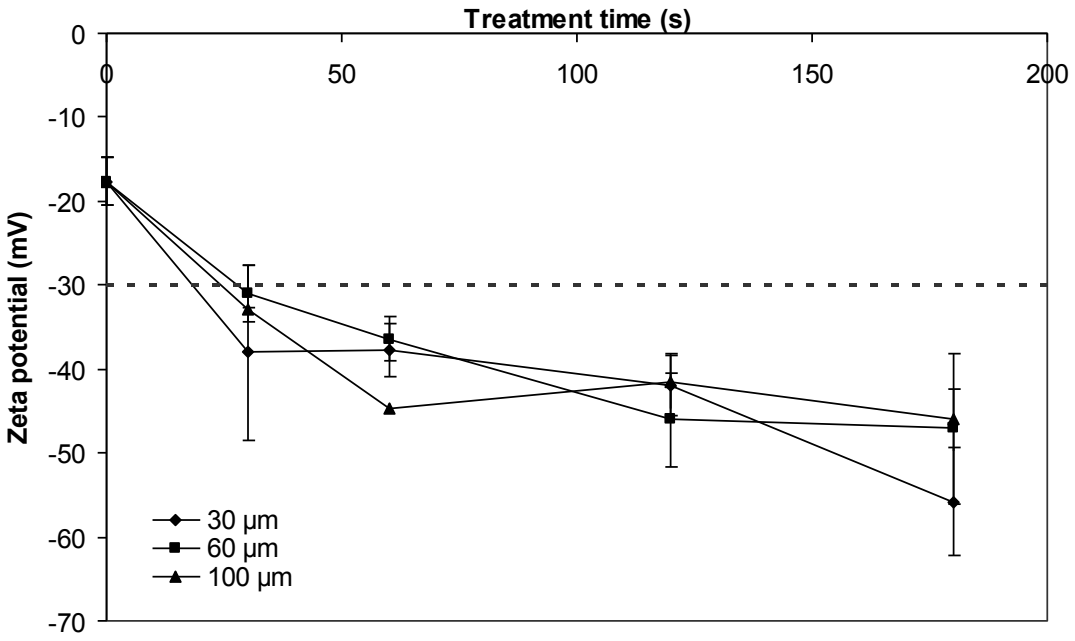

Fig. 3 Effect of ultrasound treatment time at different amplitudes (30, 60 and 100 µm) on the ζ-potential of lemongrass oil-alginate nanoemulsions. Data shown are a mean ± standard deviation.

Camino & Pilosof (2011) also reported a higher stability for ultrasound treated oil in water emulsions stabilized with hydroxypropylmethylcellulose (HPMC) compared to emulsions prepared with high shear homogenization. Similarly to the results in this study, Pongsawatmanit et al. (2006) observed a strong droplet charge of oil in water emulsions stabilized with a 0.5% (w/w) of sodium alginate, showing a ζ-potential up to -69 mV. Oil droplets disruption induced by ultrasounds is likely to promote surfactant and alginate molecules to adsorb at oil/water interface, creating a negative electrical charge surrounding oil droplets that prevents further flocculation or aggregation phenomena. It is known that, despite being non ionic surfactants, polysorbate molecules adsorbed to droplet surface lead to negative electrical charges when pH of the emulsion

system is higher than 4 (Hsu & Nacu, 2003). Moreover, anionic alginate molecules, can also adsorb to droplet surface giving a complex interface, which composition may be determined by concentration of each type of surface-active substances, their relative affinity for the interface, the method used to prepare the emulsion, the solution conditions or the order in which the emulsifiers were added (McClements, 2005).

3.3 Viscosity

The viscosity of coarse emulsion before being sonicated was 30.8 ± 0.9 mPa.s. However, the viscosity of nanoemulsions was significantly affected by the ultrasound processing parameters (Fig 4), since it decreased gradually when the sonication time and ultrasound amplitude increased. In fact, after applying ultrasound treatments during 180 s at 30, 60 or 100 μm amplitude, the viscosity of the nanoemulsion system was 12.45 ± 0.77, 11.05 ± 0.21 or 10.03 ± 0.52 mPa.s, respectively, which represents more than a 50% reduction regarding the viscosity of the coarse emulsion. Besides the properties of biopolymeric solution that constitutes the aqueous phase, there are several factors that influence emulsion viscosity, such as the disperse phase volume fraction, rheology of component phases, droplet size, colloidal interactions or droplet charge (McClements, 2005; Pal, 2011; Tadros, 1994). Therefore the contribution of changes in droplet size and ζ-potential to the differences in viscosity of the nanoemulsion systems was studied (Fig 5). A positive correlation was observed between the viscosity of nanoemulsions and their respective droplet size or ζ-potential with coefficients of correlation of 0.72 and 0.58, respectively. It is known that influence of droplet size and droplet size distribution on viscosity of an emulsion depends on the disperse phase volume fraction and the nature of colloidal interactions (Mewis & Macosko, 1995). Oppositely to the results in this study, it has been reported that the higher the droplet size the lower the emulsion viscosity, at the same dispersed phase concentration and shear stress (Chanamai & McClements, 2000; Cortés-Muñoz et al., 2009; Derkach, 2009; Pal, 2011). However, this fact was mainly observed in highly concentrated emulsions, with the oil phase above 20% (Chanamai & McClements, 2000; Cortés-Muñoz et al., 2009). Moreover, droplet size polydispersity seems to play an important role in emulsion rheology. Pal (1996) observed a decrease on the viscosity of a bimodal emulsion obtained by mixing a coarse (big droplet size) and a fine (small droplet size) emulsion, in comparison with the coarse emulsion, as it seems that, in the bimodal

emulsion, the smaller droplets serve to isolate or lubricate the large droplets. On the other hand, droplet charge may influence rheological properties of emulsions due to electroviscosus effects, since repulsive forces oppose to movement and increase emulsion viscosity (Hiemenz & Rajagopalan, 1997). Therefore, it is possible that viscosity changes observed in the present work are due to the effect of ultrasound processing on alginate molecules. In fact, Camino et al. (2009) studied the effect of ultrasound processing on the functional properties of HPMC biopolymer. They observed that apparent viscosity of HPMC solutions decreased after applying a sonication treatment revealing molecular weight changes or changes in the interaction between water and the polysaccharide.

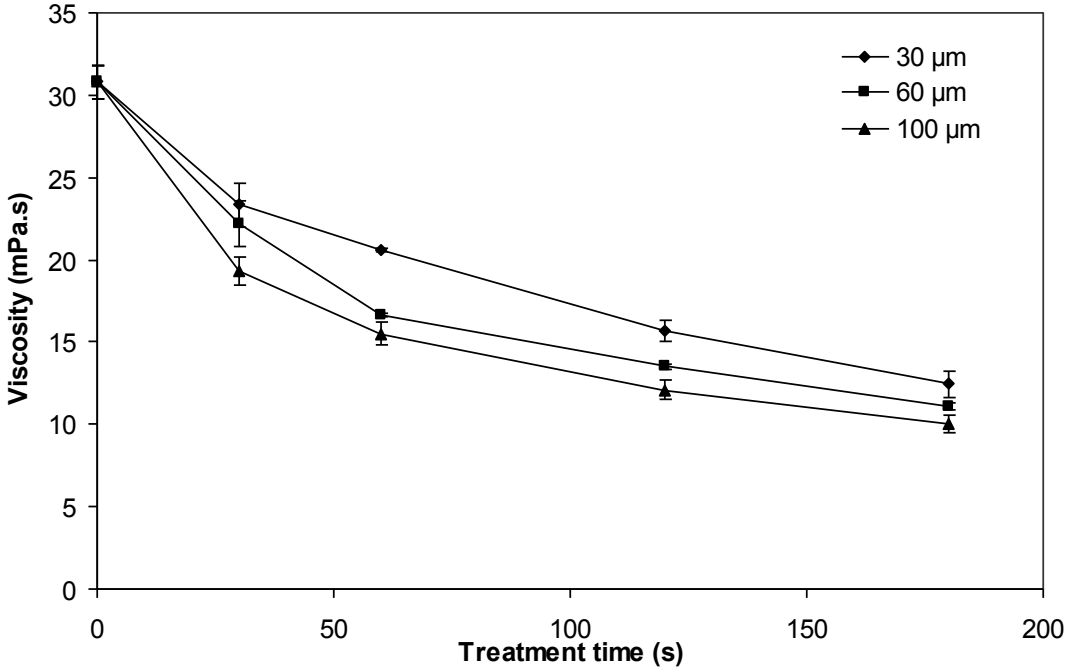

Fig. 4 Effect of ultrasound treatment time at different amplitudes (30, 60 and 100 µm) on the viscosity of lemongrass oil-alginate nanoemulsions. Data shown are a mean ± standard deviation.

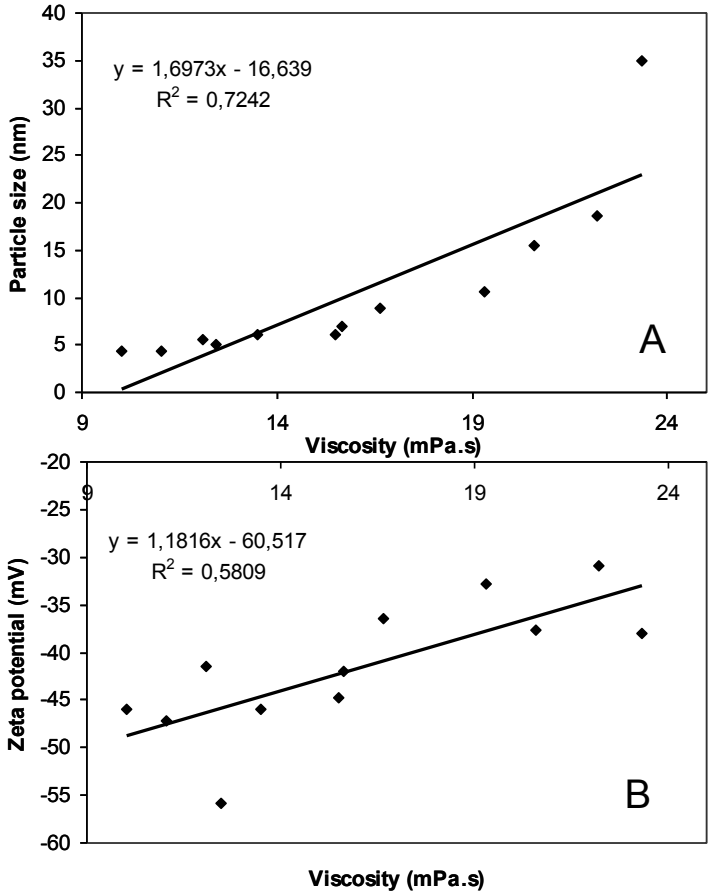

Fig. 5 Correlation of viscosity with droplet size (A) and ζ-potential (B) of lemongrass oil-alginate nanoemulsions.

3.4 Whiteness index

The WI of emulsions decreased significantly at increasing sonication time and amplitude (Fig 6). The WI of the primary emulsion was 46.78 ± 0.16, and after applying an ultrasound treatment for 30 s at 30, 60 or 100 μm of amplitude it diminished up to 39.39 ± 0.95, 35.65 ± 0.65 and 32.56 ± 0.57, respectively. Increasing the sonication treatment time up to 60 s at 30, 60 or 100 μm caused a decrease in WI to 33.17 ± 0.48, 31.13 ± 0.19 and 30.04 ± 0.06, respectively. By extending the ultrasound treatment for 120 s the WI of nanoemulsions slightly diminished, but it remained practically constant beyond this point, regardless the sonication amplitude. Regarding visual observations, emulsions started to become translucent after 60 s of ultrasound processing irrespectively of the amplitude, and presented a higher translucency after 180 s (Fig 7).

Up to our knowledge, there are no studies reporting the effect of ultrasound processing on emulsion color. However the effect of oil concentration, droplet size and droplet size

distribution on emulsion appearance has been well studied (Chanamai & McClements, 2001; Chantrapornchai et al., 1998). According to McClements (2005), lightness of emulsions increases at increasing droplet size. When a beam of white light is incident upon the outer surface of an emulsion one part of the light is transmitted and another part is reflected (Frei et al., 1975). The fraction that is reflected and its direction depend on the refractive index of the droplets and continuous phase, as well as on the size and size distribution of the droplets (McClements, 2002). It is known that sub-micron emulsions with droplet size in the range of 50-200 nm are translucent or transparent systems (Tadros et al., 2004). Likewise, these results show a strong relationship between droplet size and color of nanoemulsions, with a correlation coefficient of 0.94 (Fig 8).

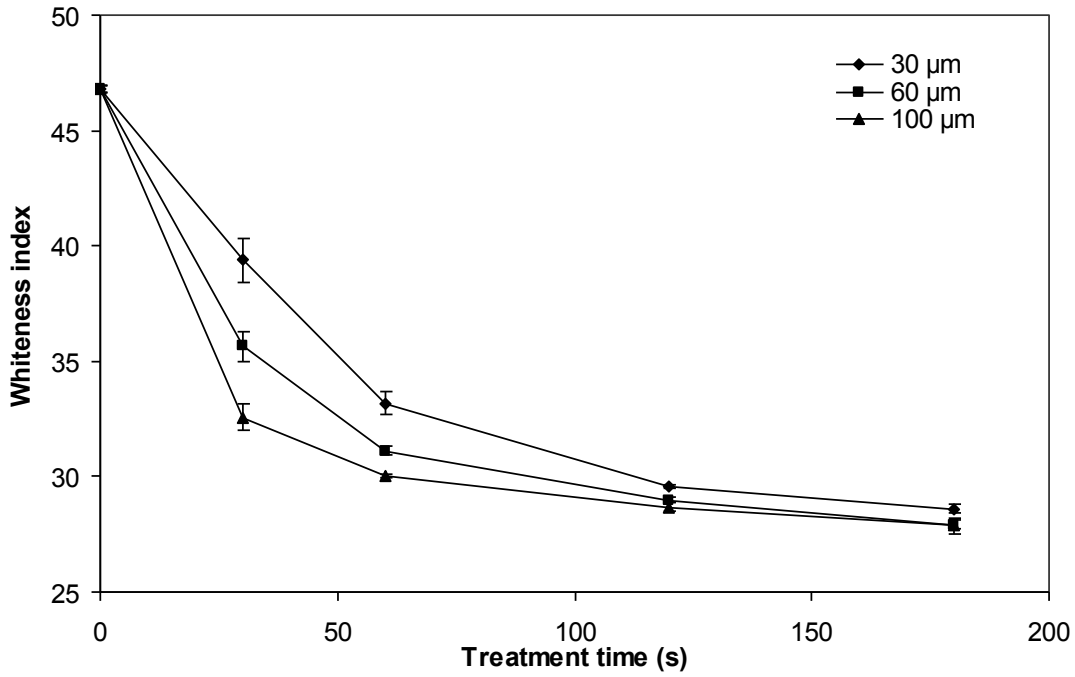

Fig. 6 Effect of ultrasound treatment time at different amplitudes (30, 60 and 100 µm) on the whiteness index of lemongrass oil-alginate nanoemulsions. Data shown are a mean ± standard deviation.

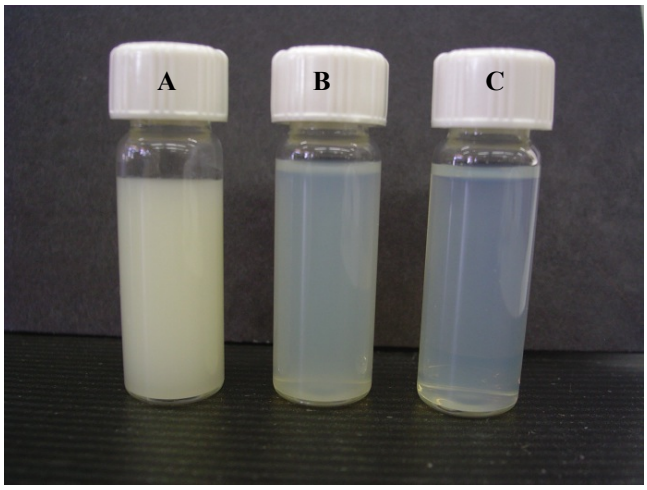

Fig. 7 Coarse emulsion (A) and nanoemulsions treated by ultrasounds for 60 s (B) and 180 s (C) at 100 μm containing lemongrass oil (1% v/v), alginate (1% w/v) and tween 80 (1% v/v).

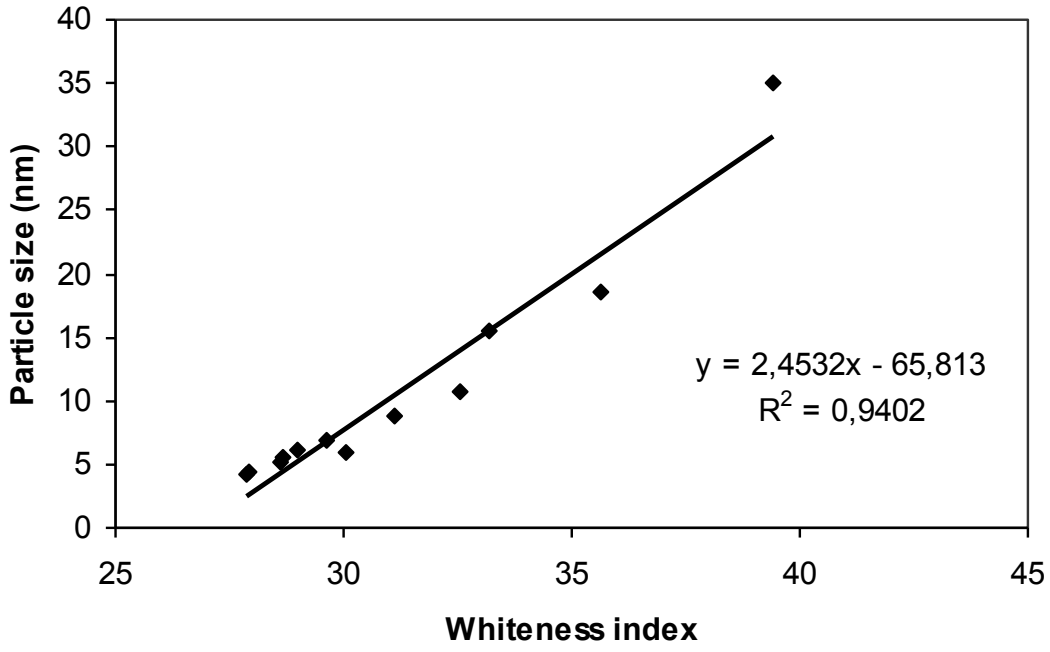

Fig. 8 Correlation of whiteness index with droplet size of lemongrass oil-alginate nanoemulsions.

4 Conclusions

The average droplet size of lemongrass oil-alginate nanoemulsions decreased gradually by increasing sonication amplitude and time, showing a minimum average droplet size

of 4.31 ± 0.18 nm after being treated at 100 μm for 180 s. Ultrasounds lead to higher electrical charge on the surface of oil droplets, thus increasing the colloidal stability of the oil droplets. Moreover, ultrasonic treatment induced changes in the thickening properties of the sodium alginate present in the continuous phase since the viscosity of the nanoemulsions significantly decreased with sonication amplitude and time. In addition, lemongrass oil-loaded nanoemulsions were optically transparent due to the weak light scattering of nano-sized oil droplets. Therefore, ultrasound processing is able to produce lemongrass oil-alginate nanoemulsions with extremely small droplet sizes, high stability and transparent to be used as delivery systems of essential oils in food products. However, there is still lacking information about the functionalization of lipids incorporated in nanoemulsions and their advantages regarding to conventional emulsions.

AKNOWLEDGEMENTS

This study was supported by the Ministerio de Ciencia e Innovación (Spain) throughout the project AGL2009-11475. Laura Salvia-Trujillo thanks the Ministry of Science and Education (Spain) for the predoctoral grant. Prof. Olga Martín-Belloso thanks the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia 2008 Award.

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PUBLICATIONS

Chapter II

Effect of processing parameters on physicochemical characteristics of microfluidized lemongrass essential oil-alginate nanoemulsions

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Food Hydrocolloids 30 (1), 401-407

Abstract

The purpose of this work was to study the effect of processing parameters (pressure and cycles) on the formation of microfluidized lemongrass oil-alginate nanoemulsions considering their average droplet size and size distribution, ζ-potential, viscosity and whiteness index. To confirm that nanoemulsions were in the nano-range, samples were also observed through transmission electron microscopy (TEM) and atomic force microscopy (AFM) techniques. Average droplet size, viscosity and whiteness index of nanoemulsions decreased by increasing the processing pressure and the cycles through the interaction chamber of the microfluidizer device. Nanoemulsions obtained at 150 MPa for 10 cycles exhibited a minimum average droplet size of 6 nm. Moreover, the droplet electrical charge of nanoemulsions ranged between -36.66 and -51.95 mV while it was -17.61 mV in the coarse emulsion. Furthermore, nanoemulsions obtained at 150 MPa for 3 times or more through the microfluidization system were almost transparent. Results obtained in the present study reveal that microfluidization is a potential technology to be used to produce nanoemulsions of essential oils. However, more information is needed about the influence of microfluidization conditions on the antimicrobial properties of essential oils dispersed in nano-sized emulsions.

Keywords

Nanoemulsion, microfluidization, sodium alginate, essential oils, droplet size, color, viscosity

Table of contents

1 Introduction

Nowadays, recent developments achieved by nanotechnology are being considered in several research areas, since the manipulation of matter at the nanoscale offers numerous opportunities. In this sense, nanoemulsions are described as nano-sized delivery systems of nanoencapsulated lipophilic ingredients in an oil matrix, with an extremely small droplet size (Solans, et al., 2005; Mason, et al., 2006; McClements, 2011). In pharmaceutical science, extensive research has been conducted on a variety of nanoemulsion-based drug delivery systems (Puri, et al., 2009). However nanoemulsions are gaining importance among the food sector due to its wide range of potential applications (Flanagan, et al., 2006; Weiss, et al., 2006) as delivery systems in liquid foods (Sagalowicz, et al., 2010) or minimally processed fruits and vegetables (Rojas-Graü, et al., 2010; Vargas, et al., 2008b; Sozer, et al., 2009) due to their unique characteristics of stability, optical transparency and enhanced functionality (McClements, et al., 2011). The use of nanoemulsions as carriers of lipophilic flavouring agents, such as essential oils, would be an interesting approach to develop new food products. In fact, the incorporation of essential oils in food still presents several technological limitations considering their low solubility in aqueous media, as well as their toxicological, organoleptical and economical aspects when they are incorporated at high doses (Sánchez-González, et al., 2011; Burt, 2004).

Despite that nanoemulsion production methods are divided in low and high-energy approaches (McClements, et al., 2011), the second group are generally more likely to be used in the food sector due to their scale up and equipment availability, in addition to their ability to produce nanoemulsified systems without the addition of organic solvents. Among high-energy emulsification technologies, microfluidization has lead to good nanoemulsion based systems in several research studies, with droplet sizes ranging from 60 to 600 nm (Jafari, et al., 2007a; Jafari, et al., 2007b; Wooster, et al., 2008; Qian, et al., 2011; Hatanaka, et al., 2010; Hatanaka, et al., 2008; Rao, et al., 2011b). Microfluidization processing implies that a coarse emulsion is passed through and interaction chamber using a high-pressure pumping device (Jafari, et al., 2007a). The interaction chamber consists of two flow channels, which are designed so that they cause two streams of the coarse emulsion to impinge on each other at high velocity, thus creating a very high shearing action that provides an exceptionally fine emulsion

(Mahdi Jafari, et al., 2006). Nevertheless, there is a lack of information about the effect of processing conditions to produce microfluidized essential oil loaded nanoemulsions as flavour delivery systems.

Therefore, the objective of the present study was to evaluate the influence of processing parameters (pressure and cycles) on the droplet size, stability, whiteness index and viscosity of microfluidized O/W nanoemulsions containing lemongrass oil as lipid phase and alginate as aqueous phase.

2 Material and methods

2.1 Primary emulsion formation

Sodium alginate (1% w/v) (FMC Biopolymers, UK) was dissolved in hot water at 70 ºC and continuous stirring until it was completely dissolved. A coarse or primary emulsion was made by mixing the sodium alginate solution as aqueous phase and lemongrass essential oil (1% v/v) (Laboratoris Dicana, Spain) as lipidic phase plus Tween 80 (1% v/v) (Scharlau, Spain) as surfactant, with a laboratory T25 digital Ultra-Turrax mixer (IKA, Staufen, Germany) working at 3400 rpm for 2 min. The lemongrass oil concentration was set according to antimicrobial purposes (Rojas-Graü, et al., 2007), and Tween 80 concentration was adjusted to achieve a surfactant to oil ratio of 1 (Rao and McClements, 2011b). All samples were prepared using ultra pure water obtained from a Mili-Q filtration system. The final volume of the coarse emulsion was 300 mL. Physico-chemical characterization of the primary emulsion is shown in Table 1.

Table 1. Physicochemical characterization of the coarse lemongrass oil-alginate emulsion obtained with a high speed blender at 3400 rpm for 2 min.

Average droplet size (nm)	1410 ± 366
ζ-potential (mV)	-18 ± 3
Whiteness index	46.8 ± 0.1
Viscosity (mPa.s)	30.8 ± 0.9
Density (g/mL)	1.005 ± 0.001
pH	6.026 ± 0.011

2.2 Nanoemulsion formation

A high pressure homogenization system (M110P, Microfluidics, Massachusetts, USA) was used to treat the coarse emulsion to obtain nanoemulsions. This device pumped the emulsion towards an interaction chamber where the product was accelerated at high

velocities, and the high-shear forces created within reduce the droplet size of the emulsion. At the outlet of the interaction chamber the product was refrigerated through an external coiling coil immersed in a water bath with ice so that the temperature of the product was always kept below 20 ºC. Coarse emulsions were passed through the system working at 50, 100 or 150 MPa for several cycles (1, 2, 3, 4, 5 and 10). The final volume of nanoemulsions was 250 mL, as 50 mL were discarded to avoid the dilution of the sample.

2.3 Particle size and ζ-potential

The oil droplet size was measured by dynamic light scattering (DLS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK) working at 633 nm at 25 ºC and equipped with a backscatter detector (173 º), which is appropriate to measure sub-micron particles. DLS measures the Brownian motion of nanosized droplets and relates this movement to an equivalent hydrodynamic diameter (Brar, et al., 2011).

The oil droplet size (nm) was characterized by distribution curves in intensity (%), average droplet size and polydispersity index. A polydispersity index near to 1 indicates a heterogeneous distribution between sizes of droplets. The lemongrass oil absorbance at 633 nm was 0.001 and its refractive index was 1.487, which were measured with a spectrophotometer and a refractometer, respectively.

The ζ-potential of oil droplets was measured by phase-analysis light scattering (PALS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK) to determine the surface charge at the interface of the droplets dispersed in the biopolymeric solution.

2.4 Viscosity

Viscosity of emulsions was measured from approximately 10 mL of product using a SV-10 vibro-viscometer (A&D Company, Tokyo, Japan) vibrating at 30 Hz and constant amplitude. The viscosity of sodium alginate (1 % w/v) as dispersant material, being 30 mPa.s, was considered with regards dynamic light scattering measurements.

2.5 Color

The color of emulsions was measured at room temperature with a Minolta CR-400 colorimeter (Konica Minolta Sensing, Inc., Osaka, Japan) set up for illuminant D_{65} and 10º observer angle and calibrated with a standard white plate. CIE L*, a* and b* values

were determined and the whiteness index (WI) was calculated with the following equation (Vargas, et al., 2008a):

$$WI = 100 - ((100 - L)^2 + (a^2 + b^2))^{0.5} \quad \text{Eq. (1)}$$

2.6 Transmission electron microscopy

Nanoemulsions were observed by negative-staining electron microscopy as a direct measure of their droplet size and shape. The sample was adsorbed onto carbon-coated copper/palladium grids for 1 min. Then, the grids were washed three times by floating it face-down on drops of sterilized, deionised water for 1 min. Finally, the sample was negatively stained by floating the grids face-down on a drop of 2% (w/v) ammonium molybdate at pH 6.5 for 1 min. The grids were observed in a Tecnai Spirit transmission electron microscope (FEI Company, Netherlands) at an acceleration voltage of 100 kV.

2.7 Atomic force microscopy

Atomic force microscopy (AFM) experiments were carried out as a complementary tool for droplet characterization. Nanoemulsions were diluted 1:10 with ultra pure mili-Q water and a 20 μL drop was placed onto freshly cleaved mica for 10 minutes. After adsorption to mica surface at room temperature, samples were rinsed with ultra pure mili-Q water and then blow-dried with nitrogen compressed air. Samples were then observed using an extended multimode® 8 AFM head with a Nanoscope V controller (Bruker, Germany). AFM images were recorded in peak force tapping mode with triangular silicon cantilevers (nominal radius of 8 nm and spring constant of 0.5 N/m) at a scan rate of 1 Hz.

2.8 Statistics

All the experiments were assayed in duplicate, and two replicate analyses were carried out on each parameter in order to obtain mean values. Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, MD) was used to perform the analysis of variance. To discriminate between treatment conditions, the least significant difference (LSD) test was run at a 5 % significance level.

3 Results and discussion

3.1 Droplet size, polydispersity index and size distribution

The microfluidizer processing pressure and number of cycles significantly influenced the average droplet size, polydispersity index and size distribution of lemongrass oil-alginate nanoemulsions (Fig. 1, Fig. 2 and Table 2). The average droplet size of nanoemulsions decreased with increasing the processing pressure and number of cycles. These results are in agreement with those obtained by other authors in curcumin or pea protein-based nanoemulsions (Donsí, et al., 2010; Donsì, et al., 2010). The average droplet size of the coarse emulsion was 1410 ± 366 nm, and it was drastically reduced up to 53 ± 5, 46 ± 7 and 23 ± 2 nm after being passed 1 cycle through the microfluidization system working at 50, 100 or 150 MPa, respectively. Beyond this point, the average droplet size of nanoemulsions decreased exponentially when increasing the number of cycles up to a certain limit. Nanoemulsions processed at 150 MPa always exhibited the lowest average droplet size, as it decreased up to 7.35 ± 1.67 nm when passed 3 times through the interaction chamber. However, it was observed that a further increase in the number of cycles did not lead to a significant reduction of the average droplet size. The same behaviour has been reported recently by other authors studying the formation of nanoemulsions by microfluidization systems. Qian and McClements (Qian, et al., 2011) observed a drastic reduction of the average droplet size of corn oil nanoemulsions processed once by microfluidization at different pressures, up to 165 nm, but further cycles through the microfluidizer had a fair effect. Similarly, Meleson, Graves and Mason (Meleson, et al., 2004) mathematically defined a "saturation droplet size" in silicone oil nanoemulsions, which corresponds to the limit when shearing the emulsion at the same pressure does not yield any additional rupturing or change in the size distribution. They attributed this phenomenon to the fact that microfluidization systems do not have a 100 % efficiency at a single pass, since droplets nearest the walls of the channels experience a smaller shear flow than the bulk of the emulsion away from the walls, resulting in a droplet size distribution that has larger particles. Passing the emulsion multiple times through the interaction chamber submits larger droplets that were not disrupted in a single pass to a higher shear rate, thus reducing its size and leading to a more uniform emulsion. In fact, this theory is corroborated by our results from polydispersity index (Table 2) and size distribution (Fig. 2). The polydispersity indices of nanoemulsions processed with one cycle at 50,

100 or 150 MPa were 0.79 ± 0.05, 0.84 ± 0.07 or 0.65 ± 0.04, respectively, indicating that larger droplets were not disrupted, as can also be observed in size distribution graph (Fig. 2). When the treatment was conducted at a pressure of 50 MPa, there was not an evident reduction of the polydispersity index. However, passing the nanoemulsion several times through the interaction chamber at 100 and 150 MPa, led to a narrower size distribution. Namely, after 3 cycles at 150 MPa the polydispersity index decreased to 0.34 ± 0.10 indicating that larger particles were disrupted, being enough to obtain a homogenous nanoemulsion.

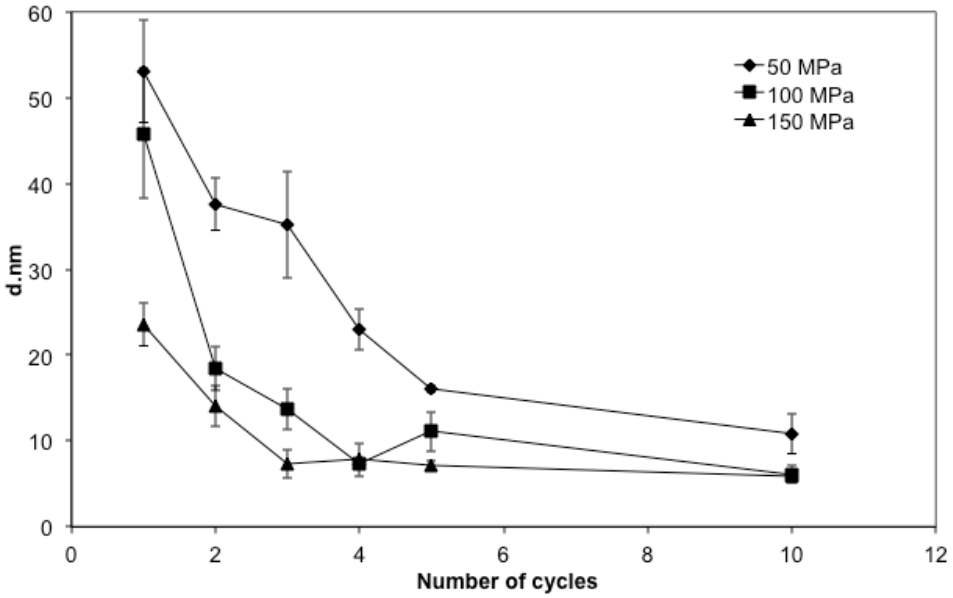

Figure 4. Effect of microfluidization pressure (50, 100 and 150 MPa) and number of cycles (1, 2, 3, 4, 5, 10) on the average droplet size of lemongrass oil-alginate nanoemulsions. Data shown are a mean ± standard deviation.

Recently, a few studies dealing with the formation of nanoemulsions incorporating essential oils by means of microfluidization have been published. Donsí, Annunziata, Sessa and Ferrari (Donsì, et al., 2011) reported droplet sizes between 74.4 and 356.7 nm in nanoemulsions of d-limonene and/or a mixture of terpenes with several formulations and processed for 10 cycles and 300 MPa. Likewise, Rao and McClements (Rao, et al., 2011a) obtained nanoemulsions of lemon oil with sucrose monopalmitate after three passes trough a microfluidizer at 62 MPa, with a minimum average droplet size lower than 15 nm.

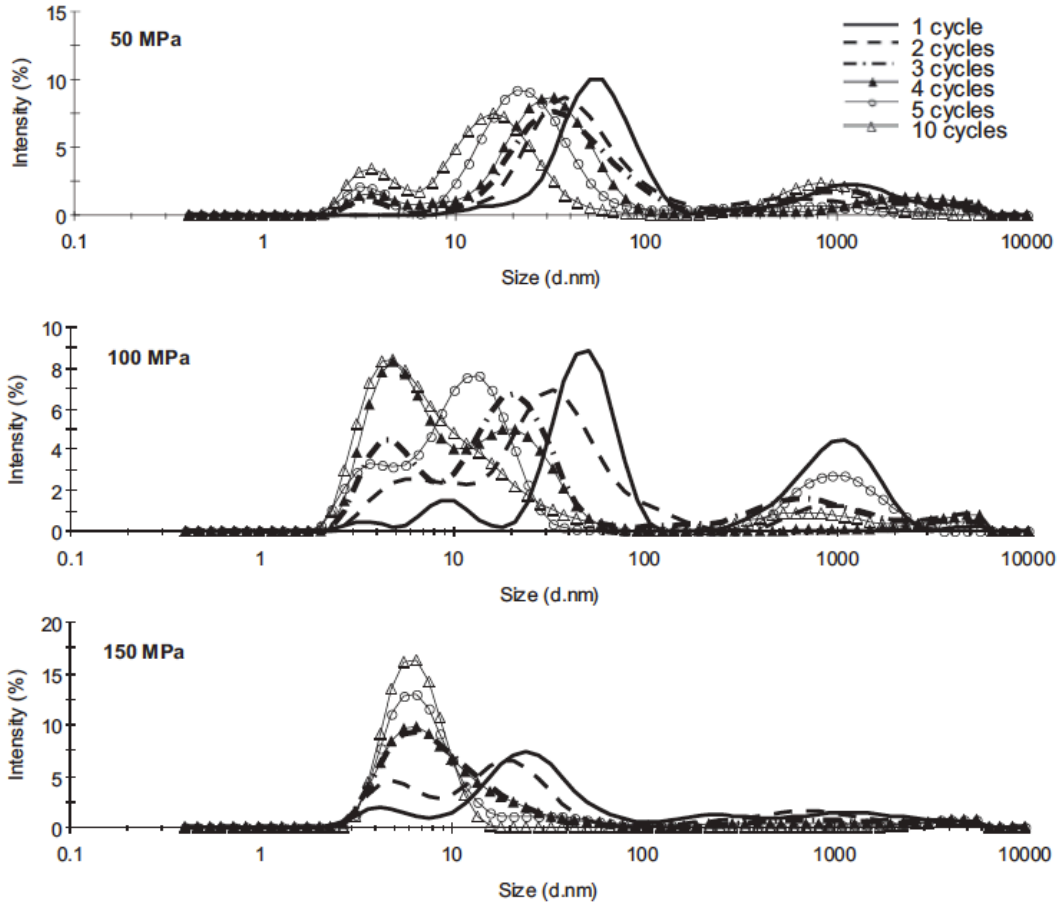

Figure 5. Effect of processing pressure (50, 100 and 150 MPa) and number of cycles (1, 2, 3, 4, 5, 10) on the size distribution of lemongrass oil-alginate nanoemulsions.

Table 2. Polydispersity index of microfluidized nanoemulsions processed several cycles at different pressures (MPa)

	Number of cycles					
Pressure	1	2	3	4	5	10
50	0,79 ± 0,05	0,65 ± 0,14	0,66 ± 0,12	0,68 ± 0,04	0,58 ± 0,10	0,64 ± 0,10
100	0,84 ± 0,08	0,68 ± 0,13	0,62 ± 0,02	0,43 ± 0,02	0,48 ± 0,03	0,43 ± 0,08
150	0,65 ± 0,04	0,60 ± 0,02	0,34 ± 0,10	0,43 ± 0,07	0,37 ± 0,06	0,24 ± 0,01

3.2 ζ-potential

The interfacial electrical charge of lipid droplets of the coarse emulsion was -17.61 ± 2.89 mV. The effect of microfluidization processing pressure and number of cycles is shown in Fig. 3. After passing the coarse emulsion through the microfluidization system a statistically significant reduction of the droplet ζ-potential was observed regardless the pressure applied, which meant an increase on the electrical net charge of lemongrass oil droplets. Nevetheless, there was not a clear effect of microfluidization processing

conditions on the ζ-potential of nanoemulsions. After microfluidization processing, the interfacial electrical charge of droplets ranged between -36.66 and -51.95 mV, irrespectively of the pressure applied and the number of cycles. Generally, particles with ζ-potential more positive than +30 mV or more negative than -30 mV are considered to be stable, since electrical charge of droplets is strong enough to assume that repulsive forces between droplets are predominant in the nanoemulsion system (Heurtault, et al., 2003). Therefore, it can be stated that lemongrass oil-alginate nanoemulsions obtained by microfluidization are more stable than the coarse emulsion produced by a conventional high speed blender.

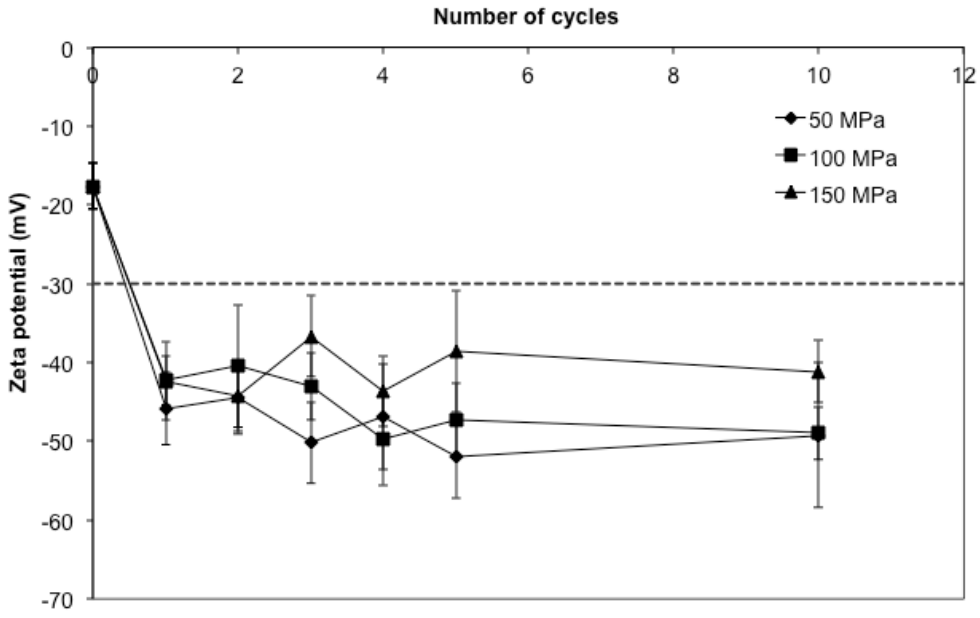

Figure 6. Effect of microfluidization pressure (50, 100 and 150 MPa) and number of cycles (1, 2, 3, 4, 5, 10) on the ζ-potential of lemongrass oil-alginate nanoemulsions. Data shown are a mean ± standard deviation.

Up to our knowledge, there are no published data about the effect of high pressure homogenization parameters on the ζ-potential of nanoemulsions. However, nanoemulsions are generally considered more stable regarding particle aggregation (flocculation and coalescence) than conventional macroemulsions due to their small droplet size (McClements, et al., 2011; Tadros, et al., 2004). Non ionic surfactants, such as tween 80, are surface-active molecules able to adsorb to droplet surfaces, facilitating droplet disruption, and protecting droplets against aggregation (Kralova, et al., 2009). During microfluidization, the newly formed droplets due to mechanical stress are rapidly surrounded by surfactant molecules which are adsorbed to droplet surface,

preventing re-coalescence (Jafari, et al., 2008). Despite that non ionic surfactants do not provide electrical charge to oil droplet surface, it has been observed that they may lead to negative electrical charges when pH of the emulsions are higher than 4 (Hsu, et al., 2003). Nevertheless, in the present work, the strong negative ζ-potential observed in nanoemulsions is due to the presence of sodium alginate, which is an anionic hydrocolloid. Food hydrocolloids are used as emulsion stabilizers, since they are able to adsorb to the interfacial layer, but their stabilizing action depends on the possible interactions and competition between previously adsorbed species (Dickinson, 2009; Dickinson, 2003). In addition, the main stabilizing action of hydrocolloids is due to viscosity modification in the aqueous continuous phase by decreasing the rate of creaming and coalescence (Dickinson, 2009; Dickinson, 2003; Garti, et al., 2001; Rodríguez-Abreu, et al., 2008).

3.3 Viscosity

The viscosity of the coarse emulsion before passing through the microfluidization system was 35.65 ± 2.33 mPa.s. A significant effect of microfluidization treatment was observed on the viscosity of nanoemulsions, since it significantly decreased with increasing the treatment pressure and the number of cycles (Fig. 4). The major reduction was observed after a single pass at 150 MPa, since the viscosity of the nanoemulsion was reduced up to 16.85 ± 0.21 mPa.s. The viscosity of nanoemulsions decreased exponentially when the number of cycles was increased showing fair changes when the process was extended up to 10 cycles, irrespectively of the processing pressure. There are several factors that influence emulsion viscosity, such as the disperse phase volume fraction, rheology of component phases, droplet size, colloidal interactions or droplet charge (McClements, 2005; Pal, 2011; Tadros, 1994). In this sense, some authors have reported that the higher the droplet size the lower the emulsion viscosity, at the same dispersed phase concentration and shear rate (Pal, 2011; Derkach, 2009; Chanamai, et al., 2000; Cortés-Muñoz, et al., 2009). However, in the case of the present work, the continuous phase plays a fundamental role in nanoemulsion characteristics and governs its behaviour during processing (Rodríguez-Abreu, et al., 2008). Other authors have studied the influence of high pressure homogenization on the rheological properties of hydrocolloids. Namely, Harte and Venegas (Harte, et al., 2010) reported a 75-85 % viscosity reduction in alginate, κ-carrageenan and xanthan gum solutions processed

with high pressure homogenization from 1 to 5 cycles up to 300 MPa, observing a viscosity plateau after sufficient homogenization pressure and number of homogenization cycles. The same response was observed by Lagouyete and Paquin (Lagoueyte, et al., 1998) when processing xanthan gum solutions by high pressure homogenization at 75 MPa for several cycles, attributing the viscosity changes to molecular weight losses, thus decreasing its thickening properties. They postulated that the turbulence, cavitation and high shear stress of the microfluidization treatment had an effect on the conformation of the molecule and, consequently, induced an ordered-desordered conformation transition and degradation of the molecule, which was irreversible (Lagoueyte, et al., 1998). This pattern has also been observed in oil in water emulsions stabilized with biopolymeric solutions as aqueous phase when they were treated by high pressure homogenization (Floury, et al., 2003; Floury, et al., 2000; Bonilla, et al., 2012). Therefore, we assumed that viscosity changes induced in nanoemulsions after the application of microfluidization treatment are due to molecular changes on sodium alginate structure. Subsequently, we confirmed the above-mentioned assumption by subjecting a sodium alginate solution to a microfluidization treatment of 150 MPa for 10 cycles. The biopolymeric solution exhibited the same behaviour than the emulsion containing lemongrass essential oil, showing a viscosity reduction from 35.42 ± 0.32 mPa.s to 9.18 ± 0.38 mPa.s.

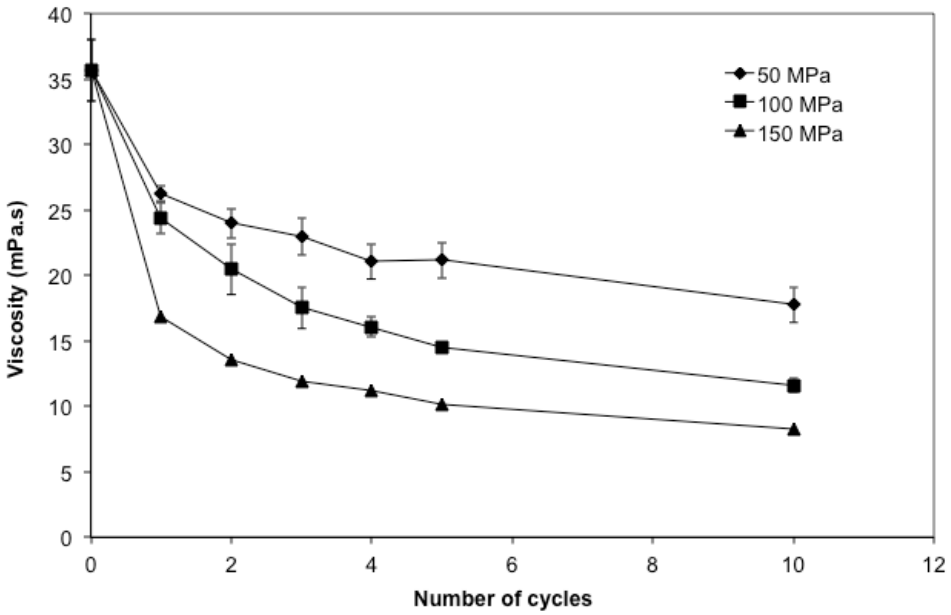

Figure 7. Effect of microfluidization pressure (50, 100 and 150 MPa) and number of cycles (1, 2, 3, 4, 5, 10) on the viscosity of lemongrass oil-alginate nanoemulsions. Data shown are a mean ± standard deviation.

3.4 Whiteness index

The whiteness index of the coarse emulsion was 54.83 ± 0.18 and it diminished in microfluidized nanoemulsions. The effect of pressure and number of cycles on the whiteness index of lemongrass oil-alginate nanoemulsions is shown in Fig. 5. The whiteness index of nanoemulsions significantly decreased when increasing the pressure and the number of cycles. It diminished up to 42.79 ± 0.04, 36.65 ± 0.08 and 35.71 ± 0.16 in nanoemulsions processed for 3 cycles at 50, 100 and 150 MPa, respectively. Beyond this point, an increase in the number of processing cycles through the interaction chamber lead to little changes on the color of nanoemulsions, observing a plateau in the whiteness index. In fact, the whiteness index of nanoemulsions processed at 150 MPa for 10 cycles was 32.63 ± 0.07, which did not differ significantly from that observed at 3 cycles and the same processing pressure. These results were also observed by visual examination of nanoemulsions, since they appeared almost transparent after being treated by microfluidization at 150 MPa for 3 cycles whereas the coarse macroemulsion was opaque, as can be seen in Fig. 6.

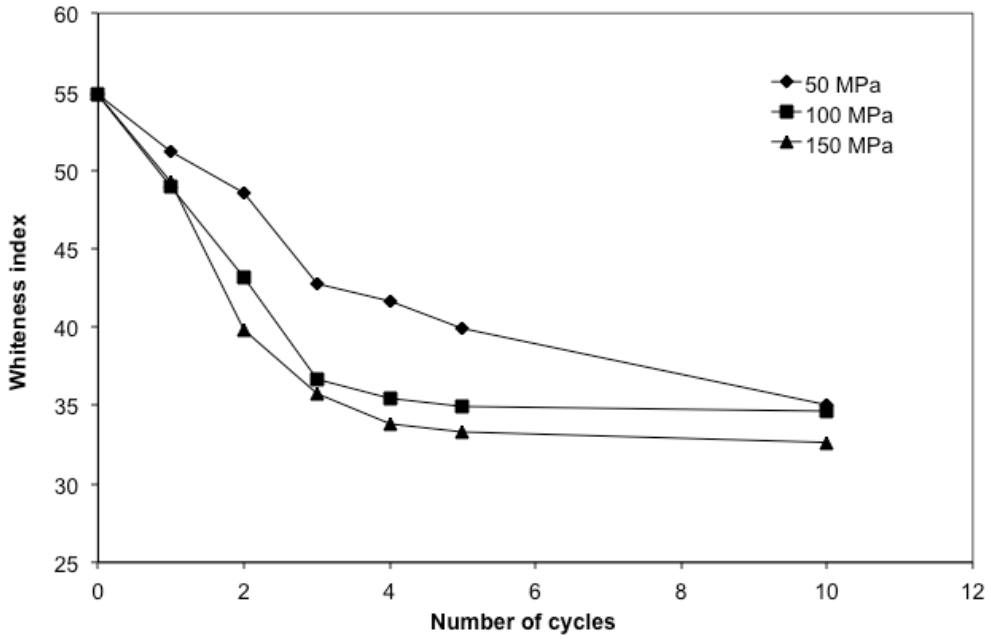

Figure 8. Effect of microfluidization pressure (50, 100 and 150 MPa) and number of cycles (1, 2, 3, 4, 5, 10) on the whiteness index of lemongrass oil-alginate nanoemulsions. Data shown are a mean ± standard deviation.

Light scattering, and therefore emulsion color, depends largely on the refractive index of continuous and dispersed phase, oil concentration and also droplet size (McClements,

2002). Large particles scatter the light more intensely than smaller ones, which cause an increase in the lightness, opacity and whiteness index of emulsions (McClements, et al., 2011). Nanoemulsions are described in previous review articles as transparent systems due to the reduced light scattering caused by their small droplet size (Solans, et al., 2005; Mason, et al., 2006; McClements, 2011; McClements, et al., 2011; Tadros, et al., 2004). Accordingly to our results, Wooster, Golding and Sanguansri (Wooster, et al., 2008) obtained optically transparent peanut oil nanoemulsions after treating the coarse emulsion 5 cycles at 100 MPa by microfluidization. Optical appearance of nanoemulsions is an important factor to take into account for the development of new products. In this way, lemongrass oil-alginate nanoemulsions might be suitable to incorporate in food products due to their optical transparency.

Figure 9. Lemongrass oil-alginate nanoemulsions microfluidized 3 cycles at 150 MPa

3.5 TEM and AFM images

TEM and AFM observations of nanoemulsions were done to confirm dynamic light scattering (DLS) measurements. TEM image of a nanoemulsion (Fig. 7) obtained by passing the coarse emulsion 3 times through the microfluidization system at 150 MPa showed droplet sizes between 10 nm and 250 nm of diameter. Electron microscopy has been described as an appropriate tool to characterize nanoemulsions (Klang, et al., 2011) and nanomaterials in foods (Dudkiewicz, et al., 2011; Blasco, et al., 2011), since it is able to provide enough resolution to see nano-sized structures which could not be detected by classical microscopy techniques. In controversy with our results, when nanoemulsion samples are prepared by negative-staining techniques they normally appear as light droplets against a darker background (Seki, et al., 2004; Kelmann, et al.,

2007; Nam, et al., 2010; Araújo, et al., 2011). However, this may vary according to the affinity of the staining agent to interfacial components, as an increased contrast has been observed in the interface of oil droplets in previous studies (Singh, et al., 2008; Ibrahim, et al., 2009).

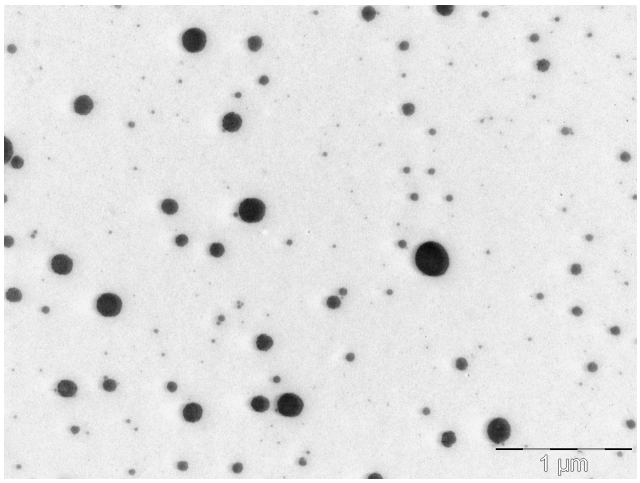

Figure 10. Transmission electron microscopy image of microfluidized lemongrass oil-alginate nanoemulsion (3 cycles at 150 MPa)

On the other hand, nano-sized droplets observed by AFM (Fig. 8) exhibited a radius between 50 and 150 nm. The higher droplet sizes observed in AFM images, regarding those observed by TEM or DLS, might be probably due to a weak adsorption of smallest oil droplets to mica surface. In addition, AFM may lead to wider nanoparticles due to the tip broadening effect, which takes place when the tip is in contact with soft or sticky biomaterials (Luykx, et al., 2008; Yang, et al., 2007). However, other authors have published AFM images of nanoemulsions with good results (Marxer, et al., 2011; Mao, et al., 2009). Accordingly to our results, Preetz, Hauser, Hause, Kramer and Mäder (Preetz, et al., 2010) have reported differences between droplet sizes of nanoemulsions and nanocapsules, since they found that the mean droplet size determined by DLS was 150 nm, whereas it ranged between 50 and 500 nm if they were observed by TEM or AFM. Therefore we can conclude that TEM or AFM approaches are valuable tools to compare and complement the information obtained by DLS measurements.

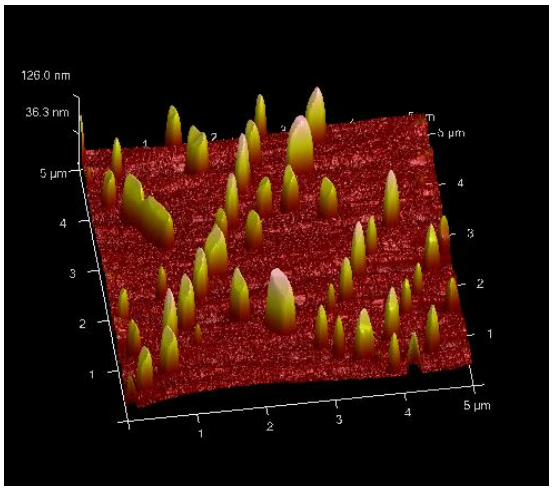

Figure 11. Atomic force microscopy image of microfluidized lemongrass oil-alginate nanoemulsion (3 cycles at 150 MPa)

4 Conclusions

It the present work, we have obtained lemongrass oil-alginate nanoemulsions with an average droplet size of 7.35 ± 1.67 nm and a polydispersity index of 0.34 when they were passed three times through a microfluidization device working at 150 MPa. At these processing conditions, nanoemulsions were almost transparent with lower viscosity and a more negative ζ-potential regarding the coarse emulsion, thus presenting an enhanced stability. Moreover, TEM and AFM observations confirmed that lipid droplets of nanoemulsions were in the nano-scale. Therefore, microfluidization could be a suitable technology for nanoemulsion production. However, more information is needed about the effect of microfluidization treatments on antimicrobial properties of essential oil-loaded nanoemulsions.

Acknwoledgements

This study was supported by the Ministerio de Ciencia e Innovación (Spain) throughout the project AGL2009-11475. Laura Salvia-Trujillo thanks the Ministry of Science and Education (Spain) for the predoctoral grant. Prof. Olga Martín-Belloso thanks the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia 2008 Award. We also thank Dr. Jordi Díaz and Dr. Carmen López-Iglesias, from the scientific-technical services of the University of Barcelona, for AFM and TEM imaging support, respectively.

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PUBLICATIONS

Chapter III

Impact of microfluidization or ultrasound processing on the antimicrobial activity against *Escherichia coli* of lemongrass oil-loaded nanoemulsions

Laura Salvia-Trujillo, M. Alejandra Rojas-Graü, Robert Soliva-Fortuny, Olga Martín-Belloso

Food Control 37 (1), 292-297.

Abstract

The effect of the ultrasonication and microfluidization processing conditions on the antimicrobial activity of lemongrass oil-alginate nanoemulsions was studied. Sonication led to less effective nanoemulsions against *Escherichia coli*, being the loss of antimicrobial potential dependent on the amplitude and treatment time applied. Namely, nanoemulsions sonicated at 100 μm for 180 s almost completely lost their bactericidal action, leading to 0.3 log-reductions of *E. coli* population after 30 min of contact time. On the contrary, nanoemulsions processed by microfluidization exhibited an enhanced antimicrobial activity, which was proportional to the number of cycles through the microfluidization chamber. In fact, whereas the coarse emulsion reduced the *E. coli* population up to 0.66, 2.25 and 5.85 log-units after 5, 15 and 30 min of contact time, the microfluidized nanoemulsions (10 cycles, 150 MPa) achieved 1.37, 5.29 and 7.07 log-reductions. Therefore, nanoemulsions with an improved functionally could be obtained by microfluidization, whereas ultrasounds seem to have a deleterious impact on the antimicrobial activity of lemongrass essential oil.

Keywords

Antimicrobial activity, nanoemulsion, sodium alginate, essential oils, ultrasounds, microfluidization

Table of contents

1 Introduction

Plant essential oils (EOs) have been widely used for many years due to their antimicrobial properties in foods and pharmaceutical products (Burt, 2004). In this sense, their well established bactericidal activity has made them object of numerous research studies dealing with food preservation (Tajkarimi, Ibrahim and Cliver, 2010, Holley and Patel, 2005), as there is a strong consumer demand for safe and high-quality foods being free from synthetic additives. Antimicrobial action of EOs relies on their hydrophobic nature, which enables them to partition in the lipids of bacterial cell membrane and mitochondria, causing damages to these structures and rendering them more permeable (Burt, 2004, Hammer, Carson and Riley, 1999, Bakkali, Averbeck, Averbeck and Idaomar, 2008, Sikkema, De Bont and Poolman, 1994). Leakage of ions followed by efflux of cytoplasmatic constituents cause the viability loss of microbial cells (Walsh, Maillard, Russell, Catrenich, Charbonneau and Bartolo, 2003, Lambert, Skandamis, Coote and Nychas, 2001). However other mechanisms may be involved in microbial inactivation by EOs since they could interfere with membrane function, altering its electron transport, nutrient uptake, proteins, nucleic acid synthesis and enzyme activity (Bajpai, Baek and Kang, 2011, Tiwari, Valdramidis, O'Donnell, Muthukumarappan, Bourke and Cullen, 2009). Thus, the incorporation of EOs to food products is a potential alternative to chemical antimicrobial compounds regarding food preservation. However, their incorporation to foods still poses several drawbacks. On one hand, the type of essential oil to be incorporated to a food system should be accurately selected taking into account the organoleptical attributes of the final product (Tiwari, Valdramidis, O'Donnell, Muthukumarappan, Bourke and Cullen, 2009). Moreover, the EO concentration needs to be reduced in order to minimize possible toxicological effects or consumer rejection (Sánchez-González, Vargas, González-Martínez, Chiralt and Cháfer, 2011). On the other hand, due to their lipophilic nature, the formulation of foods containing EOs presents technological limitations considering their low solubility in aqueous media. Among EOs, lemongrass (*Cymbopogon citratus*) essential oil has been found to be effective against several foodborne pathogens when it was incorporated in minimally processed fruit (Rojas-Graü, Raybaudi-Massilia, Soliva-Fortuny, Avena-Bustillos, McHugh and Martín-Belloso, 2007, Raybaudi-Massilia, Rojas-Graü, Mosqueda-Melgar and Martín-Belloso, 2008, Raybaudi-Massilia,

Mosqueda-Melgar and Martín-Belloso, 2008), fruit juices (Raybaudi-Massilia, Mosqueda-Melgar and Martín-Belloso, 2006, Duan and Zhao, 2009), minced meat (Barbosa, Rall, Fernandes, Ushimaru, Da Silva Probst and Fernandes Jr., 2009), chocolate (Kotzekidou, Giannakidis and Boulamatsis, 2008) or fish products (Mejlholm and Dalgaard, 2002). Therefore, it is a strong candidate to be used as natural antimicrobial in high perishable foods.

Nowadays, there is a growing interest in the use of antimicrobial delivery systems for food preservation. In this way, nanoemulsions are being regarded as an interesting approach to control the release of active ingredients in food formulations. Nano-sized emulsions with an increased active surface area may not only enhance the functionality of EOs (Flanagan and Singh, 2006, Imran et al., 2010), but also improve emulsion appearance and stability (Solans, Izquierdo, Nolla, Azemar and Garcia-Celma, 2005). However, the information about the feasibility to obtain nanoemulsions containing EOs is yet limited. In this sense, ultrasound and microfluidization treatments, as high-energy emulsification methods, have yielded emulsions with nano-sized droplets typically ranging between 30 and 600 nm (McClements and Rao, 2011). However, there is a lack of scientific evidence about the release of active compounds from nano-sized emulsions. In addition, the impact of particle size reduction treatments on the functionality of lipophilic compounds dispersed in oil-in-water nanoemulsions has not been previously studied. Thus, the purpose of the present work was to assess the influence of the lipid particle disruption method on the activity against *Escherichia coli* of lemongrass oil-alginate nanoemulsions.

2 Material and methods

2.1 Primary emulsion formation

Sodium alginate (1% w/v) (FMC Biopolymers, UK) was dissolved in hot water at 70 ºC and continuous stirring until it was completely dissolved. A coarse or primary emulsion was made by mixing the sodium alginate solution as aqueous phase and lemongrass essential oil (1% v/v) (Laboratoris Dicana, Spain) as lipidic phase plus Tween 80 (1% v/v) (Scharlau, Spain) as surfactant, with a laboratory T25 digital Ultra-Turrax mixer (IKA, Staufen, Germany) working at 3400 rpm for 2 min. All samples were prepared using ultra pure water obtained from a Mili-Q filtration system.

2.2 Nanoemulsion formation

2.2.1 Ultrasonication

A UP400S Hielscher sonifier (Hielscher Ultrasound Technology, Teltow, Germany), of 400 W nominal power and a frequency of 24 kHz equipped with a 22-mm sonotrode, was used to perform the ultrasonic treatment. The coarse emulsion was pumped into a stainless steel ultrasonic flow cell by means of a peristaltic pump (model Selecta- PR 2003) set at 100 mL/min giving a residence time of 16.5 seconds. The ultrasonic flow cell was equipped with a water-cooled jacket to avoid excessive heating of nanoemulsions. Several treatments were applied to produce nanoemulsions, varying the ultrasound amplitude (30, 60 and 100 μm) and treatment time (0, 30, 60, 120, 180 seconds). The maximum temperature of the sample registered at the outlet of the system was 47 ºC.

2.2.2 Microfluidization

A microfluidizer (M110P, Microfluidics, Massachusetts, USA) was used to treat the coarse emulsion to obtain nanoemulsions. This device pumped the emulsion towards an interaction chamber where the product was accelerated at high velocity, and the high-shear forces created inside reduced the droplet size of the emulsion. Afterwards, the product passed immediately through a coiling coil immersed in a water bath with ice, keeping the outlet temperature below 20 ºC. The coarse emulsion was passed through the system several times (1, 2, 3, 4, 5 and 10) at different pressures (50, 100 and 150 MPa).

2.3 Droplet size characterization

The emulsion and nanoemulsion droplet diameter was measured by dynamic light scattering (DLS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK). The ζ-potential of oil droplets, to determine the interfacial electrical charge of lipid nanodroplets, was measured by phase-analysis light scattering (PALS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK). The physicochemical properties of nanoemulsions produced by ultrasounds or by microfluidization were published previously in two different journal papers (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny and Martín-Belloso, 2012; Salvia-

Trujillo, Rojas-Graü, Soliva-Fortuny and Martín-Belloso, 2013). Nevertheless, the effect of ultrasound or microfluidization processing parameters on the average droplet diameter and ζ-potential nanoemulsions are shown in Table 1 and Table 2, respectively.

Table 1 Droplet diameter (nm) and interfacial ζ-potential (mV) of lemongrass oil-alginate emulsions or nanoemulsions produced by sonication at different processing amplitude (μm) and sonication times (s).

Amplitude (μm)	Sonication time (s)	Droplet diamater (nm)	ζ-potential (mV)
0	0	1410 ± 365	-17.6 ± 2.9
30	30	34.9 ± 8.5	-38.0 ± 10.4
	60	15.3 ± 2.9	-37.7 ± 3.2
	120	6.9 ± 1.3	-42.0 ± 3.6
	180	5.1 ± 0.2	-55.8 ± 6.4
60	30	18.6 ± 5.1	-30.9 ± 3.4
	60	8.8 ± 1.5	-36.4 ± 2.7
	120	6.1 ± 1.5	-46.0 ± 5.6
	180	4.3 ± 0.1	-47.1 ± 8.9
100	30	10.6 ± 1.8	-32.8 ± 0.1
	60	6.0 ± 0.1	-44.7 ± 0.1
	120	5.6 ± 0.9	-41.5 ± 3.2
	180	4.3 ± 0.2	-46.0 ± 3.7

Values are expressed as mean ± standard deviation.

2.4 Antimicrobial activity assay

The antimicrobial activity of lemongrass oil-alginate nanoemulsions was assessed by evaluating the *in vitro* inhibition of *Escherichia coli*. The method used was a modification of that previously described by Ferreira, Alves, Neves, Gibbs and Teixeira (Ferreira, Alves, Neves, Silva, Gibbs and Teixeira, 2010). *E. coli* 1.107 (Laboratoire de Répression des Fraudes, Montpellier, France) was cultured in tryptone soy broth (Bioakar Diagnostic; Beauvais, France) and incubated at 37 ºC with continuous agitation at 120 rpm for 11 h to obtain cells in stationary growth phase. The final concentration reached in the culture was 10^8-10^9 colony forming units/millilitre (CFU/mL). A 0.5 mL-aliquot of overnight bacterial culture was mixed with 0.5 mL of the lemongrass oil-alginate nanoemulsion and 4.5 mL of sterile Mili-Q water. To determine the inactivation kinetics, an aliquot was taken after 5, 15 and 30 minutes of contact time to be serially diluted and spread on McConkey agar (Biokar Diagnostics, Beauvais, France) plates. A control was performed with the same method, replacing the

nanoemulsion by sterile Mili-Q water. The antimicrobial activities of the individual components of the nanoemulsion system were not evaluated due to their negligible antimicrobial activity (Ziani, Chang, McLandsborough and McClements, 2011). Colony counts were determined after incubation of agar plates at 37 ºC for 24 hours.

Table 2 Droplet diameter (nm) and interfacial ζ-potential (mV) of lemongrass oil-alginate emulsion or nanoemulsions produced by microfluidization at different processing pressure (MPa) and number of cycles.

Pressure (MPa)	Cycles	Droplet diamater (nm)	ζ-potential (mV)
0	0	1410 ± 365	-17.6 ± 2.9
50	1	53.1 ± 5.9	-45.8 ± 4.6
	2	37.6 ± 3.1	-44.4 ± 4.7
	3	35.1 ± 6.2	-50.2 ± 5.1
	4	23.0 ± 2.4	-46.9 ± 6.7
	5	16.0 ± 0.4	-51.9 ± 5.3
	10	10.8 ± 2.3	-49.2 ± 9.1
100	1	45.8 ± 7.5	-42.3 ± 3.1
	2	18.5 ± 2.6	-40.5 ± 7.7
	3	13.6 ± 2.3	-43.0 ± 4.3
	4	7.4 ± 0.1	-49.8 ± 5.9
	5	11.1 ± 2.3	-47.4 ± 4.7
	10	6.1± 1.0	-49.0 ± 3.3
150	1	23.5 ± 2.5	-42.3 ± 4.9
	2	14.1 ± 2.4	-44.3 ± 4.5
	3	7.3 ± 1.7	-36.6 ± 5.1
	4	7.8 ± 2.0	-43.7 ± 4.5
	5	7.1 ± 0.6	-38.6 ± 7.7
	10	5.8 ± 0.1	-41.1 ± 3.9

Values are expressed as mean ± standard deviation.

2.5 Modeling

The inactivation kinetics of lemongrass oil-alginate nanoemulsions against *E. coli* were adjusted to a Weibull distribution model, by Eq. 1 (Peleg, 2006):

$$\ln S(t) = -\left(\frac{t}{\beta}\right)^{\alpha} \qquad \text{Eq. (1)}$$

where *S* is the survival fraction (N/N_0) of *E. coli*, *t* is the contact time (min), *β* is the scale factor, and α is the shape factor. β represents the contact time (min) necessary to inactivate the first log-cycle of the microbial population. α describes the shape of the inactivation curve. A shape factor < 1 indicates a concavity upwards, and > 1 a concavity downwards.

The model was fitted to experimental data by non-linear regression using the Statgraphics Plus v.5.1 Windows package. The adjusted regression coefficients (R^2 adj) and accuracy factor (A_f, Eq. 2) (Ross, 1996) were calculated to evaluate the fitting of the model to experimental data.

$$A_f = 10^{\frac{\sum\left|\log\left(\frac{predicted}{observed}\right)\right|}{n}} \qquad \text{Eq. (2)}$$

where log (predicted/observed) is the logarithmic relation between the predicted and the experimental values, and *n* is the number of observations.

The highest the R^2 adj values and the nearer the A_f values to 1, the better the fitting of the model to experimental data.

2.6 Statistics

All the experiments were assayed in duplicate, and two replicate analyses were made of each nanoemulsion sample in order to obtain the mean value. Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, MD) was used to perform the analysis of variance. The least significant difference (LSD) test was run to determine significant differences ($p \leq 0.05$) between nanoemulsions prepared under different treatment conditions at a 5 % significance level.

3 Results

3.1 Effect of ultrasound processing parameters on antimicrobial activity of nanoemulsions

The impact of ultrasound processing parameters, namely amplitude and time, on antimicrobial activity of lemongrass oil-alginate nanoemulsions is shown in Fig. 1. The survival fraction of the inoculated *E. coli* population decreased gradually with increasing contact time, regardless the amplitude or the sonication time used to prepare

the nanoemulsions. In this sense, the *E. coli* population decreased 0.66, 2.25 and 5.85 log-units after 5, 15 and 30 min of contact time with the coarse emulsion. Moreover, the inactivation kinetics of *E. coli* in contact with antimicrobial nanoemulsions was successfully adjusted to a Weibull model. Accordingly, R^2 and A_f values ranged between 98.01-99.99 and between 1.001-1.579, respectively (Table 3). The β-value of the inactivation kinetic of the coarse emulsion, which is the contact time (min) between the emulsion and the microbial population necessary to inactivate the first log-cycle, was 7.95 min. Regarding the antimicrobial activity of sonicated nanoemulsions, statistical analysis of experimental data showed that there was a clear effect of sonication time on antimicrobial activity of lemongrass oil nanoemulsions. In this sense, the higher the sonication time used to produce nanoemulsions, the lower the antimicrobial activity of the emulsified lemongrass EO. For instance, nanoemulsions sonicated at 30 μm for 30, 60, 120 or 180 s exhibited a β-value of 14.63, 13.57, 19.45 or 21.42 min, respectively, evidencing a weakening in the antimicrobial activity of nanoemulsions in comparison with that of the coarse emulsion. Moreover, the amplitude used to prepare sonicated nanoemulsions significantly affected the *E. coli* inactivation since a lower antimicrobial activity was observed in nanoemulsions produced with higher amplitudes. Hence, nanoemulsions sonicated using amplitudes of 100 μm for 180 s almost completely lost their antimicrobial potential, since only 0.3 log reductions in *E. coli* population were attained after 30 min of contact time. In contrast, 4.2 log reductions were observed after 30-min exposure to nanoemulsions sonicated at 30 μm amplitude for 180 s. Furthermore, *E. coli* inactivation kinetics ($\alpha > 1$) presented a concavity downwards. The α-value of the inactivation curve of the coarse emulsion was 1.33, and it increased with ultrasound amplitude and time up to 7.29. This means that the inactivation of *E. coli* at initial contact times was more slowly achieved in nanoemulsions produced with ultrasounds.

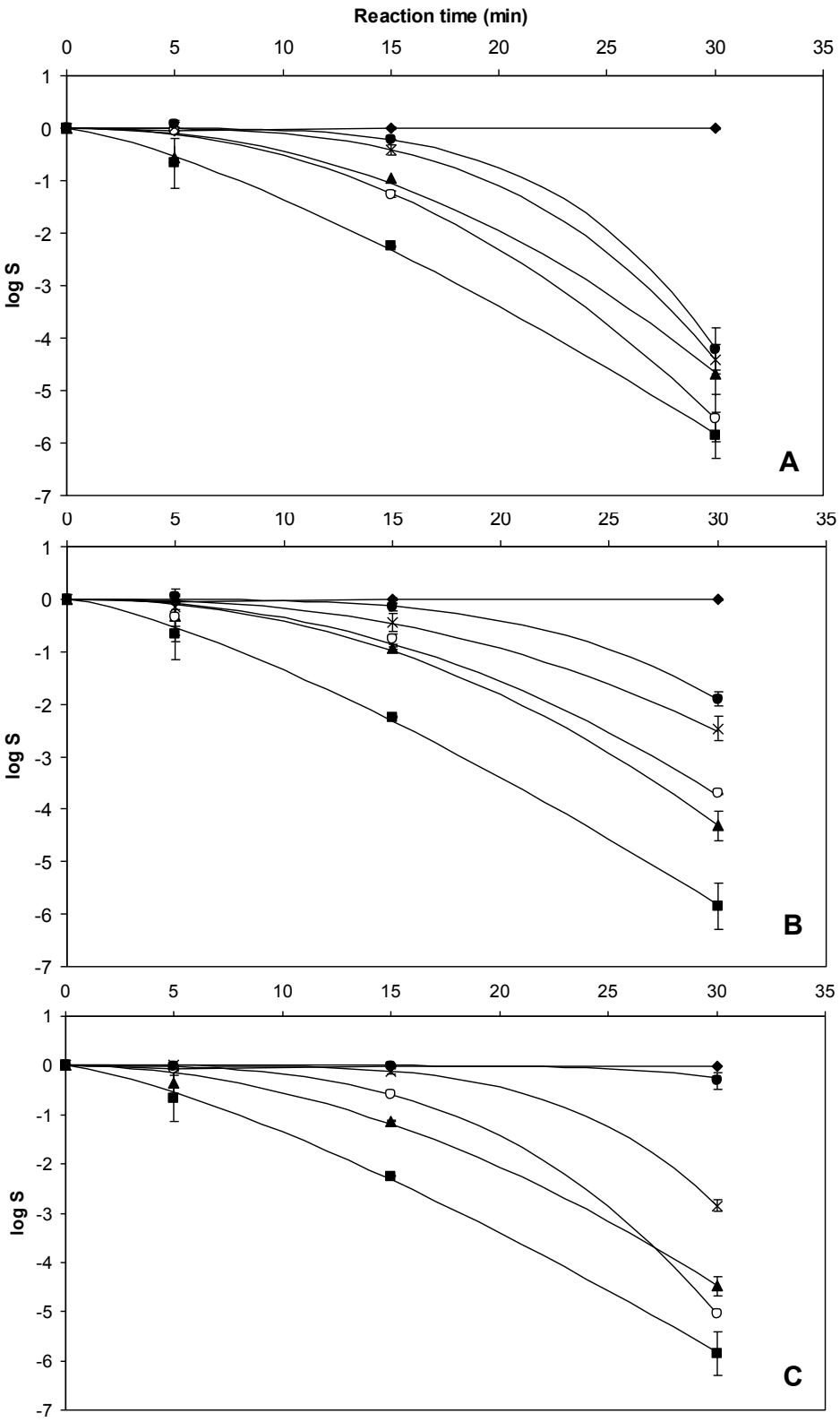

Figure 12 Survival fraction of *Escherichia coli* after contact with lemongrass oil-alginate emulsion (■) or nanoemulsions sonicated at an amplitude of 30 (A), 60 (B) or 100 μm (C) during 30 (▲), 60 (○), 120 (×) or 180 s (●). The control was water (♦). The plotted lines of log S vs. contact time (min) correspond to a Weibull equation fitted to experimental data. Data shown are a mean ± standard deviation.

Table 3 Estimated parameters of Weibull distribution function proposed to describe the inactivation kinetics of *Escherichia coli* by lemongrass oil-alginate emulsion or nanoemulsions produced by sonication at different processing amplitude (μm) and sonication times (s)

Amplitude (μm)	Sonication time (s)	β	α	R^2 (%)	A_f
0	0	7.95 ± 0.79	1.33 ± 0.05	99.40	1.062
30	30	14.63 ± 0.10	2.14 ± 0.10	98.32	1.579
	60	13.57 ± 0.35	2.15 ± 0.17	99.96	1.309
	120	19.45 ± 1.01	3.43 ± 0.26	99.99	1.004
	180	21.42 ± 0.34	4.26 ± 0.08	99.91	1.003
60	30	15.06 ± 0.86	2.12 ± 0.08	99.03	1.372
	60	16.19 ± 1.74	2.14 ± 0.37	98.01	1.488
	120	20.48 ± 2.72	2.42 ± 0.61	98.34	1.514
	180	25.31 ± 0.43	3.81 ± 0.79	99.91	1.001
100	30	13.67 ± 0.54	1.90 ± 0.04	99.48	1.258
	60	17.81 ± 0.27	3.11 ± 0.10	99.95	1.337
	120	23.89 ± 0.74	4.61 ± 0.45	99.99	1.007
	180	36.28 ± 2.06	7.29 ± 5.12	98.62	1.531

Values are expressed as mean ± standard deviation. β, is the scale factor; α, is the shape factor; A_f, is the accuracy factor.

3.2 Effect of microfuidization processing parameters on antimicrobial activity of nanoemulsions

The antimicrobial activity values of lemongrass essential oil-alginate nanoemulsions after being processed by microfluidization under different pressure and cycles are shown in Fig. 2. Both the coarse emulsion and nanoemulsions processed by microfluidization exhibited a strong antimicrobial action against *E. coli* inoculum, as the survival fraction decreased when contact time increased. *E. coli* survival fraction *versus* contact time with coarse emulsion or microfluidized nanoemulsions was successfully fitted to a Weibull equation (Table 4). The R^2 values were higher than 94.81 and accuracy factor of the equations with regard to experimental data ranged between 1.016 and 1.432. In the case of the coarse emulsion, the inactivation of *E. coli* at 5, 15 and 30 min contact time was 0.66, 2.25 and 5.85 logaritmic reductions, respectively, showing a β-value of 7.95 min.

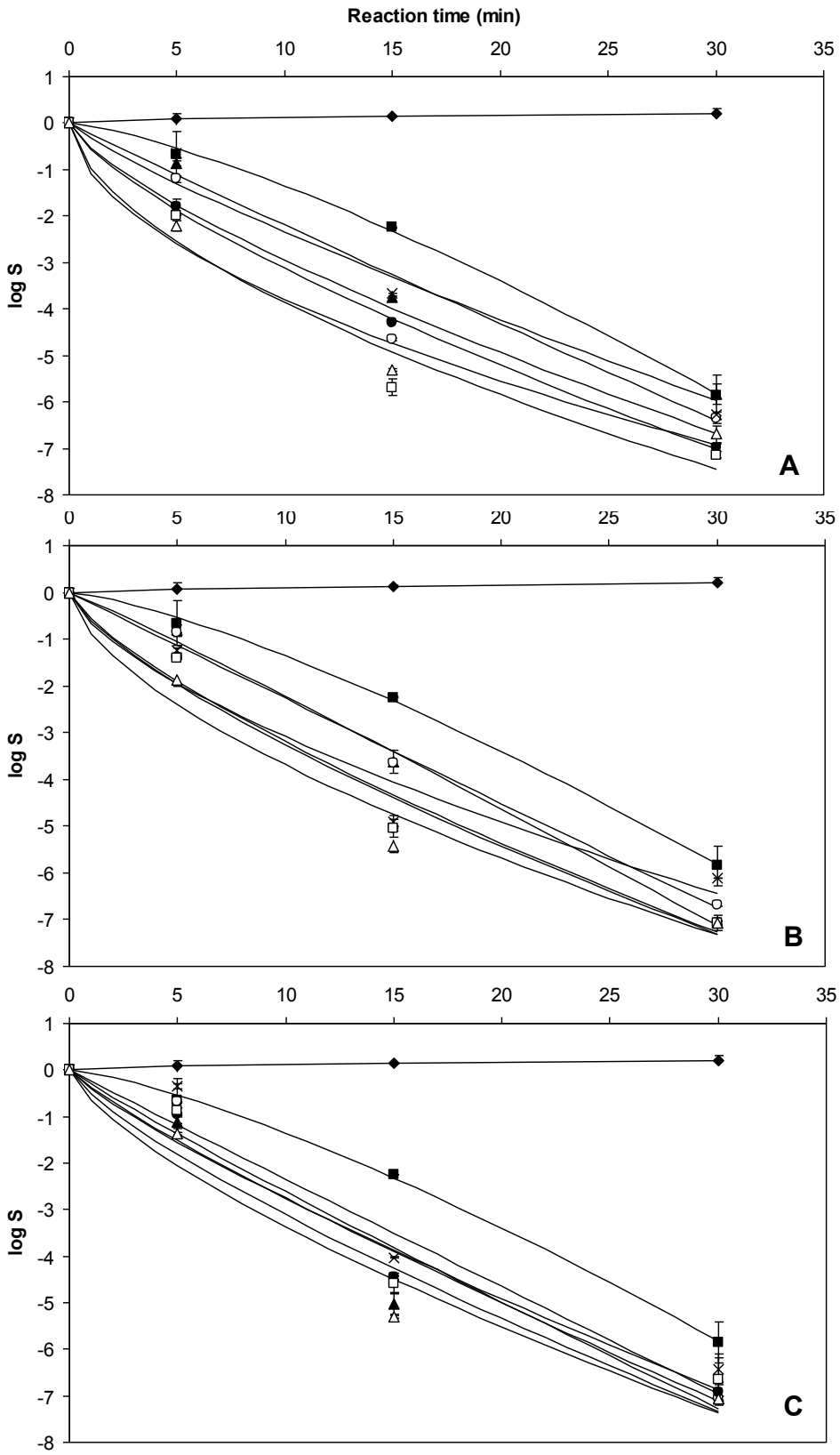

Figure 2 Survival fraction of *Escherichia coli* after contact with lemongrass oil-alginate emulsion (■) or nanoemulsions treated by microfluidization at 50 (A), 100 (B) or 150 MPa (C) for 1 (▲), 2 (○), 3 (×), 4 (●), 5 (□), or 10 (Δ) cycles. The control was water (♦). The plotted lines of log S vs. contact time (min) correspond to a Weibull equation fitted to experimental data. Data shown are a mean ± standard deviation.

Table 4 Estimated parameters of Weibull distribution function proposed to describe the inactivation kinetics of *Escherichia coli* by lemongrass oil-alginate emulsion or nanoemulsions produced by microfluidization at different processing pressure (MPa) and number of cycles

Pressure (MPa)	Cycles	β	α	R^2 (%)	A_f
0	0	7.95 ± 0.79	1.33 ± 0.05	99.40	1.062
50	1	3.68 ± 0.10	0.85 ± 0.01	98.14	1.147
	2	2.25 ± 0.13	0.73 ± 0.02	96.67	1.158
	3	4.46 ± 0.13	0.97 ± 0.02	98.39	1.184
	4	2.13 ± 0.16	0.73 ± 0.03	99.94	1.016
	5	1.04 ± 0.07	0.60 ± 0.01	96.84	1.116
	10	0.87 ± 0.03	0.54 ± 0.01	98.02	1.081
100	1	4.71 ± 0.33	1.06 ± 0.03	99.63	1.086
	2	4.38 ± 0.17	0.99 ± 0.02	99.44	1.096
	3	1.85 ± 0.18	0.67 ± 0.02	94.81	1.185
	4	1.99 ± 0.17	0.73 ± 0.02	97.46	1.135
	5	2.12 ± 0.13	0.74 ± 0.01	98.11	1.117
	10	1.23 ± 0.16	0.62 ± 0.02	97.45	1.108
150	1	2.33 ± 0.35	0.78 ± 0.04	96.61	1.182
	2	3.55 ± 0.09	0.93 ± 0.01	97.17	1.247
	3	4.42 ± 0.02	0.98 ± 0.02	96.05	1.432
	4	3.09 ± 0.11	0.86 ± 0.02	97.81	1.168
	5	2.93 ± 0.09	0.83 ± 0.04	96.53	1.213
	10	1.83 ± 0.05	0.71 ± 0.01	96.37	1.163

Values are expressed as mean ± standard deviation. β, is the scale factor; α, is the shape factor; A_f, is the accuracy factor.

The microfluidization treatment significantly influenced the antimicrobial activity of nanoemulsions. While the pressure did not affect the antimicrobial potential of nanoemulsions, an increase in the residence time in the interaction chamber seemed to improve their bactericidal action. Namely, nanoemulsions passed only once through the microfluidizer at 150 MPa exhibited a clearly enhanced bactericidal action against *E. coli*, reaching 1.13, 5.03 and 7.07 log-reductions of the inoculum at 5, 15 and 30 min of contact time. Moreover, the β-value also decreased in all nanoemulsions regardless the microfluidization pressure or the number of processing cycles. In general, the higher number of cycles, the lower the β value, which implies a higher antimicrobial potential. In fact, nanoemulsions microfluidized at 50 MPa for 10 cycles exhibited an inactivation

kinetic with a minimum β-value of 0.87 min. Regarding the shape factor, the coarse emulsion showed a concavity downwards, with α value higher than 1. However, the inactivation kinetics of *E. coli* in contact with lemongrass nanoemulsions showed a concavity upwards with α values lower than 1, of up to 0.54. In fact, this value depicts the faster inactivation of the microorganism at early contact time with nanoemulsions in comparison with the coarse emulsion.

4 Discussion

The results obtained in the present study suggest that the antimicrobial activity of nanoemulsions containing lemongrass essential oil is strongly influenced by the method used to reduce emulsion droplet size. Ultrasounds are a feasible technology to produce essential oil-loaded nanoemulsions with a very small droplet size ranging between 35 and 4 nm (Table 1) (Salvia-Trujillo, et al. 2012). However, according to the results observed in the present work, they severely compromise the bactericidal action of essential oils. Antimicrobial compounds present in EOs are extremely volatile in accordance to their aromatic hydrocarbon structure (Sell, 2010). Therefore, the loss of antimicrobial activity observed in sonicated nanoemulsions might be a consequence of the temperature reached during ultrasound treatments. Sonicated samples reached a maximum of 47 ºC at the outlet of the system when a treatment for 180 s at 100 μm amplitude was applied, and this temperature might be locally even higher inside the ultrasonic treatment chamber (Salvia-Trujillo, et al. 2012). Moreover, cavitation phenomena induced by ultrasounds produce the collapse of air bubbles within the fluid. This fact causes a local increase of the pressure and temperature, which in turn results in the dissociation of water into hydroxyl radicals and hydrogen atoms, appearing several molecular species in the media such as hydrogen peroxide (Riesz, Berdahl and Christman, 1985). It is known that the degradation of hydrophobic volatile compounds occurs via hydroxyl free radical reaction and also via a direct pyrolisis reaction in the collapsing hot bubbles and at the interface of the bubbles (Wu and Ondruschka, 2005, Nanzai, Okitsu, Takenaka, Bandow and Maeda, 2008, Jiang, Pétrier and David Waite, 2002, Jiang, Pétrier and Waite, 2002). These properties have been used as a strategy of decontamination of waste water from pollutant aromatic compounds (Gogate, 2008, Kidak and Ince, 2006). Moreover, it has also been reported as a method to improve the

functionality of food ingredients (Ashokkumar et al., 2008, Chemat, Zill-E-Huma and Khan, 2011). Nevertheless, and similarly to our results, Chemat and co-workers (Chemat, Grondin, Costes, Moutoussamy, Sing and Smadja, 2004, Chemat, Grondin, Sing and Smadja, 2004) reported the degradation of sunflower oil after emulsification by ultrasounds due to oxidation of lipid droplets. Therefore, in the present study this phenomenon might cause the undesirable degradation of aromatic compounds present in EOs, leading to a decrease in their antimicrobial activity.

Oppositely, nanoemulsions obtained by microfluidization exhibited an enhanced and faster bactericidal effect against *E. coli* in comparison with the coarse emulsion. This fact could be related to technological differences between the ultrasonicator and the microfluidizer. In this sense, the microfluidizer device allows a faster and more efficient cooling of the sample in comparison with the ultrasound equipment, maintaining the outlet temperature of the sample below 20 ºC, therefore being a more gentle technology to preserve heat sensitive volatile compounds. Moreover, it can also be explained by the small droplet size of microfluidized nanoemulsions. We observed in previous studies with the same nanoemulsion formulation a higher reduction of the particle size at increasing the processing pressure and cycles, ranging between 53 and 6 nm (Table 2) (Salvia-Trujillo, et al. 2013). The delivery mechanism of volatile compounds from nanoemulsions might be explained by the destabilization of the interfacial layer of small molecule non-ionic surfactants due to their interaction with the microbial cell since they tend to associate with some constituents of the biological membrane (Pérez-Conesa, Cao, Chen, McLandsborough and Weiss, 2011). Therefore the delivery of the aromatic compounds would occur faster in nanoemulsions rather than in macroemulsions due to their reduced droplet size, being more interfacial area exposed to the microbial cells. Despite there are no studies considering straightforward the effect of microfluidization processing parameters on the functionality of food-grade nanoemulsions, some recent investigations may support the results obtained in the present work. Namely, Donsí and co-workers (Donsì, Annunziata, Sessa and Ferrari, 2011) observed a lower minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *Lactobacillus delbrueckii*, *Saccharomyces cerevisiae* and *E. coli*, of a mixture of terpenes nanoemulsified by high pressure homogenization for 10 cycles at 300 MPa in comparison with the same emulsion produced by high shear homogenization. Furthermore, in a different work, Donsì and co-workers (Donsì, Annunziata, Vincensi and Ferrari, 2011) reported a reduction between 2 and 4 log-cycles the *E. coli*

population after being in contact during 24 h with a limonene-loaded nanoemulsion stabilized with soy lechitin or sugar esters processed by high pressure homogenization (300 MPa, 5 cycles). Lemongrass essential oil has been reported to have a strong *in vitro* antimicrobial activity against several foodborne microorganisms (Hammer, Carson and Riley, 1999, Friedman, Henika and Mandrell, 2002) and also when it has been incorporated in real food systems (Rojas-Graü, Raybaudi-Massilia, Soliva-Fortuny, Avena-Bustillos, McHugh and Martín-Belloso, 2007, Raybaudi-Massilia, Mosqueda-Melgar and Martín-Belloso, 2006, Friedman, Henika, Levin and Mandrell, 2004). Recently, other authors have reported improved functional properties of nanoemulsions with lemongrass essential oil for cosmetic applications, such as mosquito repellent (Nuchuchua, Sakulku, Uawongyart, Puttipipatkhachorn, Soottitantawat and Ruktanonchai, 2009) or for the treatment of acne (Faiyazuddina, Baboota, Ali, Ahuja and Ahmad, 2009). However, there are no previously reported data regarding the production of lemongrass oil-loaded nanoemulsions in the food sector.

5 Conclusions

Results obtained in the present work reveal that the processing method to obtain essential oil-loaded nanoemulsions determines their functionality. In spite of being a feasible technology to reduce the droplet size of emulsion up to nanometric scale, ultrasound processing diminished the antimicrobial potential of lemongrass oil-alginate nanoemulsions against *E. coli* in comparison with the coarse emulsion, with a complete loss of the antimicrobial properties of those nanoemulsions obtained at higher sonication amplitude and time. In contrast, microfluidization lead to nanoemulsions with a clearly faster and higher bactericidal action compared with the emulsion with higher droplet size. Therefore, microfluidization would be a suitable technology to improve the functionality of food ingredients. Further research would be aimed at evaluating the effectiveness of other EOs incorporated to microfluidized nanoemulsions and the antimicrobial activity of these systems when incorporated to foods.

Aknowledgements

This study was supported by the Ministerio de Ciencia e Innovación (Spain) throughout the project AGL2009-11475. Laura Salvia-Trujillo thanks the Ministry of Science and

Education (Spain) for the predoctoral grant. Prof. Olga Martín-Belloso thanks the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia 2008 Award.

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PUBLICATIONS

Chapter IV

Formulation of antimicrobial edible nanoemulsions with pseudo-ternary phase experimental design

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Food Bioprocess and Technology (under revision).

Abstract

The aim of the present work was to study the formulation of edible nanoemulsions containing lemongrass essential oil as antimicrobial agent, tween 80 as a non-ionic surfactant and sodium alginate as stabilizing and texturizing agent, by means of a pseudo-ternary phase experimental design. Polynomial models were satisfactory fitted to experimental data. Nanoemulsions with an average droplet diameter smaller than 100 nm were obtained by mixing oil and tween 80 at concentrations below 0.6% (v/v) and higher than 0.9% (v/v), respectively. However, sodium alginate played a synergistic role regarding the stabilization of oil droplets in the absence of surfactant. In this sense, the higher the sodium alginate concentration the stronger the negative surface charge of lipid droplets, as well as the higher the viscosity of the mixture. On the other hand, the emulsions whiteness decreased after increasing the surfactant and decreasing the oil phase, due to weak light scattering. As expected, the antimicrobial activity of blends was greater at higher amounts of essential oil, reaching a maximum of 7.37 log reduction of *Escherichia coli* after 30 min of contact time. Nevertheless, solubilizing and stabilizing the oil droplets by adding tween 80 and sodium alginate might enhance the bactericidal effect of essential oils due to an improved dispersion in the continuous phase. The current work presents relevant information to formulate nanoemulsions incorporating antimicrobial agents for food applications.

Keywords: Nanoemulsion, lemongrass essential oil, pseudo-ternary phase diagram, antimicrobial activity

Table of contents

1 Introduction

In recent years, the incorporation of ingredients to food formulations has gone a step forward due to the emergence of new delivery systems that are able to encapsulate, protect and release active compounds in a more efficient way (McClements, 2011). Particularly, in the case of lipophilic active ingredients such as certain vitamins, antioxidants or antimicrobials, their incorporation in foods is even more challenging due to their poor water solubility. Nanoemulsions are described as colloidal dispersions of oil droplets (radius between 10 and 100 nm) in suspension within an aqueous phase that can act as carriers of active ingredients entrapped within the lipid core (McClements & Rao, 2011). Besides the oil and aqueous phases, the formulation of nanoemulsions requires the use of a stabilizer, which is a surface-active molecule capable of adsorbing to droplet surfaces and protecting droplets against aggregation (McClements, 2005, Kralova & Sjöblom, 2009). Moreover, the addition of texture modifiers and thickening agents in the aqueous phase, such as biopolymers, is often used to improve emulsion stability, by retarding the droplet movement, as well as to provide specific textural characteristics (McClements & Rao, 2011). The characteristics and concentration of all components in the nanoemulsion system will determine its final properties. So far, several advantages have been attributed to nanoemulsions over conventional emulsions. Due to their reduced droplet size, they are considered to be more stable in terms of coalescence, gravitational separation or particle aggregation (Mason et al. 2006). Moreover, they barely scatter the light, appearing as clear or almost transparent systems that might be suitable to incorporate in clear drinks and foods (McClements, 2002a). In addition, there are evidences that the reduction of droplet size and the subsequent increase in surface area of nanodroplets might increase the functionality of the bioactive compound delivered. Therefore, the role of intrinsic characteristics of nanoemulsions is considered to be of crucial importance to design optimized delivery systems of food ingredients with poor water solubility.

Essential oils (EOs) are aromatic oily liquids obtained from natural plant products consisting of concentrated mixtures of terpenoids (Fisher & Phillips, 2008). EOs might exert different biological actions and are typically used as flavorings and preservatives in the food industry (Adorjan & Buchbauer, 2010). The use of EOs as natural antimicrobials in the food industry is a recent trend that is becoming popular due to the

consumer demand of foods free from synthetic additives (Burt, 2004). They are able to disrupt and penetrate the lipid structure of the bacterial cell membrane, causing the leakage of cytoplasmatic constituents and therefore the loss of cell viability (Burt. 2004,Tajkarimi et al. 2010,Tiwari et al. 2009,Holley and Patel. 2005). Lemongrass (*Cymbopogon citratus*) EO is an aromatic extract rich in terpenoids such as citral and limonene (Friedman et al, 2004) that has been found to be effective against a broad spectrum of microorganisms after *in vitro* tests (Hammer et al, 1999) and in food products (Rojas-Graü et al., 2007, Raybaudi-Massilia et al., 2008b, Raybaudi-Massilia et al., 2008a). However, despite the potential benefits related with the use of EOs, their incorporation to foods still presents several drawbacks due to their poor water solubility, intense aroma and potential toxicity at high doses (Sánchez-González et al., 2011). In this sense, nanoemulsions would be a useful tool to design delivery systems of EOs in order to optimize their formulation by achieving an enhanced antimicrobial activity, which in turn would allow minimizing the concentration of EO used.

The selection of the appropriate concentration of each individual ingredient in the formulation of nanoemulsions is of crucial importance in order to obtain systems with the desired physicochemical characteristics and functional properties. The construction of conventional pseudo-ternary phase diagrams as a tool to formulate nanoemulsions requires a large amount of mixtures to determine the region where nanoemulsions are formed and often does not provide any explanation of the impact of individual excipients. Response surface mixture experimental design might be an efficient methodology to evaluate the effect of multiple factors, alone or in combination, with a minimum number of experiments (Cornell, 2002). The utilization of response surface methodologies may also improve understanding the influence of the emulsion components and their interactions as well as predicting optimized formulations with the desired properties (Li & Chiang, 2012, Ren et al., 2013). Thus, the aim of the present work was to explore the effect of the lemongrass EO, surfactant and sodium alginate concentrations on the droplet size, ζ-potential, viscosity, color and antimicrobial activity of nanoemulsions. For that purpose a pseudo-ternary phase experimental design based on a response surface methodology was used as a tool to describe their effect on the selected responses.

2 Material and methods

2.1 Pseudo-ternary phase experimental design

An experimental mixture design was used to determine the effect of the concentration of sodium alginate, lemongrass EO and surfactant on the formation of oil-in-water nanoemulsions. The selected responses were emulsion droplet diameter (z-average), ζ-potential, viscosity, color and antimicrobial activity. A D-optimal design was used to build up the pseudo-ternary phase diagrams and to define the optimum number of experiments and was set up using the software Design Expert 8.0.4 (Stat Ease Inc., Minneapolis, MN). The concentration of each component was limited according to the following constraints expressed in volume basis (v/v): 0.001 < EO < 0.02; 0 < tween 80 < 0.06; 0 < sodium alginate < 0.02; 0.9 < water < 0.999; EO + tween 80 + sodium alginate + water = 1. The minimum concentration of lemongrass EO was set below its minimum inhibitory concentration (Hammer et al., 1999) to see if there is an enhancement of the antimicrobial activity after emulsification, whereas the maximum concentration was set to limit its sensorial impact. Tween 80 concentration was bound to an oil:surfactant ratio of 1:3, assuring a complete coating of lipid droplets surface with adsorbed surfactant molecules (Qian & McClements, 2011). Sodium alginate maximum concentration was set according to its viscosity, to avoid the subsequent block of the high-pressure homogenizer micro channels. The droplet diameter (z-average), ζ-potential, viscosity, color and antimicrobial activity of the mixtures were the responses measured for the different formulations. The empirical data of the response surface experimental design is shown in Table 1. All samples were prepared and analyzed in random order during the same day without further storage. Two replicate analyses of each sample and parameter were performed and the mean value was calculated. Afterwards, experimental data was represented graphically with pseudo-ternary phase diagrams and modeled with a Scheffe polynomial equation (Eq. 1) for the 4 components to predict their effect in the formulation. In those responses where the differences between experimental values were very high, a Box-Cox transformation was applied to data to normalize the data and stabilize the variance. The statistical significance of the models for each response was considered regarding their adjusted R^2 and *p*-values.

$$Y = \sum \beta_i x_i + \sum\sum \beta_{ij} x_i x_j + \sum\sum\sum \beta_{ijk} x_i x_j x_k \qquad \text{Eq. (1)}$$

Where:

Y is the response variable; β_i is the first-order coefficient, β_{ij} is the second-order coefficient; β_{ijk} is the third-order coefficient; x_1 is the lemongrass EO; x_2 is the tween 80; x_3 is the water; x_4 is the sodium alginate.

2.2 Nanoemulsion formation

First, the aqueous phase of nanoemulsions was prepared by dissolving the sodium alginate (FMC Biopolymers, UK) in hot water at 70 ºC and continuous stirring until it was completely dissolved. Primary emulsions were prepared by mixing the lemongrass (*Cymbopogon citratus*) essential oil (Laboratoris Dicana, Spain) and Tween 80 (Scharlau, Spain) as non-ionic surfactant with the aqueous phase. The blend was mixed using a laboratory T25 digital Ultra-Turrax mixer (IKA, Staufen, Germany) working at 3400 rpm for 2 min. Then, primary emulsions were passed though a microfluidizer (M110P, Microfluidics, Massachusetts, USA) 3 cycles at 150 MPa. The product was refrigerated through a cooling coil immersed in an iced-water bath, placed at the outlet of the interaction chamber, so that the temperature of the product was always kept below 20 ºC.

2.3 Nanoemulsion characterization

2.3.1 Particle size and ζ-potential

The emulsion droplet size was measured by dynamic light scattering (DLS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK) working at 633 nm at 25 ºC and equipped with a backscatter detector (173 º) (Brar & Verma, 2011). DLS measures the Brownian motion of nanosized droplets and relates this movement to an equivalent hydrodynamic diameter (nm). Average droplet diameter (nm) and size distribution curves in intensity (%) were used to characterize oil droplets dispersion in nanoemulsions. The lemongrass oil absorbance at 633 nm was 0.001 and its refractive index 1.487.

The electrophoretic mobility of oil droplets, also reported as ζ-potential, was measured by phase-analysis light scattering (PALS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK). It determines the surface charge at the interface of the droplets dispersed in the aqueous solution.

Table 1. Mixture design formulations of nanoemulsions and experimental values of droplet size, ζ –potential, viscosity, whiteness index and antimicrobial activity against *Escherichia coli* at 5, 15 and 30 min contact times

Run	LEO	Tween 80	Sodium alginate	Water	Droplet diameter (nm)	ζ-potential (mV)	Viscosity (mPa.s)	Whiteness index	logS 5'	logS 15'	logS 30'
1	0.020	0	0	0.980	1305.9 ± 387.1	0.0 ± 0.0	1.1 ± 0.1	53.1 ± 16.8	-2.66 ± 0.15	-7.24 ± 0.01	-7.24 ± 0.01
2	0.010	0.030	0	0.960	12.8 ± 0.3	-7.2 ± 1.1	1.1 ± 0.1	25.5 ± 0.4	-0.23 ± 0.03	-0.13 ± 0.07	-1.27 ± 0.29
3	0.001	0.030	0.010	0.959	12.4 ± 2.7	-36.9 ± 5.1	19.7 ± 1.9	26.1 ± 1.5	0.29 ± 0.03	0.12 ± 0.12	0.21 ± 0.08
4	0.015	0.015	0.015	0.955	5.8 ± 0.9	-47.2 ± 4.2	42.2 ± 3.5	37.6 ± 6.2	-1.98 ± 0.02	-4.31 ± 0.48	-6.76 ± 0.01
5	0.001	0	0.020	0.979	162.8 ± 18.8	-98.7 ± 19.4	135.0 ± 24.7	28.0 ± 2.7	0.22 ± 0.07	0.28 ± 0.11	0.25 ± 0.12
6	0.020	0	0.020	0.960	380.7 ± 82.9	0.0 ± 0.0	129.8 ± 20.2	72.2 ± 6.1	-6.93 ± 0.01	-6.93 ± 0.01	-6.93 ± 0.01
7	0.020	0.060	0	0.920	13.0 ± 0.4	-6.9 ± 1.7	1.3 ± 0.1	25.7 ± 0.3	-0.97 ± 0.09	-3.64 ± 0.28	-6.49 ± 0.19
8	0.001	0	0	0.999	196.0 ± 6.6	-45.0 ± 0.8	1.0 ± 0.2	31.5 ± 0.7	-5.37 ± 0.01	-6.37 ± 0.01	-6.37 ± 0.01
9	0.010	0.030	0	0.960	13.9 ± 1.3	-6.5 ± 1.1	1.3 ± 0.2	28.3 ± 0.2	-0.51 ± 0.02	-0.87 ± 0.02	-2.80 ± 0.04
10	0.001	0	0.020	0.979	151.6 ± 17.3	-94.2 ± 15.3	120.7 ± 31.9	27.2 ± 1.9	-0.28 ± 0.12	-0.27 ± 0.01	-0.29 ± 0.11
11	0.020	0.030	0.020	0.930	2.2 ± 0.7	-68.4 ± 9.0	112.0 ± 18.5	31.7 ± 2.4	-4.59 ± 0.24	-5.87 ± 0.71	-7.37 ± 0.01
12	0.006	0.045	0.015	0.934	4.8 ± 1.5	-43.0 ± 3.0	44.3 ± 3.7	26.1 ± 1.6	0.34 ± 0.04	0.39 ± 0.01	0.41 ± 0.03
13	0.020	0	0	0.980	1352.7 ± 342.3	0.0 ± 0.0	1.1 ± 0.3	58.1 ± 8.5	-2.68 ± 0.05	-7.37 ± 0.01	-7.37 ± 0.01
14	0.006	0.015	0.010	0.969	125.2 ± 33.8	-50.6 ± 3.8	18.7 ± 2.6	26.5 ± 1.8	0.37 ± 0.04	0.43 ± 0.11	0.53 ± 0.18
15	0.010	0.060	0.020	0.910	1.8 ± 0.3	-45.7 ± 6.7	98.7 ± 10.4	25.9 ± 1.8	-6.14 ± 0.07	-7.37 ± 0.01	-7.37 ± 0.01
16	0.015	0.030	0.010	0.945	2.9 ± 0.8	-36.1 ± 4.9	18.1 ± 1.0	28.3 ± 1.8	-1.98 ± 0.09	-3.63 ± 0.01	-6.93 ± 0.01
17	0.020	0.060	0	0.920	30.8 ± 5.1	-5.1 ± 1.2	1.4 ± 0.2	28.6 ± 0.3	-0.22 ± 0.03	-1.23 ± 0.08	-3.89 ± 0.09
18	0.001	0.060	0	0.939	20.4 ± 7.3	-13.6 ± 0.9	1.4 ± 0.2	27.7 ± 0.3	0.17 ± 0.19	0.03 ± 0.03	-0.05 ± 0.13
19	0.001	0	0	0.999	170.7 ± 48.3	-41.6 ± 3.3	1.0 ± 0.2	31.6 ± 0.3	0.02 ± 0.06	-0.02 ± 0.08	0.06 ± 0.01
20	0.010	0	0.010	0.980	919.8 ± 94.1	-49.7 ± 4.2	16.4 ± 1.6	62.0 ± 3.4	-5.41 ± 0.02	-6.67 ± 0.01	-6.40 ± 0.52

Values are expressed as mean ± standard deviation. LEO: lemongrass essential oil.

2.3.2 Viscosity

A SV-10 vibro-viscometer (A&D Company, Tokyo, Japan) vibrating at 30 Hz was used to measure the viscosity of 10 mL-aliquots of the emulsions. Moreover, the viscosity of sodium alginate solutions of different concentration, according to the experimental design, was also measured. The viscosity of the sodium alginate solutions at 1%, 1.5% and 2% (w/v) was 54.1, 81.3 and 256.0 mPa.s, respectively. The observed values were considered the viscosity of the dispersant phase during the measurement of the particle size by dynamic light scattering.

2.3.3 Whiteness index

The color of emulsions and nanoemulsions was measured with a Minolta CR-400 colorimeter (Konica Minolta Sensing, Inc., Osaka, Japan) at room temperature set up for illuminant D65 and 10º observer angle and calibrated with a standard white plate. CIE L*, a* and b* values were determined and the Whiteness Index (WI) was calculated with equation 2 (Vargas et al. 2008):

$$WI = 100 - ((100 - L)^2 + (a^2 + b^2))^{0.5} \quad \text{Eq. (2)}$$

2.3.4 Antimicrobial activity

The antimicrobial activity of nanoemulsions was assessed evaluating the *in vitro* inactivation kinetics of *Escherichia coli*. The method used was a modification of a technique previously described by Ferreira, Alves, Neves, Gibbs & Teixeira (2010). *E. coli* 1.107 (Laboratoire de Répression des Fraudes, Montpellier, France) was cultured in tryptone soy broth (Bioakar Diagnostic; Beauvais, France) and incubated at 37 ºC with continuous agitation at 120 rpm for 11 h to obtain cells in stationary growth phase. The final concentration reached in the culture was 108-109 colony forming units/millilitre (CFU/mL). A 0.5 mL-aliquot of overnight bacterial culture was mixed with 0.5 mL of the essential oil-alginate emulsion or nanoemulsion and 4.5 mL of sterile Mili-Q water. To determine the inactivation kinetics, an aliquot of the homogenate was taken at 5, 15 and 30 minutes of reaction time to be serially diluted and spread on McConkey agar (Biokar Diagnostics, Beauvais, France) plates. A control was performed with the same method, replacing the emulsion or nanoemulsion by sterile Mili-Q water. Colony count was determined after incubation of agar plates at 37 ºC for 24 hours.

3 Results and discussion

3.1 Particle size

The experimental results of droplet diameter of oil-in-water emulsions are shown in Table 1. The average droplet size of emulsions ranged between 2 and 1300 nm depending on the concentration of lemongrass essential oil, tween 80 and sodium alginate. Contour plots and 3D surface response graphs throughout the manuscript are represented setting the concentration of water constant at 0.98 to facilitate the representation of the data in a two-dimensional plane. It was observed that the droplet size of the emulsions was smaller with decreasing concentrations of oil and increasing amounts of tween 80 (Figure 1). In this sense, mixtures with concentrations of oil and tween 80 lower than 0.6 and higher than 0.9 % (v/v), respectively, led to nanoemulsions with particle sizes smaller than 100 nm. However, in the region of the mixture design representing higher concentrations of lemongrass essential oil and lower concentrations of surfactant the droplet size of emulsions was above 500 nm. Low-mass surfactants, such as tween 80, are known to rapidly coat the surface of the created oil-water interface during emulsification (Kralova & Sjöblom, 2009). Therefore, there must be a sufficient amount of surface-active molecules to cover the surface area of oil droplets generated during the formation of nanoemulsions. Moreover, the concentration of alginate had a less pronounced influence on the droplet size of the obtained nanoemulsions. However, mixtures with absence of a surfactant (tween 80) and a concentration of sodium alginate dissolved in the aqueous phase higher than 1.5 % (w/v) exhibited droplet sizes between 200 and 300 nm, thus confirming the role of sodium alginate as emulsifier in the formation of nanoemulsions. This effect might be due to the increase of the continuous phase viscosity produced by the addition of sodium alginate. This effect has also been observed by other authors (Qian & McClements, 2011) who reported a reduction on the nanoemulsion droplet size as the viscosity of the continuous phase increased. When nanoemulsions are produced by microfluidization, the viscosity of the continuous phase may affect the droplet size throughout an increased droplet fragmentation due to an increased disruptive shear stress, or a decreased droplet re-coalescence due to a decreased droplet collision frequency.

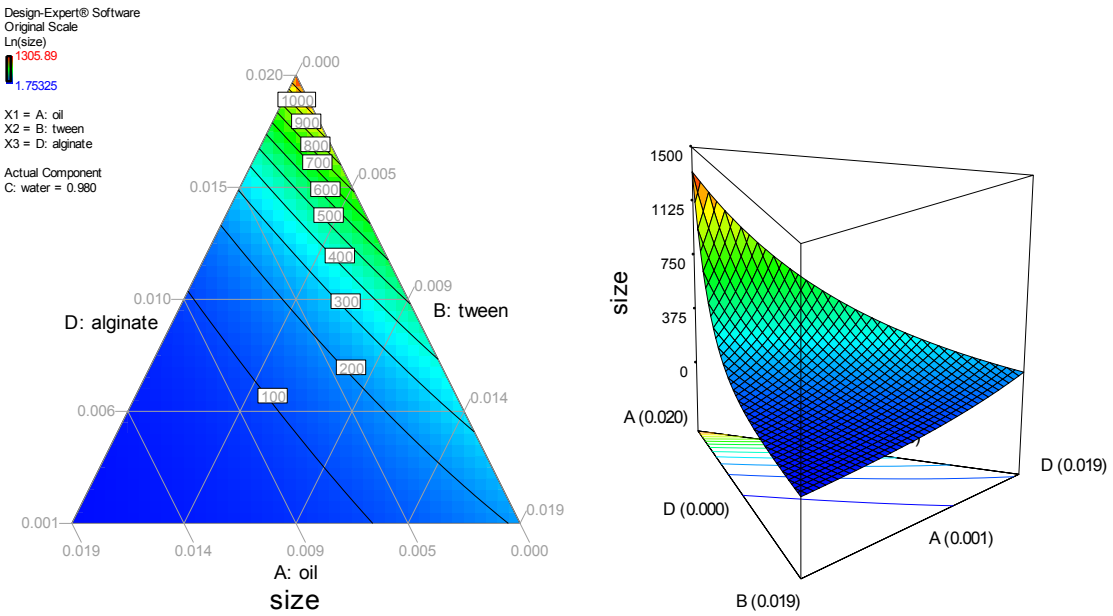

Figure 1. Contour and surface response plots of average droplet diameter (nm) of mixtures containing lemongrass essential oil (A), tween 80 (B) and sodium alginate (D). The water (C) content was set at 0.98.

Table 2. Coded polynomial equation coefficients of the Scheffe model for L-pseudo-components and statistical significance of models.

	Particle size	ζ-potential	Viscosity	Whiteness index	log S 5'	log S 15'	log S 30'
A	22.94	2024.94	3.20	-119.28	99.61	9.37	27.59
B	11.62	-74.76	0.17	98.51	-10.43	-11.60	-7.07
C	5.46	-43.26	0.00952	31.83	-2.89	-3.44	-3.23
D	-32.63	-928.00	-3.87	-475.95	9.75	56.03	65.73
AB	-53.74	-2581.95	-3.96	-85.53	-100.62	3.71	-56.76
AC	-10.45	-2281.26	-372	347.42	-126.24	-40.82	-64.18
AD	-4.66	-104.71	12.83	1321.27	-295.80	-196.17	-243.10
BC	-24.50	203.65	0.18	-168.24	30.59	36.30	26.02
BD	9.29	2420.57	16.35	367.88	3.19	-71.77	-79.27
CD	46.46	779.16	17.87	618.98	1.79	-51.87	-63.77
ABC	-	988.97	-	-	-	-	-
ABD	-	-10704.7	-	-	-	-	-
ACD	-	-58.60	-	-	-	-	-
BCD	-	-2813.47	-	-	-	-	-
R^2	0.9103	0.9982	0.9990	0.9246	0.7887	0.8187	0.7856
p-value	0.0004	<0.0001	<0.0001	0.0002	0.0184	0.0095	0.0196

A: lemongrass EO; B: tween 80; C: water; D: sodium alginate

The coefficients of the polynomial equation to predict the droplet size and the statistic significance of the model are shown in Table 2. A quadratic model was successfully fitted to experimental data, which was previously transformed using a logarithmic power law as recommended by the Box-Cox method, being significant with a *p*-value of 0.0004 and a R^2 of 0.91 (Table 2). It is considered that the higher the magnitude of the

coefficient parameter of the linear components in the fitted equation the higher the response (Cornell, 2002). However, in this particular case, instead of considering the effect of single components on the droplet size of the oil-in-water emulsion, it is more useful to consider the possible interactions of oil (component A) with other components, which are given by the estimate coefficients of the quadratic terms. In this sense, positive (>0) coefficients of the quadratic terms indicate a synergistic effect, whereas negative coefficients indicate an antagonistic effect (Cornell, 2002). Thus, the large negative coefficient for the AB term (Table 2) shows the effect of the concentration of oil and tween 80 on the droplet size of nanoemulsions, meaning that by combining both ingredients, the droplet size is reduced. Recently, two other research works by Hessein and co-workers (2011) and Maher and co-workers (2011) have used ternary-phase diagrams as a tool to formulate nanoemulsions.

3.2 ζ -potential

The electrical charge at oil-water interface of lipid droplets of the different formulations is shown in Table 1. The ζ-potential of oil droplets with different formulations varied significantly according to their composition, ranging between 0 and -98.65 mV. The surface electrical charge of lipid droplets was clearly influenced by the addition of sodium alginate in the mixture. In the contour plot and 3D surface response graph (Figure 2) it can be observed that blends with a low concentration of sodium alginate and high concentration of oil phase resulted in ζ-potential close to 0. However, slightly increasing the concentration of sodium alginate beyond 0.1 % (w/v) led to oil droplets with ζ-potential around -30 mV. In general, particles with ζ-potential more positive than +30 mV or more negative than -30 mV are considered to be stable, since electrical charge of droplets is strong enough to assume that repulsive forces between droplets are predominant in the system (Heurtault et al., 2003). Increasing the concentration of sodium alginate up to 2 % (w/v) decreased the surface electrical charge of lipid particles up to -90 mV. Alginates are linear polymers of mannuronic acid and guluronic acids, which are monomers with a low dissociation constant (Tønnesen & Karlsen, 2002). Therefore, alginic acid and its salts tend to be negatively charged in a wide range of pH. However, oil droplets exhibited negative charges even in the absence of sodium alginate (axis of component D, Figure 2). This fact could be due to that, despite being non-ionic surfactants, polysorbate molecules (tween 80) adsorbed to droplet surface might lead to negative electrical charges under certain conditions (Hsu and Nacu. 2003). This effect

was confirmed after fitting a cubic model to experimental data with a *p*-value <0.0001 and a R^2 of 0.9982 (Table 2). Consistently, the negative coefficients for tween 80 (-74.76) and sodium alginate (-928.00) indicate their contribution to lower the ζ-potential of the blend, being the latter the one exerting the larger influence. However, the interaction of tween 80 and sodium alginate seem to be antagonistic in terms of the ζ-potential, as shown by the positive coefficient BD (2420.57). As can be observed by the curvature of the contour lines (Figure 2), at constant concentration of alginate the addition of a moderate amount of tween 80 increases the ζ-potential of the mixture. This fact suggests competitive adsorption of polymer and surfactant molecules on the oil/water interface, which in turn might be concentration-dependent. Low surfactant concentrations might not be enough to cover the oil/water interface. This could lead to the adsorption of polymer molecules, thus conferring a more negative ζ-potential (Goddard, 2002).

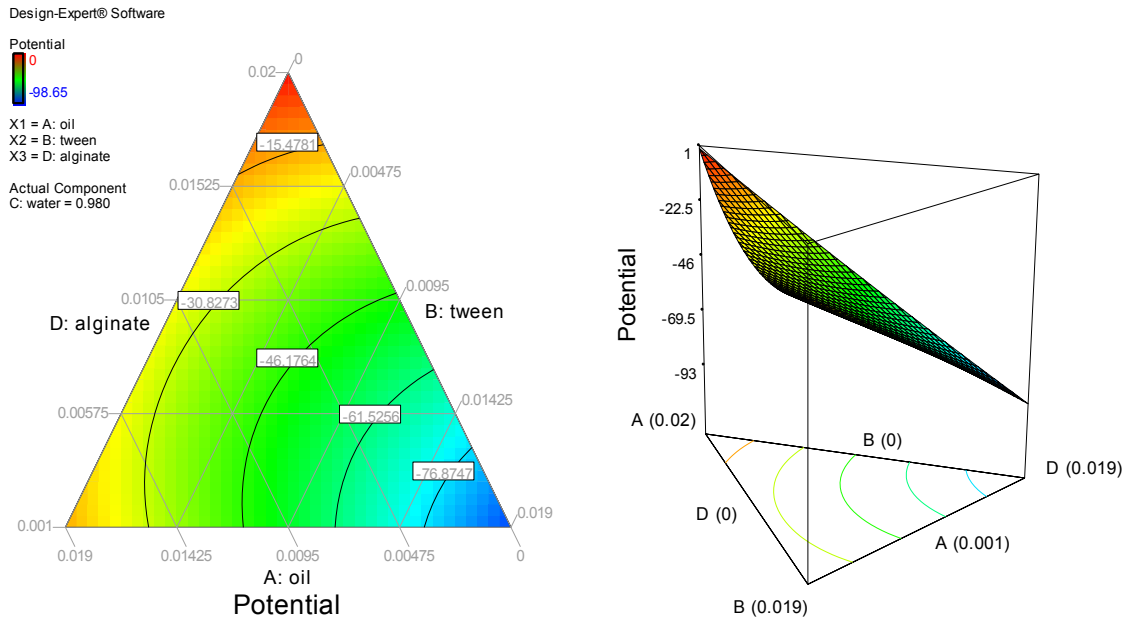

Figure 2. Contour and surface response plots of ζ-potential (mV) of mixtures containing lemongrass essential oil (A), tween 80 (B) and sodium alginate (D). The water content (C) is set at 0.98.

The behaviour observed at higher surfactant concentrations might be explained by two reasons: (i) the surfactant molecules adsorbed at the oil/water interface might repel polymer molecules (De Gennes, 1990); (ii) the hydrophilic sites of polymer molecules might bind surfactant molecules thus lowering the adsorption of sodium alginate to the oil surface (Neumann et al., 2003). Nevertheless, the large negative coefficient ABD (-

10704.75) shows that mixing lemongrass essential oil, tween 80 and sodium alginate causes a decrease in the ζ-potential of emulsions, thus conferring a higher stability to the oil-in-water system (Table 2).

3.3 Viscosity

The viscosity values of the mixtures ranged between 0.97 mPa.s and 135.00 mPa.s depending on the formulation (Table 1). As expected, viscosity of blends was mainly governed by the concentration of sodium alginate dissolved in the aqueous phase. A quadratic model was fitted to experimental data, which was transformed using a log10 power law, recommended by the Box-Cox method. The fitting of the quadratic model was statistically significant with a R^2 of 0.999 and *p*-value of <0.0001 (Table 2). As observed in the contour plot and 3D surface graph (Figure 3), the higher the concentration of sodium alginate the higher the viscosity. The horizontal trend of the contour lines regarding the alginate axis indicates a weak influence of other compounds on the emulsion viscosity. The positive coefficients of the quadratic terms of alginate with oil, tween or water (AD, BD, CD) indicate the increase of viscosity after adding alginate to the mixtures. It can be observed (Figure 3) that concentrations of sodium alginate lower than 1% (w/v) led to emulsions with viscosities lower than 15 mPa.s. However, increasing the concentration of sodium alginate in the aqueous phase from 1% (w/v) to 2% (w/v) caused a remarkable increment of the viscosity. The thickening properties of sodium alginate have been previously described (Tønnesen & Karlsen, 2002, Rinaudo, 2008) and, more recently, its use as stabilizer and texturizer in the formulation of nanoemulsions has been pointed out (McClements & Rao, 2011). Previously reported data concluded that alginates present non-newtonian behaviour in most cases and exhibited typical pseudo-plastic behaviour (King, 1994, Mancini et al., 1996). Also the viscosity of sodium alginate depends on the molecular weight and increases with increasing the concentration (Mancini et al., 1996, Gómez-Díaz & Navaza, 2003). The vibro-viscometer used to determine the viscosity of emulsions in the present study does not allow varying the shear rate of the determination. Therefore, due to the non-newtonian behaviour of sodium alginate, the viscosity increase is more pronounced at higher concentrations.

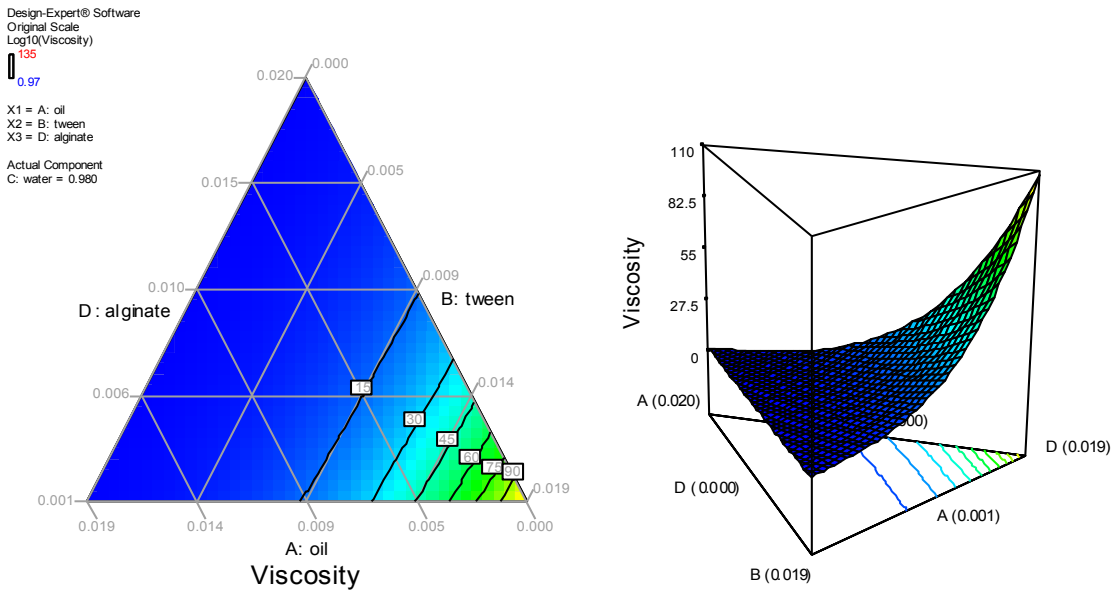

Figure 3. Contour and surface response plots of viscosity (mPa.s) of mixtures containing lemongrass essential oil (A), tween 80 (B) and sodium alginate (D). The water (C) content was set at 0.98.

3.4 Whiteness index

The whiteness index of oil-in-water emulsions was determined as an indicator of their milky appearance. Therefore, the lower the whiteness index, the more transparent the emulsions are. The whiteness index of blends ranged between 25.48 and 72.20 (Table 1) depending on the formulation. A quadratic model was fitted to experimental results, which was statistically significant with a R^2 and *p*-value of 0.9246 and 0.0002, respectively (Table 2). In this sense, in the contour plot and 3D surface graph (Figure 4) it can be observed that the concentration of tween 80 and lemongrass essential oil significantly affected the colour of the blends. In this regard, the higher the concentration of oil the higher the whiteness index was. This was confirmed by the positive coefficients of the quadratic terms in the polynomial equation that include the lemongrass EO component (AC, AD) (Table 2). Moreover, in general, at increasing concentration of surfactant, the whiteness index of the mixture is decreased. This fact was reflected by the negative coefficients of the quadratic terms in the polynomial equation (AB, BC) (Table 2). Emulsion color is given as a consequence of the scattering of light colliding with droplets. It is described that the light scattering of oil droplets depends on the refractive index of continuous and dispersed phase, oil concentration and also droplet size (McClements, 2002a, McClements, 2002b). Large particles scatter

the light more intensely than smaller ones, which cause an increase in the lightness, opacity and whiteness index of emulsions (McClements, 2011). In this sense, oil and surfactant concentrations might affect emulsion whiteness index due to their influence on droplet size. Similarly to our results, Rao & McClements (2012) also reported an increase on the turbidity of lemon oil nanoemulsion when increasing the oil content or decreasing the tween 80 concentration.

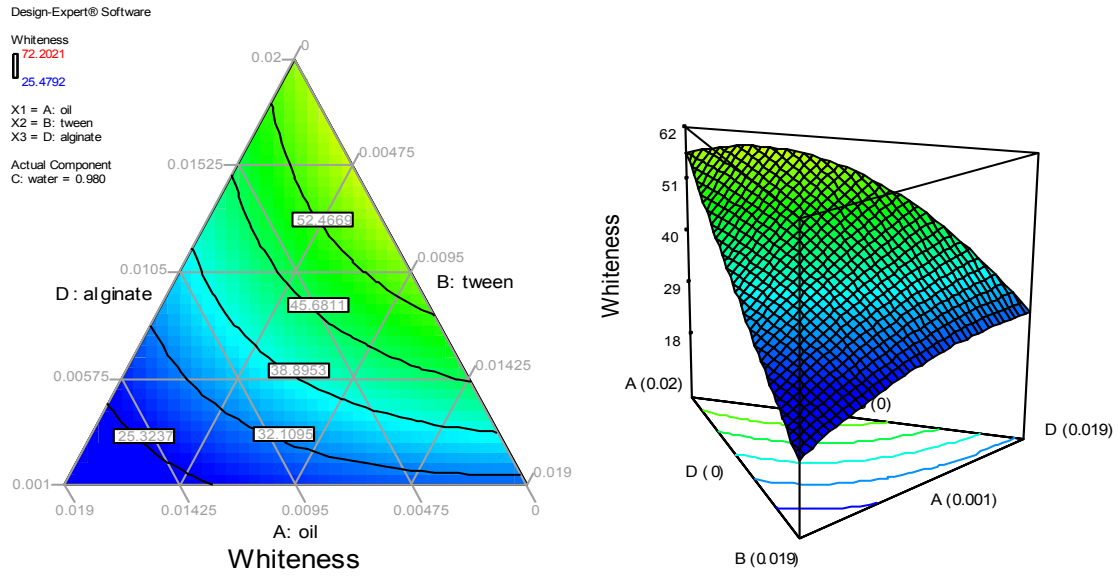

Figure 4. Contour and surface response plots of whiteness index of mixtures containing lemongrass essential oil (A), tween 80 (B) and sodium alginate (D). The water (C) content was set at 0.98.

3.5 Antimicrobial activity

The inactivation of *Escherichia coli* population after 5, 15 or 30 min of contact with the mixtures containing lemongrass essential oil is shown in Table 1. The reduction of log-units strongly depended on the formulation of emulsions and nanoemulsions, being the concentration of oil the ingredient with the greatest effect on the antimicrobial activity. In this sense, blends with an oil concentration lower than 0.06 % (v/v) did not reduce the microorganism after 30 min of contact time. On the contrary, a maximum reduction of 7.37 log-units was observed after 30 min of contact with formulations containing a concentration of oil higher than 1 % (v/v). Quadratic models were fitted to the inactivation experimental results. In all cases the fitting of the model was statistically significant with R^2 values higher than 0.78 (Table 2).

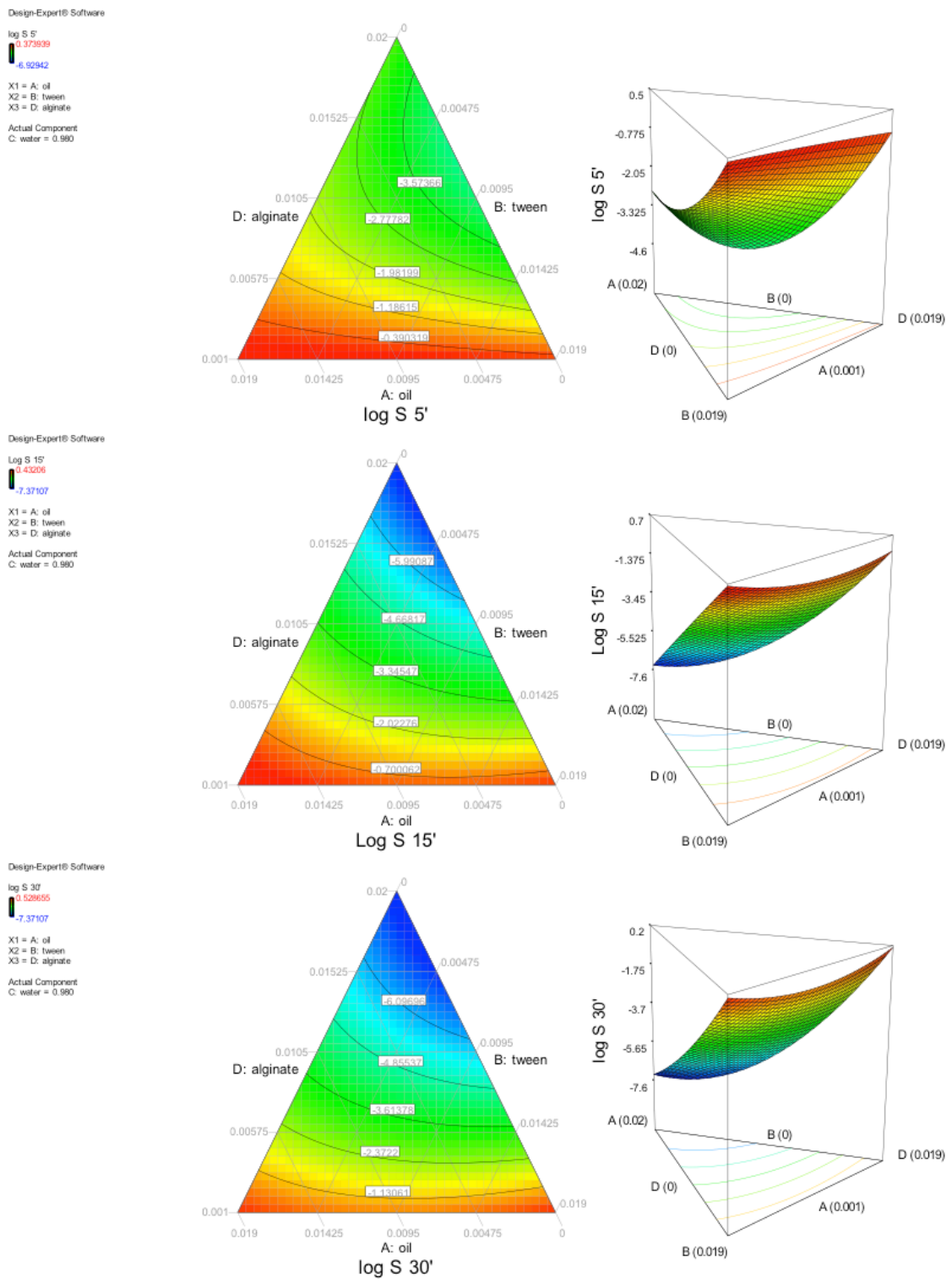

Figure 5. Contour and surface response plots of antimicrobial activity against *Escherichia coli* (log S after 5, 15 or 30 min of contact time) of mixtures containing lemongrass essential oil (A), tween 80 (B) and sodium alginate (D). The water (C) content was set at 0.98.

In the contour plots and 3D surface response graphs (Figure 5) it can be observed that, as expected, the higher the concentration of essential oil, the higher the antimicrobial activity, and this behavior was more pronounced after 15 or 30 min of contact time rather than 5 min. Moreover, the negative coefficients of the quadratic terms of the polynomial equation indicate a sinergistic interaction between the oil and the other ingredients (AB, AC, AD) (Table 2). This indicates that the addition of a surface-active compound, such as tween 80, or a texturizing agent, such as sodium alginate, may significantly enhance the bactericidal action of essential oils. This fact could be related with the improved dispersion and stabilization of nanosized oil droplets within the continuous phase due to the action of small molecule surfactants and biopolymers. Recently, and similarly to our results, Liang and co-workers (2012) observed an increased and prolonged antimicrobial activity against *Listeria monocytogenes* or *Staphylococcus aureus* of nanoemulsions containing peppermint essential oil and stabilized with starch in comparison with the bulk peppermint oil, which was attributed to the increased solubility and stability of the essential oil. There are scientific evidences to think that the smaller the droplet size, the higher the functionality of the active compounds encapsulated (Donsì et al., 2011, Ziani et al., 2011). However, Terjung and co-workers (2012) recently reported that the antimicrobial activity of essential oils might no only be determined by emulsion droplet size, since the location of the active compounds in the emulsion system as well as the concentration of the active compound may also be key factors affecting their bactericidal action. Therefore, it can be stated that the overall antimicrobial activity of nanoemulsified essential oils might be determined not only by the oil droplet size, but also by the oil distribution and stability within the continuous phase.

4 Conclusions

The pseudo-ternary phase experimental design was a useful tool to study the formulation of oil-in-water emulsions and nanoemulsions containing lemongrass essential oil. In all cases, quadratic or cubic models were successfully fit to experimental data to describe the behavior of the blends. Mixtures with concentrations of oil and tween 80 lower than 0.6 and higher than 0.9 % (v/v) respectively led to nanoemulsions with particle sizes smaller than 100 nm. However, it was observed a fair

stabilizing activity of sodium alginate, since a concentration of biopolymer above 1.5% (w/v) and absence of surfactant led to droplet sizes below 500 nm. The ζ-potential and viscosity of the mixtures was mainly governed by the concentration of sodium alginate in the continuous phase due to its anionic nature and texturizing properties. The color of the emulsions was basically determined by the oil and tween 80 concentrations; namely the lower the oil and the higher the tween 80 amount, the lower the whiteness index. Regarding the functionality of the mixtures, an increase on the lemongrass essential oil concentration led to a greater antimicrobial activity. Moreover, a synergism between oil, tween 80 and sodium alginate was observed, indicating the importance of stabilizing the lipid droplets within the continuous phase to enhance its functionality. The present work reveals significant information to formulate nanoemulsions with controlled characteristics for food applications.

Acknowledgements

This study was supported by the Ministerio de Ciencia e Innovación (Spain) throughout the project AGL2009-11475. Laura Salvia-Trujillo thanks the Ministry of Science and Education (Spain) for the predoctoral grant. Prof. Olga Martín-Belloso thanks the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia 2008 Award.

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PUBLICATIONS

Chapter V

Physicochemical characterization and antimicrobial activity of food-grade emulsions and nanoemulsions incorporating essential oils

Laura Salvia-Trujillo, M. Alejandra Rojas-Graü, Robert Soliva-Fortuny, Olga Martín-Belloso

Food Hydrocolloids (under revision)

Abstract

Coarse emulsions containing essential oils (lemongrass, clove, tea tree, thyme, geranium, majoram, palmarosa, rosewood, sage or mint) and stabilized with tween 80 and sodium alginate were prepared by high shear homogenization. Nanoemulsions were obtained by microfluidization of coarse emulsions. In general, the average droplet size of coarse emulsions was dramatically reduced after microfluidization up to a few nanometers, with the exception of palmarosa and rosewood oil emulsions, which were already in the nano-range before being treated. The ζ-potential of nanoemulsions exhibited values more negative than -30 mV, indicating a high stability of the dispersed oil droplets in the aqueous phase. The viscosity of nanoemulsions significantly decreased after microfluidization, with at least a 30% drop in their initial values. The whiteness index of nanoemulsions diminished after being treated. In fact, nanoemulsions containing tea tree, geranium or marjoram essential oils became completely transparent after microfluidization. Lemongrass, clove, thyme or palmarosa-loaded nanoemulsions were those with a higher *in vitro* bactericidal action against *Escherichia coli*, as they achieved 4.1, 3.6, 2.8 or 3.9 log-reductions after 30 min of contact time. In addition, a faster and enhanced inactivation kinetic was observed in the case of nanemulsions containing lemongrass or clove essential oils in comparison with their respective coarse emulsions. Thus, the present work evidences the promising advantages of using nanoemulsions as delivery systems of flavoring and preservative agents in the food industry.

Keywords: Antimicrobial activity, *Escherichia coli*, nanoemulsion, alginate, essential oils, microfluidization, droplet size, ζ-potential, viscosity.

Table of contents

1 Introduction

Essential oils contain a complex mixture of non-volatile and volatile compounds produced by aromatic plants as secondary metabolites (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Burt, 2004). They are used in a wide variety of applications in food, pharmaceutical and cosmetics industries due to their flavoring, antioxidant and antimicrobial properties (Adorjan & Buchbauer, 2010; Brud, 2010; Tajkarimi, Ibrahim, & Cliver, 2010). In particular, the antimicrobial action of essential oils has been attributed to their phenolic compounds and their interaction with microbial cell membranes. They are known to penetrate through the microbial membrane and cause the leakage of ions and cytoplasmatic content thus leading to cellular breakdown (Burt, 2004). The interest of incorporating essential oils in foods as preservatives is related to their recognition as safe natural compounds, being a potential alternative to produce foods free of synthetic additives. However, the incorporation of antimicrobial essential oils to foods still presents several drawbacks due to their poor water solubility as well as to toxicological and economical considerations (Burt, 2004; Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2011). In addition, their strong flavor makes incorporation at high doses difficult in certain types of food products due to possible objectionable sensory characteristics. Hence, there is a need to investigate new delivery systems to encapsulate and release essential oils in food products.

In the food sector, the incursion of nanotechnological advances is still discreet but it is gaining more and more interest by both the scientific and industrial community (Rashidi & Khosravi-Darani, 2011). Recently, emulsions with small droplet size, typically from 10 to 100 nm, also called nanoemulsions, are being investigated as lipophilic drug delivery systems in food, cosmetic and pharmaceutical products (Bernardi et al., 2011; He et al., 2011). Due to their intrinsic properties, they may present several advantages for encapsulating functional lipophilic compounds over conventional emulsions. On the one hand, their reduced droplet size might not only enhance the transport of active molecules through biological membranes but also increase the surface area/volume ratio, thus leading to improved functionality. On the other hand, nanoemulsions are kinetically stable and transparent colloidal dispersions, being suitable for a wide range of practical applications (Solans, Izquierdo, Nolla, Azemar, & Garcia-Celma, 2005).

To produce nanoemulsions with droplet sizes in the nano-range a high shear rate is required in order to generate a high interface area (Delmas et al., 2011). Among the few methods that can produce nanoemulsions, microfluidization has been shown to generate extremely fine emulsions with droplet sizes between 60 and 600 nm (Hatanaka et al., 2010; Hatanaka, Kimura, Lai-Fu, Onoue, & Yamada, 2008; Jafari, He, & Bhandari, 2007a, 2007b; Qian & McClements, 2011; Rao & McClements, 2011; Wooster, Golding, & Sanguansri, 2008). The microfluidizer device uses a pump to force a coarse emulsion pre-mix to an interaction chamber under high pressure (Jafari et al., 2007b; McClements, 2011). In the interaction chamber the coarse emulsion is split in two streams that further impinge on each other at high velocity, providing an exceptionally fine emulsion (Mahdi Jafari, He, & Bhandari, 2006; McClements, 2011).

The use of nanoemulsions as potential delivery systems of lipophilic food ingredients is arising with promising expectations. However, there is a lack of scientific evidence about the enhanced functionality of nanoemulsions in comparison with similar non-nano-formulated delivery systems (Bouwmeester et al., 2009). Therefore, the purpose of the present research work was to characterize microfluidized nanoemulsions incorporating essential oils in terms of droplet size, size distribution, ζ-potential, viscosity and color in comparison with conventional emulsions. Moreover, the *in vitro* antimicrobial activity against *Escherichia coli* of nanoemulsions was assessed and compared with that exhibited by conventional emulsions.

2 Material and methods

2.1 Primary emulsion formation

Sodium alginate (FMC Biopolymers, UK) was dissolved in hot water at 70 ºC (1% w/v) under continuous stirring until complete homogenization. Coarse emulsions were prepared by mixing the resulting sodium alginate aqueous solution with essential oils (1% v/v). Lemongrass (*Cymbopogon citratus*) essential oil was purchased at Laboratoris Dicana (Spain) and clove (*Eugenia caryophyllata*), tea tree (*Melaleuca alternifolia*), thyme (*Thymus vulgaris*), geranium (*Pelargonium graveolens*), marjoram (*Origanum majorana*), palmarosa (*Cymbopogon martinii*), rosewood (*Aniba rosaedora*), sage (*Salvia officinalis*) and mint (*Mentha spicata*) were purchased to Dietetica Intersa (Spain). Tween 80 (1% v/v) (Scharlau, Spain) was added as surfactant with a laboratory T25 digital Ultra-Turrax mixer (IKA, Staufen, Germany) working at 3400 rpm for 2

min. All samples were prepared using ultra pure water obtained from a Mili-Q filtration system.

2.2 Nanoemulsion formation

A microfluidization system (M110P, Microfluidics, Massachusetts, USA) was used to obtain nanoemulsions from the coarse emulsions. Coarse emulsions were passed through the system 3 times at a constant pressure of 150 MPa. At the outlet of the interaction chamber the product was refrigerated through an external cooling coil immersed in an ice-water bath so that the temperature of the product was always kept below 20 ºC.

2.3 Emulsion and nanoemulsion characterization

2.3.1 Particle size and ζ-potential

The oil droplet size was measured by dynamic light scattering (DLS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK) working at 633 nm at 25 ºC and equipped with a backscatter detector (173 º), which is appropriate to measure sub-micron particles (Brar & Verma, 2011). DLS measures the Brownian motion of nanosized droplets and relates this movement to an equivalent hydrodynamic diameter (nm). Average droplet size, size distribution curves and polydispersity index were used to characterize oil droplets dispersion in nanoemulsions. The refractive index of essential oils and their absorbance at 633 nm is presented in Table 1.

Table 1 Refractive index and absorbance (λ=633 nm) of essential oils

Essential oil	Plant	Refractive index	Abs_{633}
Lemongrass	*Cymbopogon citratus*	1.453	0.022
Clove	*Eugenia caryophyllata*	1.499	0.023
Tea Tree	*Melaleuca alternifolia*	1.465	0.001
Thyme	*Thymus vulgaris*	1.471	0.094
Geranium	*Pelargonium graveolens*	1.462	0.157
Marjoram	*Origanum majorana*	1.472	0.002
Palmarosa	*Cymbopogon martinii*	1.461	0.003
Rosewood	*Aniba rosaedora*	1.468	0.002
Sage	*Salvia officinalis*	1.479	0.071
Mint	*Mentha spicata*	1.477	0.002

The electrophoretic mobility of oil droplets, also reported as ζ-potential, was measured by phase-analysis light scattering (PALS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK). It determines the surface electrical charge of the droplets dispersed in the continuous phase.

2.3.2 Viscosity

Viscosity of emulsions and nanoemulsions was measured from approximately 10 mL of sample using a SV-10 vibro-viscometer (A&D Company, Tokyo, Japan) vibrating at 30 Hz and constant amplitude. The viscosity of the sodium alginate solution (30 MPa.s) was considered the viscosity of the dispersant material with respect to the dynamic light scattering measurements.

2.3.3 Whiteness index

The color of emulsions and nanoemulsions was measured with a Minolta CR-400 colorimeter (Konica Minolta Sensing, Inc., Osaka, Japan) at room temperature set up for illuminant D65 and 10º observer angle and calibrated with a standard white plate. CIE L*, a* and b* values were determined and the Whiteness Index (WI) was calculated with equation 1 (Vargas, Cháfer, Albors, Chiralt, & González-Martínez, 2008):

$$WI = 100 - ((100 - L)^2 + (a^2 + b^2))^{0.5} \qquad \text{Eq. (1)}$$

2.3.4 Antimicrobial activity assay

The antimicrobial activity of essential oil-alginate emulsions and nanoemulsions was assessed evaluating the *in vitro* inhibition of *Escherichia coli*. The method used was a modification from a previously described by other authors (Ferreira et al., 2010). *E. coli* 1.107 (Laboratoire de Répression des Fraudes, Montpellier, France) was cultured in tryptone soy broth (Bioakar Diagnostic; Beauvais, France) and incubated at 37 ºC with continuous agitation at 120 rpm for 11 h to obtain cells in stationary growth phase. The final concentration reached in the culture was 10^8-10^9 colony forming units/millilitre (CFU/mL). A 0.5 mL-aliquot of overnight bacterial culture was mixed with 0.5 mL of the essential oil-alginate emulsion or nanoemulsion and 4.5 mL of sterile Mili-Q water. Aliquots of 0.1 mL were taken after 5, 15 and 30 minutes and were serially diluted and spread on McConkey agar (Biokar Diagnostics, Beauvais, France) plates to determine the inactivation kinetics. A blank determination was performed with the same method,

replacing the emulsion or nanoemulsion by sterile Mili-Q water. Colony counts were determined after incubation of agar plates at 37 ºC for 24 hours.

2.4 Kinetic modelling

The inactivation kinetics of essential oil-alginate emulsions or nanoemulsions against *E. coli* was adjusted to a Weibull distribution model, by Eq. 2 (Peleg, 2006):

$$\ln S(t) = -\left(\frac{t}{\beta}\right)^{\alpha} \qquad \text{Eq. (2)}$$

where S is the survival fraction (N/N_0) of *E. coli*, t is the reaction time (min), β is the scale factor, and α is the shape factor. The scale factor represents the contact time (min) necessary to inactivate the first log-cycle of the microbial population, whereas the shape factor is related to the trend of the inactivation curve. A shape factor below 1 indicates a concavity upwards while a value greater than 1 means a concavity downwards.

The model was fitted to experimental data by non-linear regression using the Statgraphics Plus v.5.1 Windows package. The adjusted regression coefficients (R^2 adj) and accuracy factor (A_f, Eq. 3) (Ross, 1996) of the model were calculated.

$$A_f = 10^{\frac{\sum\left|\log\left(\frac{predicted}{observed}\right)\right|}{n}} \qquad \text{Eq. (3)}$$

where log (predicted/observed) is the logarithmic relation between the predicted and the experimental values, and n is the number of observations.

The higher the values of R^2 adj and the nearer the values of A_f to 1, the better the fitting of the model to experimental data.

2.5 Statistics

All the experiments were assayed in duplicate, and two replicate analyses of each sample were carried out in order to calculate a mean value. Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, MD) was used to perform the analysis of variance. The least significant difference (LSD) test was run to determine significant differences ($p \leq 0.05$) between emulsions and nanoemulsions containing different essential oils at a 5 % significance level.

3 Results

3.1 Particle size and particle size distribution

The results of the average droplet size and polydispersity index of essential oil-loaded emulsions and nanoemulsions are shown in Table 2, and their droplet size distribution is presented in Fig. 1.

The method used to prepare the mixtures significantly influenced the emulsion droplet size. The further processing of blends by microfluidization after a previous high shear homogenization rendered nanoemulsions with a reduced droplet size. Moreover, strong differences among emulsion droplet size and size distribution were observed depending on the essential oil used in the formulation of nanoemulsions. Namely, microfluidized lemongrass oil-loaded nanoemulsions exhibited a dramatic reduction of their average droplet size, up to 5.49 ± 0.28 nm, in comparison with the values of the coarse emulsion, which averaged 1121.43 ± 228.93 nm. Oppositely, emulsions incorporating palmarosa and rosewood oils exhibited average droplet sizes already in the nano-scale range (32.91 ± 6.92 or 13.82 ± 0.48 nm, respectively) before microfluidization and with a narrow size distribution. Therefore, in both cases the microfluidization treatment had a slight effect on the average droplet size, which was confirmed by size distribution data (Figure 1).

Regarding the rest of essential oils (clove, tea tree, thyme, geranium, marjoram, sage or mint), the average droplet size of coarse emulsions was also in the nano-scale range, with values between 13.16 ± 0.92 and 86.49 ± 4.31 nm. Nonetheless, a multimodal size distribution was observed with major intensity peaks positioned at 1000 nm and with minor peaks at the nano-range. Those minor peaks might be surfactant micelles that were not adsorbed in the oil droplets interface, which are typically around 10 nm (Heydenreich, Westmeier, Pedersen, Poulsen, & Kristensen, 2003; Ménard et al., 2011). In fact, the particle size distribution graphs of microfluidized emulsions (Figure 1) show a significant decrease in the intensity peaks of particles above 100 nm. Consequently, the intensity peaks of smaller particles increased, indicating a clear particle size reduction. The average droplet size (z-average) of emulsions corresponds to the mean hydrodynamic diameter and is calculated according to the ISO-13321 (1996), but it may lead to misinterpretation in the case of non-monodispersed emulsions. In general, from the results described above it can be observed a clear droplet size disruption caused by microfluidization treatment, leading to nanoemulsions with small droplet sizes. During

the microfluidization treatment, the process stream is pressurized and passed through an interaction chamber thus creating shear and impact forces as well as cavitation phenomena within the product that reduce the emulsion droplet size (Maa & Hsu, 1999). However, the final droplet size of nanoemulsions is a complex interplay between the processing conditions and the adsorption of surfactant onto oil droplets (Wooster et al., 2008).

Table 2 Average droplet size (z-average) (nm) and polydispersity index (PDI) of essential oil-alginate emulsions or nanoemulsions produced by High Shear Homogenization (3400 rpm, 2 min) or microfluidization (150 MPa, 3 cycles).

Essential oil	Homogenization system	Z-average (nm)	PDI
Lemongrass	HSH	1121.43 ± 228.93	0.49 ± 0.08
	MF	5.49 ± 0.28	0.51 ± 0.04
Clove	HSH	23.23 ± 10.51	0.94 ± 0.09
	MF	20.88 ± 5.10	0.82 ± 0.07
Tea Tree	HSH	16.20 ± 5.24	1.91 ± 0.70
	MF	2.65 ± 0.68	0.10 ± 0.01
Thyme	HSH	13.16 ± 0.92	0.23 ± 0.03
	MF	12.86 ± 0.25	0.36 ± 0.01
Geranium	HSH	29.24 ± 9.73	0.38 ± 0.18
	MF	2.88 ± 0.16	0.28 ± 0.09
Marjoram	HSH	35.14 ± 8.26	0.21 ± 0.18
	MF	7.71 ± 0.64	0.10 ± 0.01
Palmarosa	HSH	32.91 ± 6.92	1.02 ± 0.01
	MF	13.82 ± 0.31	0.71 ± 0.08
Rosewood	HSH	13.82 ± 0.48	1.02 ± 0.32
	MF	12.26 ± 0.09	1.08 ± 0.11
Sage	HSH	86.49 ± 4.31	0.27 ± 0.09
	MF	8.56 ± 1.29	0.34 ± 0.04
Mint	HSH	13.95 ± 1.97	0.78 ± 0.01
	MF	2.22 ± 0.06	0.12 ± 0.01

Values are expressed as mean ± standard deviation.

On the one hand, the ability of flavor oil molecules to be incorporated into surfactant micelles depends on their molecular characteristics, such as molecular weight, polarity and conformation (Djekic & Primorac, 2008). It has been reported that essential oils with higher concentration of polar compounds might reduce the interfacial tension and facilitate droplet disruption during high-pressure homogenization (Ziani, Fang, & McClements, 2012). In this sense, low molecular weight or high polarity compounds tend to be solubilized in the aqueous phase rather than in the micelle interior. On the other hand, the emulsifier type and concentration are important in determining the

emulsion droplet size, since they should adsorb to oil droplet surface to prevent re-coalescence (Jafari, Assadpoor, He, & Bhandari, 2008). Moreover, viscosity and concentration of the dispersed phase are determining factors when considering the formation of nano-sized emulsions (Jafari et al., 2008; McClements, 2005; Qian & McClements, 2011; Seekkuarachchi, Tanaka, & Kumazawa, 2006; Wooster et al., 2008).

It has been observed that the higher the viscosity of the oil phase, the higher the emulsion droplet size (Qian & McClements, 2011). In fact, as the oil viscosity increases, the disruption forces needed to deform the oil droplets during homogenization are higher (Håkansson, Trägårdh, & Bergenståhl, 2009; McClements, 2005). Namely, Wooster, Golding & Sanguansri (2008) observed droplet sizes of around 120 nm in nanoemulsions formulated with high viscosity oils, whereas nanoemulsions containing low viscosity oils presented droplet sizes of around 80 nm. Usually, plant essential oils are constituted of low molecular weight compounds and present low viscosity values (Sell, 2010).

In agreement to our results, Rao & McClements (2012) and Ziani, Chang, McLandsborough, & McClements (2011) have used microfluidization approaches for the generation of lemon oil and thyme oil-loaded nanoemulsions, respectively. In addition, Donsì, Annunziata, Sessa, & Ferrari (2011) obtained d-limonene nanoemulsions stabilized with tween 20 and glycerol monooleate through high pressure homogenization (10 cycles at 300 MPa) with an average droplet size of 154.6 ± 1.4 nm. Differences in droplet size observed in the present work between various essential oil-loaded nanoemulsions might be attributed to their molecular structure, concentration of volatile compounds, viscosity, interfacial tension or surfactant affinity to each type of flavor oil.

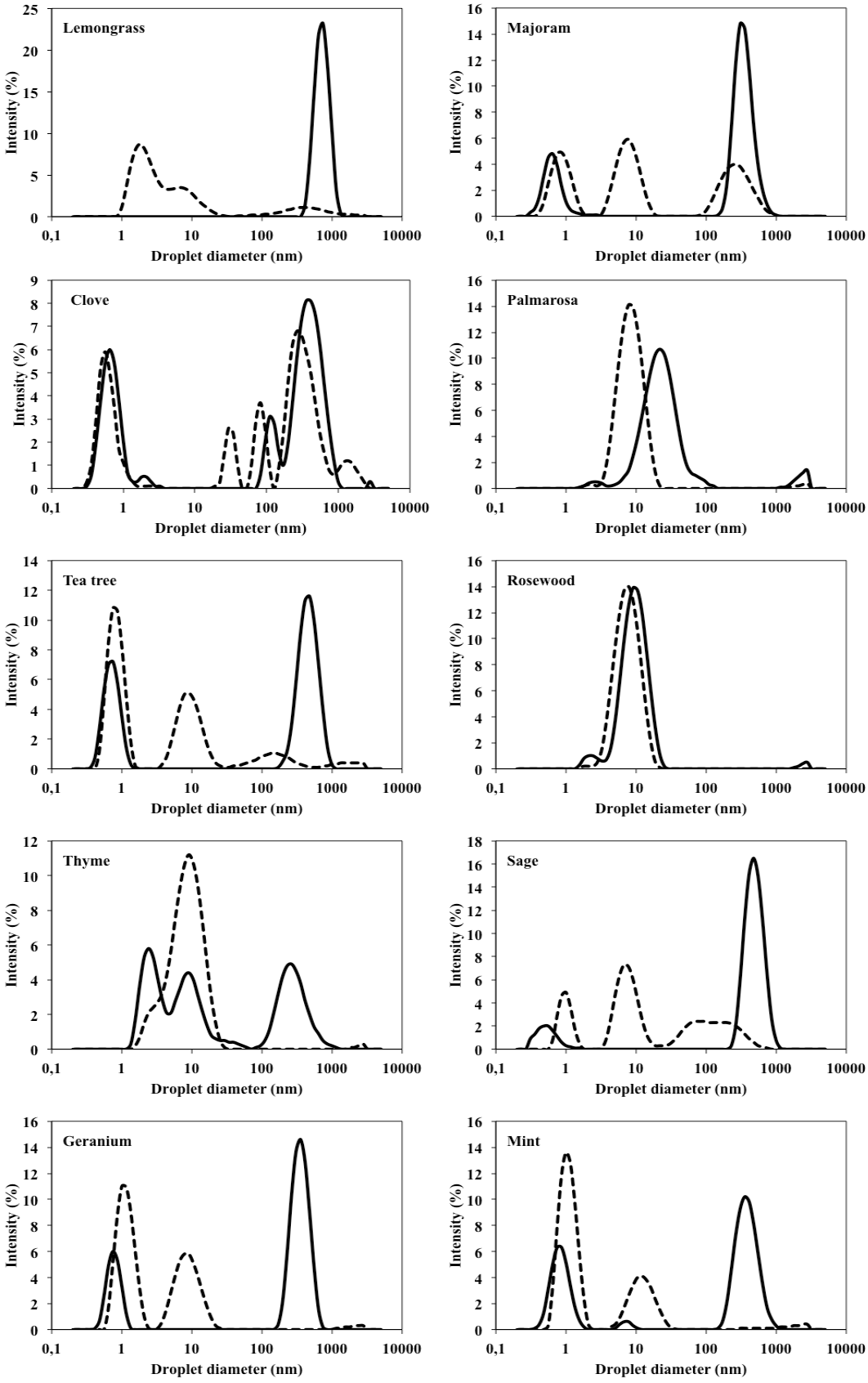

Figure 1 Droplet size distribution of essential oil-alginate emulsions or nanoemulsions produced by High Shear Homogenization (3400 rpm, 2 min) (continuous line) or microfluidization (150 MPa, 3 cycles) (dotted line).

3.2 ζ-potential

The surface charge of oil droplets in emulsions and nanoemulsions incorporating several essential oils is shown in Fig. 2. Particles with ζ-potential more positive than +30 mV or more negative than -30 mV are usually considered to be stable, since electrical charge of droplets is strong enough to assume that repulsive forces between droplets are predominant in the nanoemulsion system (Heurtault, Saulnier, Pech, Proust, & Benoit, 2003). On one hand, coarse emulsions formed by high shear homogenization exhibited a weak surface charge with values less negative than -30 mV. In fact, the coarse emulsion incorporating mint essential oil exhibited the weaker ζ-potential (-10.0 mV). In contrast, clove, tea tree or marjoram-loaded emulsions treated by high shear homogenization exhibited a strong electrical charge of -52.6, -30.8 and -37.4 mV, respectively. On the other hand, nanoemulsions obtained by microfluidization showed a significant decrease in their ζ-potential regardless of the oil type used in the formulation. In fact, most of the nanoemulsions had ζ-potential values more negative than -30 mV, with the exception of palmarosa-loaded nanoemulsions (-20.9 mV). According to Bonilla, Atarés, Vargas, & Chiralt (2012), differences observed in the ζ-potential of emulsions and nanoemulsions formulated with different essential oils might be attributed to differences in the dissociation degree and number of ionisable compounds of oils.

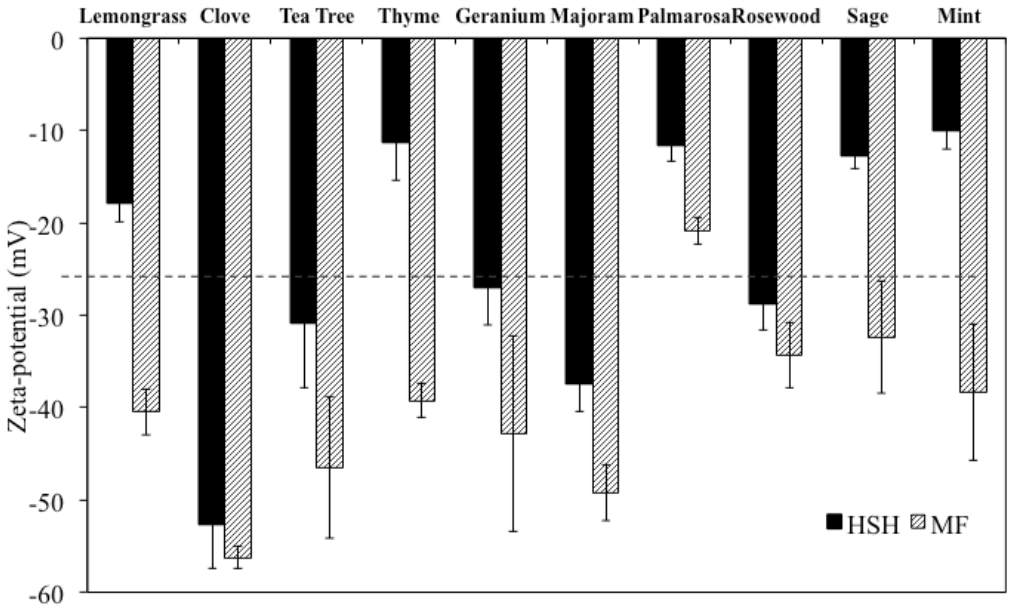

Figure 2 ζ-potential of essential oil-alginate emulsions or nanoemulsions produced by High Shear Homogenization (3400 rpm, 2 min) (HSH) or microfluidization (150 MPa, 3 cycles) (MF).

Tween 80 is a non-ionic low-mass surfactant that rapidly coats the newly created droplets during emulsification (Kralova & Sjöblom, 2009). It is preferably adsorbed on the oil surface in the co-presence of polymeric stabilizers (Nambam & Philip, 2012), giving a neutral electrical charge on oil-water interface (Kralova & Sjöblom, 2009). Thus, the negative charge of oil droplets observed in the present work might be due to the anionic sodium alginate molecules dispersed in the aqueous phase. Even though alginates, as well as most food hydrocolloids, do not possess hydrophobic moieties (Dickinson, 2003, 2009), it has recently been observed that can present surface activity under certain conditions (Garti & Leser, 2001; Yang, Jiang, He, & Xia, 2012). The mechanical stress during microfluidization can lead to break up sodium alginate (see viscosity discussion 3.3) thus increasing the number of molecules to be potentially adsorbed on the oil-water interface. This fact could explain the ζ-potential strengthening observed in microfluidized nanoemulsions.

3.3 Viscosity

The viscosity of emulsions or nanoemulsions incorporating different essential oils before and after microfluidization at 150 MPa for 3 cycles is presented in Fig. 3. The viscosity of coarse emulsions ranged between 22.95 and 36.05 mPa.s depending on the type of essential oil used in the formulation. Mint oil led to more viscous emulsions, whereas thyme oil rendered the lowest viscosity values. The characteristics of the dispersed phase have a significant impact on the rheological properties of emulsions (McClements, 2005; Pal, 2011; T. F. Tadros, 1994). Similarly to our results, Bonilla and co-workers (2012) found that thyme oil-loaded emulsions stabilized with chitosan showed lower viscosity values in comparison with emulsions containing basil essential oil. They attributed these variations to differences in the dissociation degree and number of ionisable compounds of the different essential oils, since biopolymeric molecules would be adsorbed at the oil droplet surface and lead to changes in the effective thickening concentration in the aqueous phase.

In addition, a dramatic reduction of emulsion viscosity has been observed after microfluidization, regardless the essential oil used in the formulation. Coarse emulsion viscosity values decreased up to a 60.66 % after microfluidization. In this sense, nanoemulsions presented viscosities ranging between 9.77 and 14.95 mPa.s. Furthermore, there were no significant differences in viscosity among microfluidized

nanoemulsions with different essential oils. The droplet size influences the emulsion viscosity, as it has been demonstrated in template oil-in-water emulsions (Chanamai & McClements, 2000; Cortés-Muñoz, Chevalier-Lucia, & Dumay, 2009; Derkach, 2009; Pal, 2011). However, in the present work the thickening properties of the continuous phase play a fundamental role to determine the rheological behaviour of the nanoemulsion system. It is known that microfluidization processing may induce conformational changes on the molecular structure of biopolymers, as well as a significant reduction on their molecular weight (Lagoueyte & Paquin, 1998).

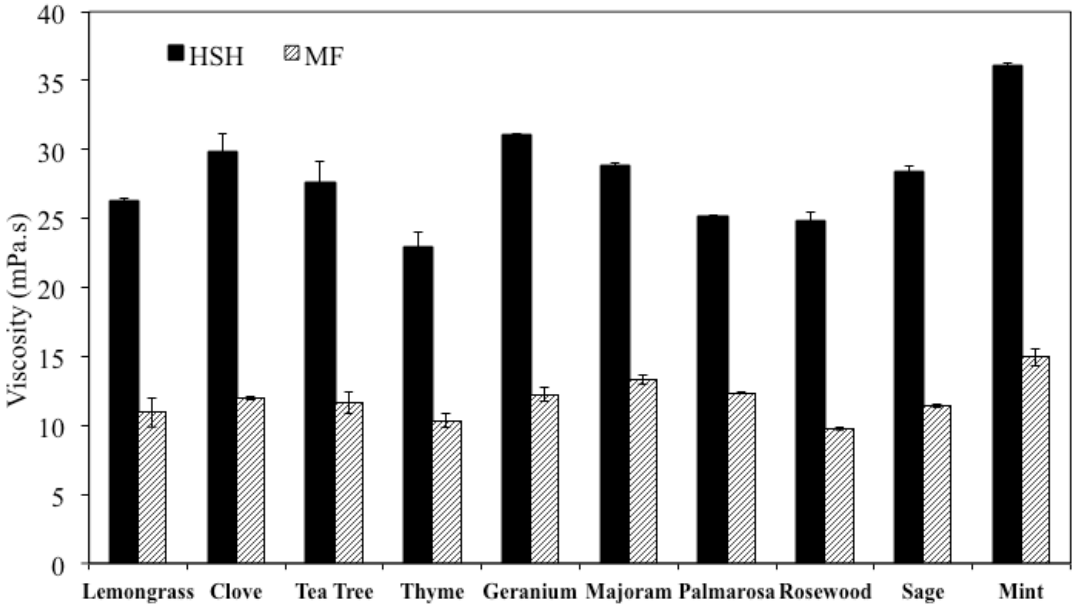

Figure 3 Viscosity (mPa.s) of essential oil-alginate emulsions or nanoemulsions produced by High Shear Homogenization (3400 rpm, 2 min) (HSH) or microfluidization (150 MPa, 3 cycles) (MF).

Recently, Villay and co-workers (2011) observed a viscosity reduction on sodium alginate solutions when they were treated with high pressure homogenization at 195 MPa and several cycles. They attributed this effect to a decrease in the molar mass of sodium alginate due to a depolymerisation of chains caused by covalent bond disruption. Similarly, Harte & Venegas (2010) reported a 75-85 % viscosity reduction in alginate, κ-carrageenan and xanthan gum solutions processed with high pressure homogenization from 1 to 5 cycles up to 300 MPa. Hence, in basis of the results observed in the present work, it can be assumed that sodium alginate may undergo molecular changes in its molecular structure during microfluidization process, leading

to a decrease in its thickening properties and therefore in the viscosity of nanoemulsions.

3.4 Whiteness index

The type of oil used in the formulation significantly influenced the whiteness index of coarse emulsions and nanoemulsions (Figure 4). Coarse emulsions containing lemongrass, thyme, geranium or palmarosa essential oils were those presenting higher whiteness index, with values of 47.9, 49.6, 57.8 or 51.3, respectively. On the contrary, coarse emulsions formulated with clove, tea tree or marjoram essential oils exhibited the lowest whiteness index, with values of 24.4, 29.9 or 27.8, respectively.

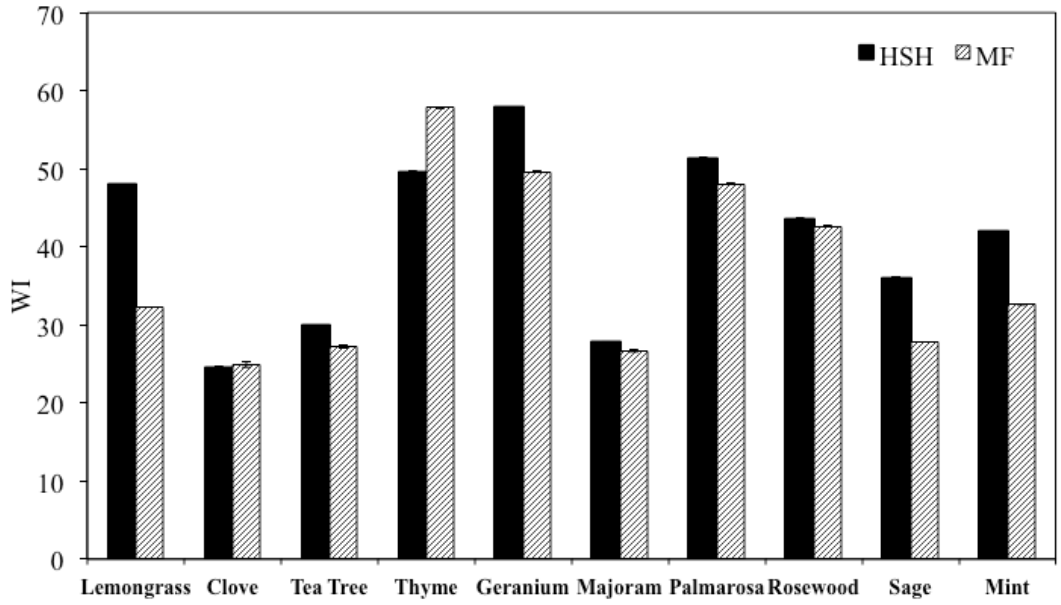

Figure 4 Whiteness index (WI) of essential oil-alginate emulsions or nanoemulsions produced by High Shear Homogenization (3400 rpm, 2 min) (HSH) or microfluidization (150 MPa, 3 cycles) (MF).

In general, microfluidization significantly affected the whiteness index of mixtures generating nanoemulsions with lower whiteness index. Nonetheless, the extent of whiteness index decrease after microfluidization depended on the oil type used in the formulation. For instance, nanoemulsions incorporating lemongrass, sage or geranium essential oils showed a drastic decrease in their whiteness index from 47.9, 36.0 and 57.8 up to 32.2, 27.7 and 49.6, respectively. Nevertheless, emulsions incorporating thyme essential oil exhibited an opposite behaviour, as the whiteness index of microfluidized nanoemulsions was higher in comparison with the coarse emulsion. It is

known that oil droplet size significantly influences the optical properties of emulsions (McClements, 2002). Large particles scatter the light more intensely than smaller ones, which causes an increase in the lightness, opacity and whiteness index of emulsions (McClements, 2011). In general, nanoemulsions are described as transparent systems due to their reduced droplet size (Mason, Wilking, Meleson, Chang, & Graves, 2006; McClements, 2011; Solans et al., 2005; T. Tadros, Izquierdo, Esquena, & Solans, 2004). However, in spite that all coarse emulsions of the present study exhibited a significant decrease in their average droplet size and size distribution after being microfluidized, not all of them became completely transparent (Fig. 5). In fact, only tea tree, geranium and marjoram nanoemulsions became completely transparent after 3 microfluidization cycles, which is consistent with the light scattering measurements showing that these nanoemulsions presented the lowest average droplet size and narrowest size distribution (Table 2, Figure 1). Moreover, some essential oils might be water-soluble due to their polar compounds, thus being able to solubilize in the aqueous phase and leading also to transparent systems (Ziani et al., 2012). In line with our results, Wooster and co-workers (2008) obtained transparent nanoemulsions containing peanut oil by microfluidization (100 MPa and 5 cycles), with a droplet size lower than 60 nm.

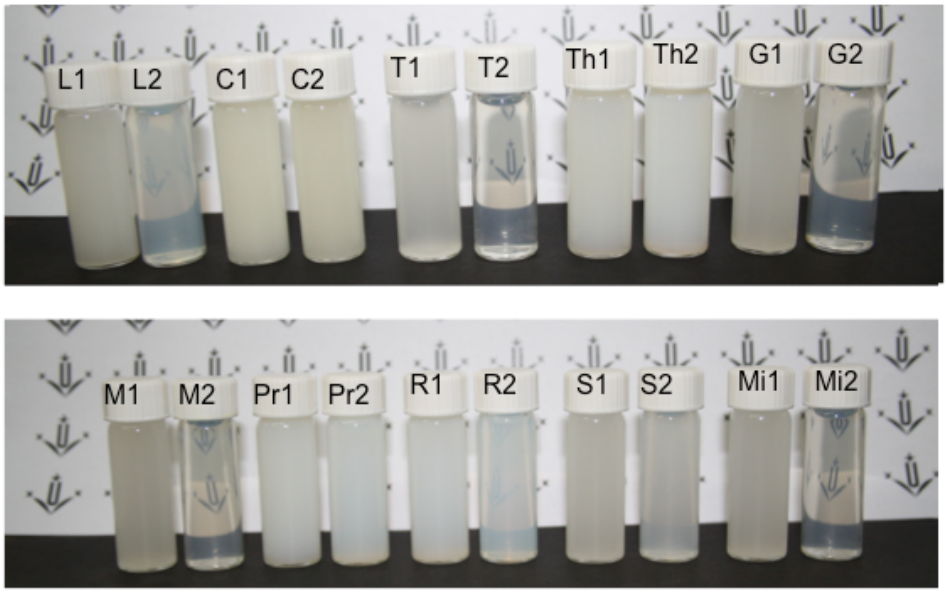

Figure 5 Images of essential oil-alginate emulsions or nanoemulsions produced by High Shear Homogenization (3400 rpm, 2 min) (1) or microfluidization (150 MPa, 3 cycles) (2). Lemongrass (L), clove (C), tea tree (T), thyme (Th), geranium (G), marjoram (M), palmarosa (Pr), rosewood (R), sage (S), mint (Mi).

3.5 Antimicrobial activity

The inactivation kinetics of emulsions and nanoemulsions against *Escherichia coli* is shown in Figure 6. A Weibull equation was fitted to experimental data of the survival curves, with accuracy factors near to 1 and R^2 values higher than 86 (Table 3).

Emulsions and nanoemulsions containing lemongrass, clove, thyme or palmarosa essential oil showed the strongest antimicrobial activity. Namely, lemongrass or clove-loaded emulsions exhibited 3.98 ± 0.01 or 3.52 ± 0.02 log reductions after being in contact 30 min with the inoculated microorganism, respectively. On the contrary, coarse emulsions containing tea tree, geranium, marjoram, rosewood, sage or mint essential oils presented less than 1 log reduction of *E. coli* after 30 min of contact time.

It is known that the antimicrobial activity of each essential oil is due to their phenolic compounds and it largely depends on their concentration as well as on their chemical structure (Dorman & Deans, 2000). In general, the mechanism of action of essential oils against microorganisms involves the interaction of phenolic compounds with the proteins (porins) in the cytoplasmatic membrane that can precipitate and lead to leakage of ions and other cell content causing the cell breakdown (Nychas, Skandamis, & Tassou, 2000). Those essential oils containing carvacrol and thymol terpenes, such as thyme essential oil, are reported to have a strong bactericidal action (Lambert, Skandamis, Coote, & Nychas, 2001). Nevertheless, eugenol and citral, the major components of clove and lemongrass or rosewood essential oil, respectively (Friedman, Henika, Levin, & Mandrell, 2004), have been found to inactivate a broad spectrum of microorganisms (Hammer, Carson, & Riley, 1999). Other aromatic compounds such as linalool (tea tree), pinene (mint), geraniol (geranium), or borneol (sage), which can be found in essential oils, exhibit a lower inhibitory influence against bacteria (Zachariah & Leela, 2006). Therefore, the final antimicrobial activity of a determined essential oil is a combination of composition and concentration of each volatile compound, which in turn are governed by the plant variety, growing conditions and method of extraction of the essential oil.

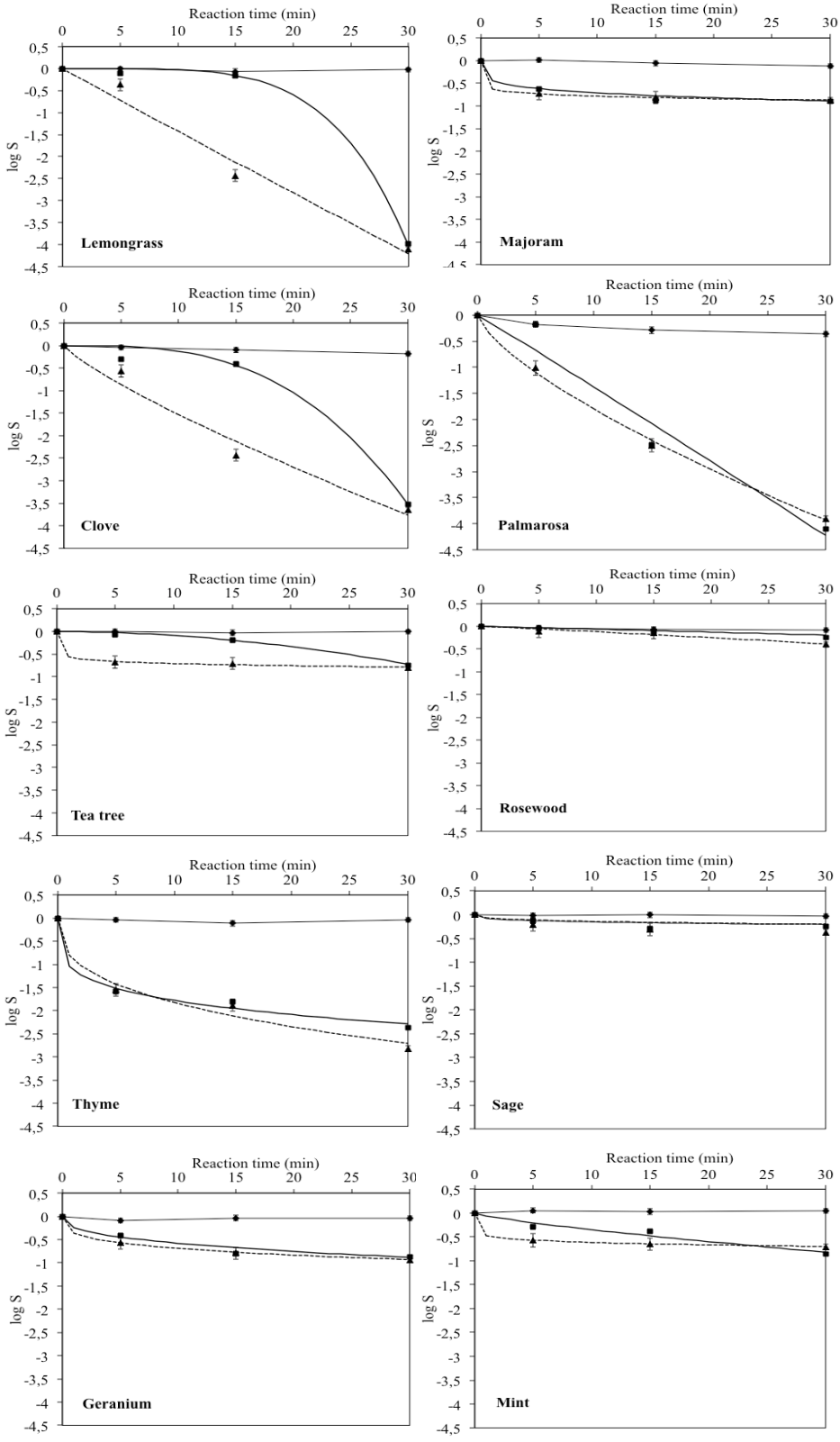

Figure 6 Survival fraction of *Escherichia coli* through 30 min of reaction time with essential oil-alginate emulsions or nanoemulsions produced by High Shear Homogenization (3400 rpm, 2 min) (■) or microfluidization (150 MPa, 3 cycles) (▲). A control with water is represented (♦). The plotted lines of log S vs. reaction time (min) correspond to the data fitted to a Weibull equation. Data shown are a mean ± standard deviation

In our experiments it was observed that microfluidization of coarse emulsions significantly affected their antimicrobial activity, since nanoemulsions exhibited an increased and faster inactivation of *E. coli*. This behaviour was clearly noticeable in the case of lemongrass or clove oil-loaded nanoemulsions. In particular, while the coarse emulsion formulated with lemongrass essential oil presented a β of 22.30, its respective microfluidized nanoemulsion exhibited a β of 6.97 (Table 3). Similarly, the β factor of the inactivation kinetics of *E. coli* with clove oil decreased from 19.71 with the coarse emulsion to 5.97 with the nanoemulsion. In addition, a concavity upwards was observed in the inactivation kinetics presented by both microfluidized nanoemulsions with α lower than 1, whereas coarse emulsions presented inactivation curves with α values higher than 1, which implies a slow release of phenolic compounds at early contact times. Therefore, microfluidized nanoemulsions containing lemongrass or clove essential oils showed a faster release of antimicrobial compounds in comparison with coarse emulsions, presenting a faster inactivation of inoculated *E. coli* population. The rest of microfluidized nanoemulsions containing other essential oils did not exhibit a significant enhancement of their antimicrobial potential compared to their corresponding coarse emulsion, since the β values of the inactivation curves were slightly reduced or even were increased in some cases. It is generally assumed that nanoencapsulated lipophilic antimicrobials would be able to penetrate more easily though microbial membranes thus leading to an improved bactericidal action. In the case of Gram-negative bacteria, such as *E. coli*, the presence of proteins (porins) that form channels (pores) in the outer membrane allow the influx of hydrophilic particles, being a barrier for lipophilic compounds and also restricting the entry of compounds by size (Schweizer, 2012; Taber, 2008). Therefore, it would be reasonable to assume that the solubilization of lipophilic antimicrobials within nanoemulsions would lead to an increase of binding sites able to interact with porins. In agreement with our results, recent research papers report an enhancement on the antimicrobial activity of nanoemulsions containing flavor oils such as thyme oil (Chang, McLandsborough, & McClements, 2012), peppermint oil (Liang et al., 2012), limonene (Donsì et al., 2011) or eugenol and cinnamaldehyde (Gomes, Moreira, & Castell-Perez, 2011). In line with our results, Buranasuksombat, Kwon, Turner, & Bhandari (2011) found that emulsion droplet size had no influence on the antimicrobial properties of lemon myrtle oil. These apparently controversial results indicate that a significant droplet size reduction does not necessarily imply an increase in the functionality of essential oil nanoemulsions, as their

antimicrobial activity might be given by the whole delivery system and its particular ability to react with microbial membranes. In this sense, it is also known that the membrane permeability and cellular absorption is surfactant-dependent since the droplet charge is known to influence the efficacy of nanoemulsified antimicrobials. Our results show that nanoemulsions with different essential oils had significantly different droplet charge, which indicates a distinct interaction between oil and surfactant or biopolymer molecules. In our case, negative droplet charges could have blocked the entrance of antimicrobial molecules though the microbial pores. Ziani and co-workers (2011) an antagonistic effect of cationic and anionic surfactants on the antimicrobial activity of thyme oil nanoemulsions, which was attributed to the partition of surfactant molecules between the oil droplet and microbial surfaces.

4 Conclusions

In the present work, a significant influence of microfluidization on the physicochemical properties of nanoemulsions with essential oils was evidenced. On the one hand, there was an appreciable decrease in the droplet size of emulsions after microfluidization, up to values below 20 nm. The droplet size of nanoemulsions could explain the whiteness index results, being transparent those nanoemulsions with the smallest droplets. Nonetheless, the optical properties of nanoemulsions were highly dependent on the oil type used in the formulation. On the other hand, the mechanical stress during microfluidization caused a decrease in the nanoemulsions viscosity. This fact could be related with the stronger ζ-potential of nanoemulsion in comparison with coarse emulsions. The antimicrobial activity of nanoemulsions was rather determined by the essential oil type used in the formulation than by their droplet size. Only in the case of nanoemulsions with lemongrass or clove essential oil an enhanced antimicrobial activity against *E. coli* could be observed. These results evidence the potential advantages of nanoemulsions as delivery systems of essential oils as preservatives in foods but also indicate the need to elucidate the mechanism of interaction of lipid nanoparticles with biological membranes.

Aknowledgements

This study was supported by the Ministerio de Ciencia e Innovación (Spain) throughout the project AGL2009-11475. Laura Salvia-Trujillo thanks the Ministry of Science and

Education (Spain) for the predoctoral grant. Prof. Olga Martín-Belloso thanks the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia 2008 Award.

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PUBLICATIONS

Chapter VI

Use of antimicrobial nanoemulsions as edible coatings: impact on safety and quality attributes of fresh-cut *Fuji* apples

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Journal of Agricultural and Food Chemistry (submitted)

Abstract

This work aims to study the effect of nanoemulsion-based edible coatings with lemongrass essential oil (LEO) at several concentrations (0.1, 0.5 or 1% v/v) on the safety and quality parameters of fresh-cut *Fuji* apples along storage time, and compare it with edible coatings formed from conventional emulsions. Edible coatings with LEO droplets in the nano range exhibited a faster and greater inactivation of *Escherichia coli* during storage time compared with conventional emulsions. Also, nanoemulsion-based edible coatings with LEO at 0.5% or 1% (v/v) completely inhibited the natural microflora of fresh-cut *Fuji* apples during 2 weeks and it was significantly slowed down with LEO at 0.1% (v/v). The droplet size of emulsions incorporated in edible coatings did not have a significant influence on the quality parameters during storage time. High LEO concentration led to reduced respiration rates and ethylene production. Significant browning was observed during storage time on fruits coated with high LEO concentrations (0.5 or 1% v/v), but not on those coated with 0.1% (v/v) of oil. Flesh firmness was maintained practically constant during storage time regardless the coating applied. These results contribute to evidence the potential benefits of incorporating nanoemulsions as delivery systems of antimicrobial compounds in edible coatings for minimally processed fruit.

Keywords

Nanoemulsions, edible coatings, fresh-cut apple, cold storage, essential oil

Table of contents

1 Introduction

The use of edible nanoemulsion, as a method to encapsulate lipophilic active ingredients dispersed in aqueous media, is emerging as a potential tool to design new food products with an optimal functionality. Oil-in-water nanoemulsions consist on lipid nano droplets (between 10 and 100 nm diameter) dispersed in an aqueous solution with unique physicochemical and functional characteristics (Rao & McClements, 2011). On the one hand, nanoemulsions present higher resistance to destabilization phenomena such as particle aggregation or gravitational separation (Tadros, Izquierdo, Esquena, & Solans, 2004). Also, nanosized droplets weakly scatter light, which provides a transparent or slightly turbid appearance (Mason, Wilking, Meleson, Chang, & Graves, 2006) and allows uses for specific food products. On the other hand, it has been recently pointed out that nanoemulsions may enhance the transport of active ingredients through biological membranes, thus intensifying the bioavailability of bioactive compounds (Acosta, 2009) or the bactericidal activity of antimicrobials (Donsì, Annunziata, Sessa, & Ferrari, 2011).

Even though the potential benefits of using nanoemulsions over conventional emulsions seem to justify their potential application in food products, there is a lack of research in this regard. The use of nanoemulsions as delivery systems in drinks and beverages is humble but growing in the food market (Silva, Cerqueira, & Vicente, 2012). However, using these systems in solid foods still remains unexplored due to the challenge that the immobilization of nano droplets on the surface of foods poses. In this respect edible coatings formed from nanoemulsion-based solutions would represent an effective approach to place active ingredients on the surface of minimally processed foods. Typically, edible coatings are used in fresh-cut fruit as a strategy to reduce the deleterious effects of minimal processing (Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2009). Moreover, edible coatings may contribute to extend the shelf-life of fresh-cut fruits by reducing moisture and solute migration, gas exchange, respiration and oxidative reaction rates, as well as reducing or even suppressing physiological disorders (Baldwin, Nisperos-Carriedo, & Baker, 1995; Debeaufort, Quezada-Gallo, & Voilley, 1998; Miller & Krochta, 1997). In addition, edible coatings have also been described to be good carriers of active ingredients such as antioxidants, flavors, bioactive compounds or antimicrobials (Campos, Gerschenson, & Flores, 2011; Rojas-

Graü et al., 2009). Particularly, the incorporation of essential oils (EOs) in edible coatings has been described as a good alternative to preserve minimally processed fruit products such as fresh-cut apple (Rojas-Graü, Tapia, Rodríguez, Carmona, & Martin-Belloso, 2007), melon (Raybaudi-Massilia, Rojas-Graü, Mosqueda-Melgar, & Martín-Belloso, 2008) or pear (G Oms-Oliu, Soliva-Fortuny, & Martín-Belloso, 2008). The antimicrobial activity of EOs is attributed to their hydrophobic nature, since they have the ability to disrupt the lipid structure of the bacteria cell membrane (Turina, Nolan, Zygadlo, & Perillo, 2006), causing the leakage of cytoplasmatic constituents and therefore the cell collapse (Burt, 2004). However, the use of EOs for fresh-cut fruit preservation presents several drawbacks due to their strong flavor or potential toxicity at high doses (Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2011). Thus, the design of new delivery systems to optimize or even enhance the effectiveness of EOs is a current challenge in order to reduce the dose to be incorporated in foods. In this sense, embedding EOs nanoparticles within edible coatings could be an interesting strategy towards a more rationalized application in foods.

Therefore, the aim of the present work was to study the effect of nanoemulsion-based edible coatings with lemongrass essential oil (LEO) on the safety and quality parameters of fresh-cut *Fuji* apples during cold storage. Moreover, the influence of incorporating edible coatings formed from conventional emulsions compared with nanoemulsions was assessed in order to establish a possible enhancement of LEO functionality due to the reduced droplet size.

2 Material and methods

2.1 Materials

Food-grade sodium alginate (Manucol® LD) was purchased in FMC BioPolymers (UK) and used to form edible coatings. Lemongrass (*Cymbopogon citratus*) essential oil, provided by Laboratoris Dicana (Spain), was used as natural antimicrobial agent. Tween 80 was purchased from Scharlau (Spain) and was the non-ionic surfactant agent in the emulsions. Calcium chloride (Sigma-Aldrich Chemic, Steinhein, Germany) was used to induce crosslinking reaction of sodium alginate and form the edible coating on the fruit surface. *Fuji* apples (*Malus domestica* Borkh) were obtained in a commercial ripening stage from a local grocery store (Lleida, Spain). Reagents and culture medium for

microbiology analysis were obtained from Biokar Diagnostics (Beauvais, France). All solutions were prepared using ultra pure water obtained from a Mili-Q filtration system.

2.2 Preparation of coating-forming nanoemulsions

First, the aqueous phase of nanoemulsions was prepared by dissolving the sodium alginate (2% w/v) with stirring in hot water at 70 ºC until complete dissolution. Coarse emulsions were prepared by mixing the LEO at different concentrations (0.1%, 0.5% or 1% v/v) with the sodium alginate solution and tween 80. The mixture was blended using a laboratory T25 digital Ultra-Turrax mixer (IKA, Staufen, Germany) working at 34000 rpm for 2 min. Coarse emulsions with LEO were used as coating-forming solutions in the non-nano form, to be compared with the nanoemulsions. Then, primary emulsions were passed three times though a microfluidizer (M110P, Microfluidics, Massachusetts, USA) working at 150 MPa to obtain nanoemulsions. The product was passed through a cooling coil placed at the outlet of the interaction chamber and immersed in an ice-water bath so that the temperature of the nanoemulsion was kept below 20 ºC. Emulsions and nanoemulsions were degassed under vacuum and further used as film-forming solutions to form the edible coating onto fruit surface.

2.3 Nanoemulsion characterization

2.3.1 Particle size and ζ-potential

The emulsion droplet size of the nano and non-nano coating-forming solutions was measured by dynamic light scattering (DLS) with a Zetasizer NanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK) working at 633 nm at 25 ºC and equipped with a backscatter detector (173 º) (Brar et al., 2011). DLS measures the Brownian motion of lipid particles and relates this movement to an equivalent hydrodynamic diameter (nm). Average droplet diameter (nm) and size distribution curves in intensity (%) were used to characterize the dispersion of oil droplets in the coating-forming solutions. The LEO absorbance at 633 nm was 0.001 and its refractive index 1.487, which were measured with a refractometer and spectrophotometer, respectively. Samples were diluted prior to analysis with mili-Q water (1:10) to avoid multiple scattering effects.

The electrophoretic mobility of oil droplets, also reported as ζ-potential, was measured by phase-analysis light scattering (PALS) with a Zetasizer NanoZS laser diffractometer

(Malvern Instruments Ltd, Worcestershire, UK). It determines the surface charge at the interface of the droplets dispersed in the aqueous solution.

2.3.2 Viscosity

A SV-10 vibro-viscometer (A&D Company, Tokyo, Japan) vibrating at 30 Hz was used to measure the viscosity of 10 mL-aliquots of the coating-forming solutions at room temperature.

2.3.3 Color

The color of coating-forming solutions was measured with a Minolta CR-400 colorimeter (Konica Minolta Sensing, Inc., Osaka, Japan) at room temperature set up for illuminant D65 and 10º observer angle and calibrated with a standard white plate. CIE L*, a* and b* values were determined and the whiteness index (WI) was calculated with equation 1 (Vargas, Cháfer, Albors, Chiralt, & González-Martínez, 2008):

$$WI = 100 - ((100 - L)^2 + (a^2 + b^2))^{0.5} \quad \text{Eq. (1)}$$

2.4 Fresh-cut fruit coating

Apples were washed with a sodium hypochlorite solution and rinsed with tap water. The excess of water was removed from the surface with absorbent paper prior to processing. Both poles of sanitized fruits were cut off with a sharp knife and then six pieces (1.4 cm diameter per 2.0 cm height) were taken out from each apple with a hollow stainless steel sampling tube. A maximum of four fruits were processed at the same time to minimize excessive exposure to environmental conditions. Apple pieces were dipped for 2min into water (control), into the alginate solution (with tween 80 but without LEO) or into the coating-forming primary emulsions or nanoemulsions with the different concentrations of LEO. Residual solutions were allowed to drip off for 1 min, before submerging the coated fresh-cut fruits for 2 min in a solution of calcium chloride solution (2% w/v) containing citric acid (1% w/v). Citric acid was used to avoid surface browning of fresh-cut *Fuji* apples (Gemma Oms-Oliu et al., 2010). Ten uncoated or coated apple pieces were placed in polypropylene trays of 500 cm^3 (Mcp Performance Plastic LTD, Kibbutz Hamaapil, Israel) and wrap-sealed using a 64 μm thickness polypropylene film with a permeability to oxygen of 110 cm^3 O_2 m^{-2} bar^{-1} day^{-1} at 23 ºC and 0% RH (Tecnopack SRL, Mortara, Italy) using a packaging machine (Ilpra

Foodpack Basic V/G, Ilpra, Vigenovo, Italy). Trays were heat-sealed and stored in darkness at 4 ºC. Analyses were carried out periodically during 14 days of storage for randomly sampled pairs of trays.

2.5 Fresh-cut fruit microbial evaluation

2.5.1 *Escherichia coli* inactivation

To assess the bactericidal effect of edible coatings, *E. coli* was inoculated on the apple pieces prior to coating procedure. *E. coli* 1.107 (Laboratoire de Répression des Fraudes, Montpellier, France) was cultured in tryptone soy broth (Bioakar Diagnostic; Beauvais, France) and incubated at 37 ºC with continuous agitation at 120 rpm for 11 h to obtain cells in stationary growth phase. The final concentration reached in the culture was 10^8-10^9 colony forming units/milliliter (CFU/mL). Afterwards, a 500µL aliquot was spread homogeneously over 10 pieces of fruit. Then, fresh-cut was coated as described previously without being used for any analysis other than *E. coli* determination. For *E. coli* counts evaluation, 10 g of fresh-cut fruit were taken from each tray, placed in a sterile plastic bag and mixed with 90 mL of saline peptone water (0.1 g peptone/100 ml water + 0.85g NaCl/100 ml water) for 1 min with a stomacher blender (IUL Instruments, Barcelona, Spain). Serial dilutions were prepared and spread on McConkey agar plates, which in turn were incubated at 37 ºC for 24 hours. Counts were expressed as $\log_{10}$ CFU/mL.

2.5.2 Microbial stability

Counts of psychrophilic bacteria or molds and yeasts on fresh-cut *Fuji* apples were determined during cold storage. A 10 g sample of fresh-cut fruit was taken of each tray in aseptic conditions and placed in a sterile plastic bag. Then, 90 mL of sterile saline peptone water (0.1 g peptone/100 ml water + 0.85g NaCl/100 ml water) were added and homogenized for 1 min in a stomacher blender (IUL Instruments, Barcelona, Spain). Serial dilutions were made and 0.1 mL was spread over previously prepared plate count agar (PCA) or chloramphenicol glucose agar (CGA) and incubated at 5 ± 1 ºC for 10 days or for at 25 ± 1 ºC for 3 days for determination of psychrophilic bacteria or molds and yeasts, respectively. Plate counts were expressed as $\log_{10}$ CFU/mL.

2.5.3 Microbial growth modeling

The growth of molds, yeasts and psychrophilic bacteria were adjusted to a Gompertz model with Eq. 2 (McKellar & Lu, 2004):

$$\log_{10} X(d) = A + C\exp(-\exp(-B(d-\mu))) \quad \text{Eq. (2)}$$

where X is the number of cells (CFU/mL), d is the time of storage expressed in days, A is the asymptotic count as d decreases to 0, C is the difference in value of upper and lower asymptote, B is the relative growth rate at μ, and μ is the day at which the absolute growth rate is maximum.

2.6 Fresh-cut fruit quality evaluation

2.6.1 Headspace gas analysis

The composition of the headspace of each tray was analyzed with a gas chromatograph equipped with a thermal conductivity detector (Micro-GC CP 2002 gas analyzer, Chrompack International, Middelburg, The Netherlands). A 1.7ml sample was automatically withdrawn from the headspace atmosphere. Portions of 0.25 and 0.33 mL were injected for O_2 and CO_2 determination, respectively. The O_2 content was analyzed with a CP-Molsieve 5 Å packed column (Chrompack International, Middelburg, The Netherlands) (4 m×0.32 mm, df=10 mm) at 60 ºC and 100kPa. For quantification of CO_2, ethylene (C_2H_4) and ethanol (C_2H_5OH), a Pora- PLOT Q column (Chrompack International, Middelburg, The Netherlands) (10 m×0.32 mm, df=10mm), held at 70 ºC and 200 kPa, was used. Two trays were taken at each sampling time to perform the gases analysis and two replicates were carried out for each one.

2.6.2 Color measurements

Fresh-cut apples surface color was directly measured with a Minolta chroma meter (Model CR-400, Minolta, Tokyo, Japan). The equipment was set up for illuminant D65 and 10 º observer angle and calibrated using a standard white reflector plate. Twenty apple cylinders were analyzed for each pair of trays. Three readings were made in each replicate by changing the position of the apple pieces. Color was measured through changes in L and h* values. Numerical values of a* and b* parameters were employed to calculate the hue angle (h*) according to Eq. 3:

$$h^* = \arctan\frac{b^*}{a^*}$$ Eq. (3)

2.6.3 Firmness determination

The texture of apple pieces was measured using a TA-XT2 Texture Analyzer (Stable Micro Systems Ltd, England, UK) by measuring the maximum penetration force required for a 4-mm-diameter probe to penetrate into apple cylinders (20 mm high) to a depth of 10 mm at a speed rate of 5 mm s^{-1}. Apple pieces were placed perpendicularly to the probe to allow the penetration in their geometric center.

2.7 Statistical analysis

All parameters were assayed in duplicate (2 trays per day), and results were expressed as the mean and the standard deviation. A statistical analysis software program (JMP 8, SAS Institute Inc.) was used to perform the analysis of variance. The Student's t test was run to determine significant differences at a 5% significance level ($p < 0.05$).

3 Results and discussion

3.1 Emulsion and nanoemulsion characterization

Conventional emulsions and nanoemulsions with different concentrations of LEO were characterized in terms of droplet size distribution (Figure 1), average droplet size, ζ-potential, whiteness index and viscosity (Figure 2, A, B, C and D).

The average droplet diameter of the alginate solution containing tween 80 was 135 nm (Figure 2, A), which can be attributed to the size of micelles formed by the small molecule surfactant. The droplet size of conventional emulsions decreased from 1775 to 494 nm when decreasing the oil content in the formulation from 1% to 0.1%. Similarly, after being processed by microfluidization, the droplet diameter ranged between 364 and 62 nm, being smaller at lower concentrations of oil. This trend might be expected since the surfactant molecules are adsorbed in the lipid surface stabilizing the dispersed lipid droplets (Rao & McClements, 2011). In this sense, the higher the oil concentration the more surface-active molecules are needed to assure the complete coverage of the interfacial area (Qian & McClements, 2011). Therefore, it is more difficult to reduce the droplet size up to the nano-range. This behavior was also confirmed by observing

droplet size distribution (Figure 1), since an increase in the particles in the nano-range can be observed when decreasing the oil concentration in the formulation.

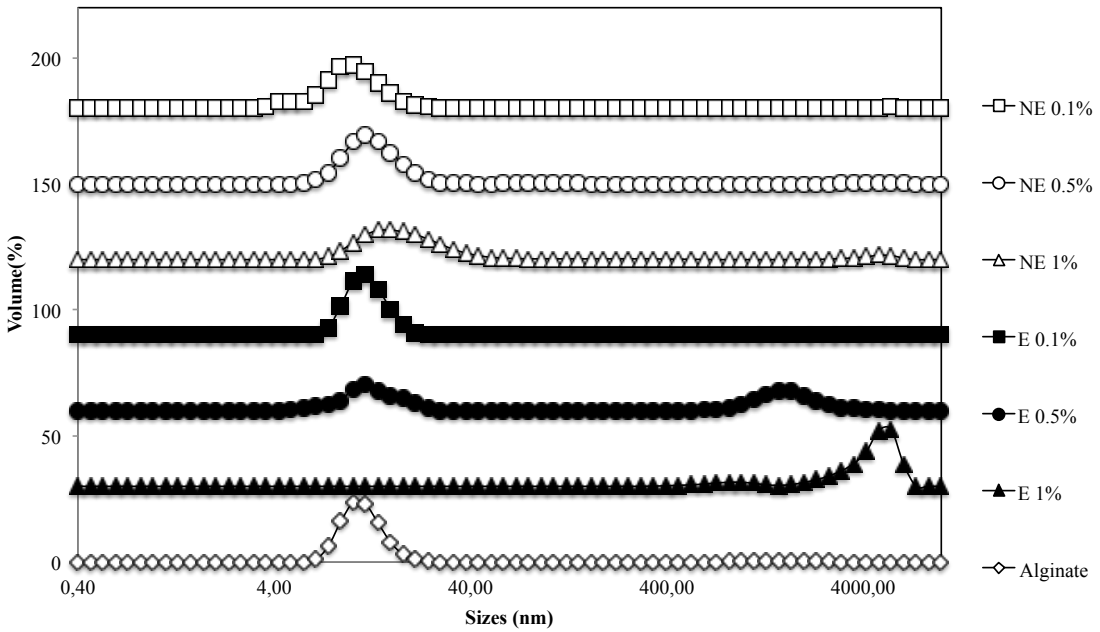

Figure 1 Droplet size distribution of emulsions (E) or nanoemulsion (NE) (microfluidized 3 cycles at 150 MPa) containing different concentrations (1, 0.5 or 0.1% v/v) of lemongrass essential oil. Data shown are the means ± standard deviation.

The ζ-potential of the sodium alginate solution was -55 mV (Figure 2, B), which was due to the anionic nature of the biopolymer. Alginates are linear polymers of mannuronic acid and guluronic acid, which are monomers with a low dissociation constant so they tend to be negatively charged in a wide range of pH (Tønnesen & Karlsen, 2002). In the case of conventional emulsions, adding LEO at 1% decreased the ζ-potential of the solution up to -35 mV. It is believed that some biopolymers can adsorb at the oil-water interface depending on possible interactions and competitive adsorption between previously adsorbed species, such as small molecule surfactants (Dickinson, 2003, 2009). In this sense, the higher the concentration of oil in the emulsion system, the more biopolymer molecules would be adsorbed on the oil-water interface. This fact would lead to the neutralization of the adsorbed species, thus reducing the droplet electrical charge. Moreover, it was observed that nanoemulsions exhibited a more negative ζ-potential in comparison with conventional emulsions regardless of the concentration of oil used in the formulation, with values between -71 and -73 mV. These results are in agreement with previous studies (Salvia-Trujillo, Rojas-Graü, Martín-Belloso, & Soliva-Fortuny, 2013), which confirmed the breakage

effect of microfluidization on the sodium alginate molecules, rendering lipid nanoparticles with a more negative charge and therefore with a higher stability.

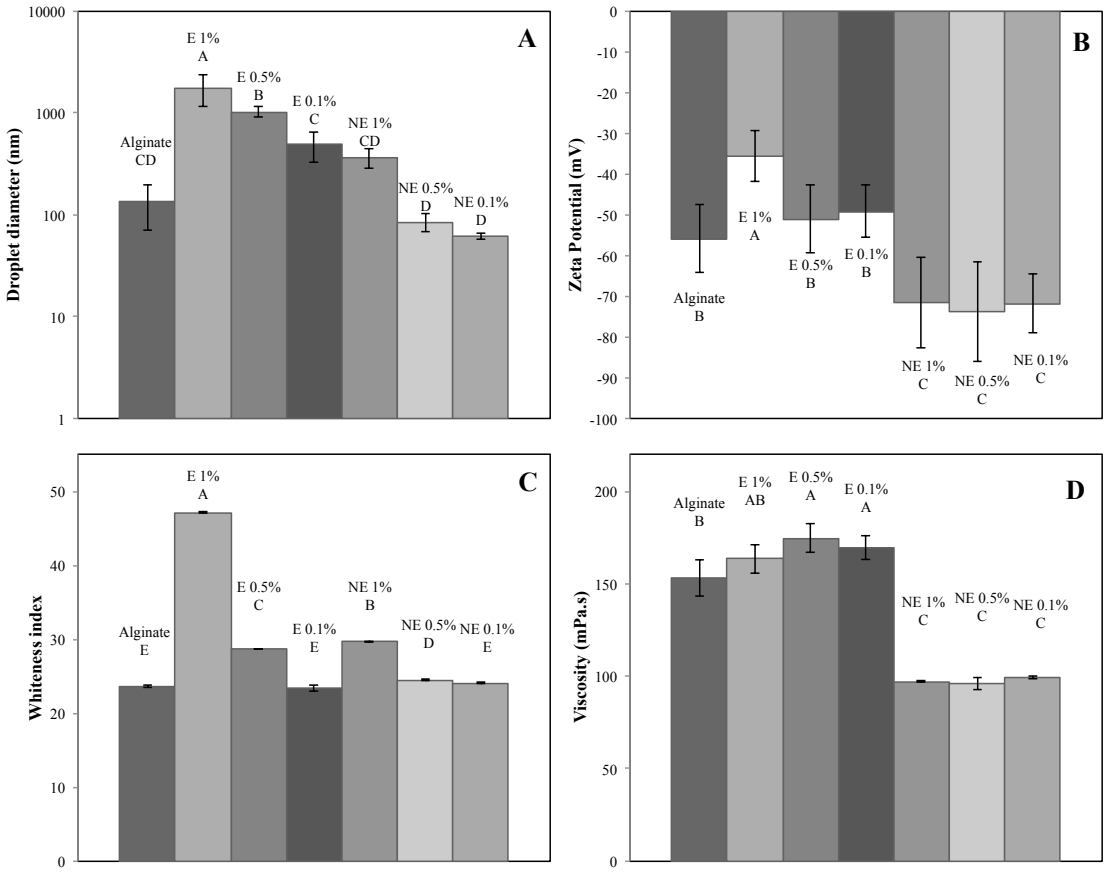

Figure 2 Droplet size (A), ζ-potential (B), whiteness index (C) and viscosity (D) of sodium alginate solution (containing tween 80), emulsions (E) or nanoemulsion (NE) (microfluidized 3 cycles at 150 MPa) containing different concentrations (1, 0.5 or 0.1% v/v) of lemongrass essential oil. Data shown are the means ± standard deviation.

The whiteness index of conventional emulsions increased when increasing the oil concentration in the formulation (Figure 2, C). Emulsions with a minimum LEO concentration of 0.1% exhibited a whiteness index of 23.3, which was close to the color of a sodium alginate solution without oil. Emulsion color is given by the light scattering that lipid droplets exert when a beam of light collides and largely depends on the oil concentration (McClements, 2002). However, it is also known that emulsion droplet size significantly affects the emulsion appearance. Smaller particles scatter the light more weakly than larger ones, leading to emulsions with lower opacity being almost transparent systems (McClements, 2011). This is in agreement with our results, where a decrease in the whiteness index of nanoemulsions was observed in comparison with

conventional emulsions (Figure 2, C), and this effect was more pronounced in emulsions with a higher lipid concentration.

Finally, the viscosity of sodium alginate increased after adding LEO in the mixture, regardless of the lipid concentration (Figure 2, D). The viscosity of conventional emulsions ranged between 153 and 174 mPa.s. Nevertheless, the viscosity of nanoemulsions was significantly lower in comparison with conventional emulsions, with values below 99 mPa.s. It is known that microfluidization processes affects the molecular structure of biopolymers, breaking the polymeric chain, rendering solutions with lower thickening properties (Bonilla, Atarés, Vargas, & Chiralt, 2012; Salvia-Trujillo et al., 2013).

3.2 Microbial inhibition in fresh-cut apple

3.2.1 *Escherichia coli* inactivation

The effect of the antimicrobial edible coatings on the *E. coli* counts during refrigerated storage of fresh-cut *Fuji* apples is shown in Figure 3. Initially, bacterial culture was spread over apple slices at a concentration of 10^6 CFU/g. Microbial counts were reduced by 0.8 log units just after applying the sodium alginate coating without LEO. The LEO concentration used in the formulation of edible coatings had a significant effect on their bactericidal activity against *E. coli*. In those samples coated with solutions containing 0.5% or 1% (v/v) of LEO the *E. coli* population was inactivated up to undetectable levels (2 log units) practically immediately after coating, and remained undetected during 2 weeks of refrigerated storage. On the other hand, coatings with a LEO concentration of 0.1% (v/v) exhibited a significantly different behavior depending on the initial droplet size of the coating-forming emulsion. In this sense, nanoemulsion coatings exhibited a faster and enhanced inactivation of *E. coli* on apple pieces during storage time in comparison with coatings formed from conventional emulsions. Nanoemulsion-based edible coatings with 0.1% (v/v) LEO reduced the *E. coli* inoculum until undetectable levels after 11 days of storage time, whereas fresh-cut apples coated with conventional emulsions with the same LEO concentration still presented 3.3 log units of *E. coli* after 14 days.

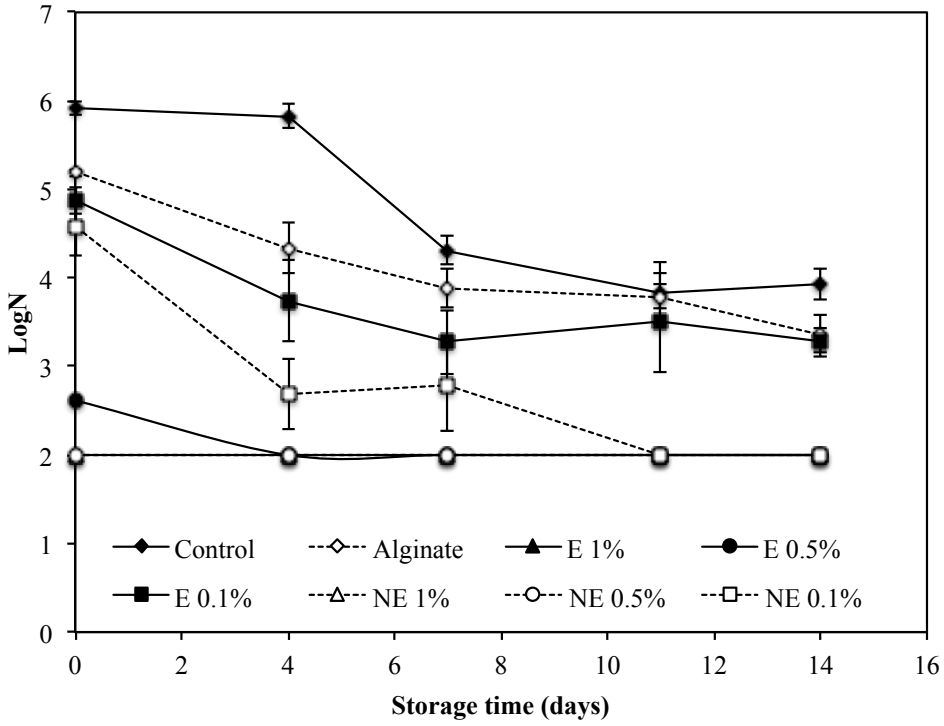

Figure 3 Effect of the edible coatings from emulsions or nanoemulsions containing different concentrations of lemongrass essential oil on *Escherichia coli* (log CFU/g) inoculated onto apple pieces. Data shown are the means ± standard deviation. Control: uncoated; Alginate: coated without lemongrass essential oil; E: emulsion; NE: nanoemulsion

It is believed that a reduction in the oil droplet size would allow antimicrobial compounds to penetrate faster in the bacterial cell (Weiss, Takhistov, & McClements, 2006), thus explaining the behavior observed. Even though there are no previously reported data about the use of nanoemulsion-based edible coatings, there are evidences that would support this hypothesis. In this sense, Donsí and co-workers (Donsì et al., 2011) used antimicrobial nanoemulsions containing a mixture of terpenes to enhance the shelf life of orange and pear juices, observing that the effect was dose-dependent. Moreover, Chang and co-workers (Chang, McLandsborough, & McClements, 2012) also developed antimicrobial nanoemulsions containing thyme essential oil for food applications, observing that ripening inhibitors in the formulation of nanoemulsions may have a detrimental effect in their antimicrobial activity, increasing the minimum inhibitory concentration (MIC). The enhanced antimicrobial activity of nanoemulsions over conventional emulsions would allow reducing the concentration to be incorporated in foods.

3.2.2 Microbial stability

Figure 4 displays the growth of molds and yeasts (A) and psychrophilic bacteria (B) in uncoated and coated apple pieces during refrigerated storage. As expected, the concentration of LEO of the emulsion coatings significantly affected the spoilage of fresh-cut apples. Microbial counts in uncoated apple pieces increased gradually during 14 days of storage time, reaching 5.2 and 5.5 log CFU/g for molds and yeasts and bacteria respectively. The growth of natural microbiota was completely inhibited during 2 weeks of refrigerated storage when apple pieces were coated with coatings with LEO concentrations of 0.5% and 1% (v/v). However, even though the addition of 0.1% (v/v) of LEO in the coating solution slowed down the microbial growth in comparison with control samples, it was not enough to completely inhibit it. Moreover, emulsion droplet size of coating-forming solutions did not have a categorical effect on their antimicrobial activity. Nevertheless, fresh-cut apples with nanoemulsion-based edible coating with 0.1% (v/v) LEO showed a final bacterial concentration of 4.6 log CFU/g, whereas the conventional emulsion coating at the same LEO concentration exhibited 5.4 log CFU/g (Figure 4 B). This behavior was also reflected by the fitted Gompertz equation to experimental values (Table 1).

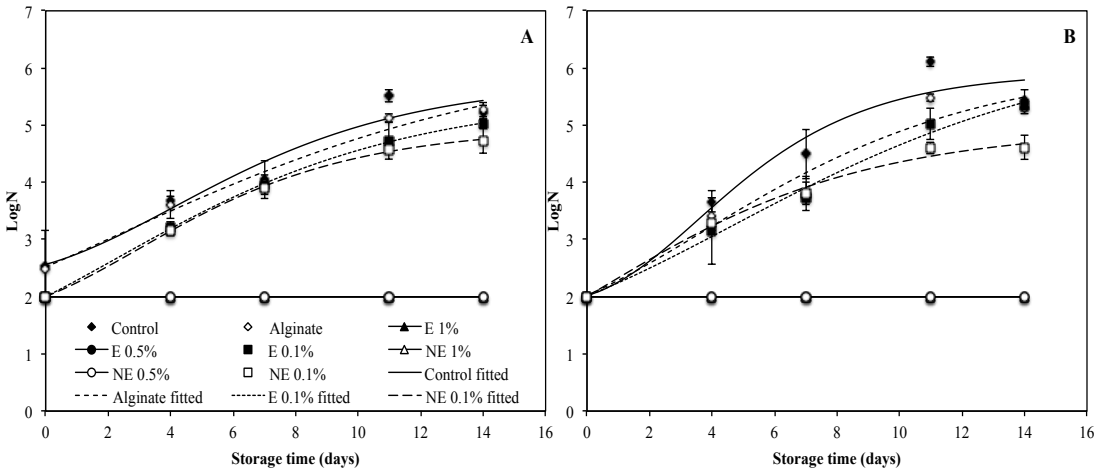

Figure 4 Effect of the edible coatings from emulsions or nanoemulsions containing different concentrations of lemongrass essential oil on the microbial growth (log CFU/g) of apple pieces: moulds and yeasts (A) and psychrophilic bacteria (B). Data shown are the means ± standard deviation. Control: uncoated; Alginate: coated without lemongrass essential oil; E: emulsion; NE: nanoemulsion

In the case of molds and yeasts growth in apple pieces coated with nanoemulsions or conventional emulsions with LEO 0.1% (v/v), the growth curve parameters did not present significant differences. However, psychrophilic bacteria in samples coated with nanoemulsions at 0.1% (v/v) LEO exhibited a lower growth rate (B-value) compared with fresh-cut apples coated with conventional emulsions at the same LEO concentration, which were 4.33 and 5.28, respectively. Recent papers evidenced the shelf life extension of fresh-cut apples (Raybaudi-Massilia, Rojas-Graü, et al., 2008; Rojas-Graü et al., 2007), melon (Raybaudi-Massilia, Mosqueda-Melgar, & Martín-Belloso, 2008) or grapes (Sanchez-Gonzalez et al., 2011) by applying edible coatings with EOs. However, as far as we know there is no reported data about the incorporation of nanoemulsions of EOs as edible coating in food products.

Table 1 Estimated parameters of Gompertz equation proposed to describe the growth of molds and yeasts and psychrophilic bacteria in fresh-cut Fuji apples uncoated (Control), coated with alginate (alginate), with emulsions (E) or nanoemulsions (NE) containing lemongrass at different concentrations (1, 0.5 or 0.1%)

		A	**C**	**B**	**μ**
Molds and yeasts	Control	2.29	0.23	3.50	4.12
	Alginate	1.15	0.13	5.24	2.29
	E 1%	-	-	-	-
	E 0.5%	-	-	-	-
	E 0.1%	1.03	0.18	4.51	2.31
	NE 1%	-	-	-	-
	NE 0.5%	-	-	-	-
	NE 0.1%	1.59	0.25	3.37	2.95
Psychrophilic bacteria	Control	1.84	0.32	4.09	3.55
	Alginate	1.42	0.19	4.68	3.71
	E 1%	-	-	-	-
	E 0.5%	-	-	-	-
	E 0.1%	1.28	0.15	5.28	4.60
	NE 1%	-	-	-	-
	NE 0.5%	-	-	-	-
	NE 0.1%	0.62	0.20	4.33	0.64

A, is the asymptotic count as time decreases to 0; C, is the difference in value of upper and lower asymptote; B, is the relative growth rate at μ; μ, is the time (days) at which the absolute growth rate is maximum.

3.3 Fresh-cut apple quality parameters

3.3.1 Headspace gas analysis

The respiration, in terms of O_2 consumption and CO_2 production, of fresh-cut *Fuji* apples uncoated or coated with the different emulsions or nanoemulsions is shown in

Figure 5. Coatings with emulsions or nanoemulsions with a LEO concentration of 0.5 (v/v) or 1% (v/v) significantly decreased the respiration of fresh-cut apples compared with the uncoated apple pieces, decreasing the O_2 consumption and CO_2 production.

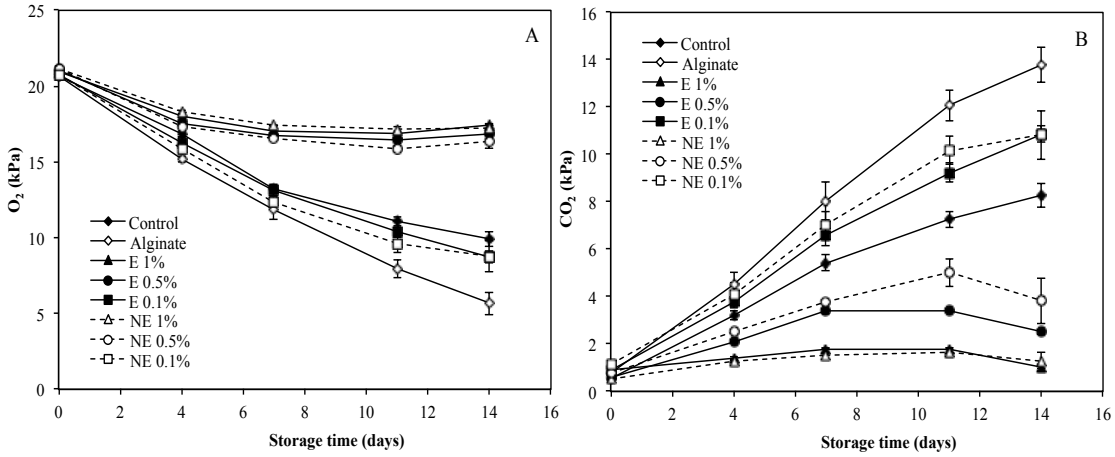

Figure 5 O_2 (A) and CO_2 (B) headspace gas concentration (kPa) in trays containing uncoated or coated (with emulsions or nanoemulsions) apple pieces during storage at 4ºC. Data shown are the means ± standard deviation. Control: uncoated; Alginate: coated without lemongrass essential oil; E: emulsion; NE: nanoemulsion

The decrease in the respiration of coated fresh-cut apples due to EOs was also described by other authors (Raybaudi-Massilia, Rojas-Graü, et al., 2008; Rojas-Graü et al., 2007), being attributed to a major resistance by the coating to gas diffusion resulting from the lipophilic nature of EOs. However, apple pieces coated without LEO or coated with nanoemulsions or conventional emulsions containing 0.1% (v/v) LEO exhibited an increased respiration (higher O_2 consumption and CO_2 production) during storage time in comparison with the uncoated apple slices. Minimal processing of fruits involves operations like peeling and cutting, which causes a physiological stress on plant tissues, thus triggering the respiration metabolism (R. C. Soliva-Fortuny & Martín-Belloso, 2003). Aerobic respiration consists of oxidative breakdown of organic reserves of carbohydrates, lipids and organic acids to simpler molecules, including CO_2 and water, with release of energy (Fonseca, Oliveira, & Brecht, 2002). Therefore, the higher respiration rate observed in fresh-cut apples coated with sodium alginate in comparison with those uncoated might be due to the higher availability to metabolize carbohydrate molecules from the biopolymer. This trend was also observed in fresh-cut apples coated with emulsions or nanoemulsions containing LEO at 0.1% (v/v). This fact might also be related to the contribution of microbial respiration in fresh-cut apples on the headspace

gas composition, since apple pieces coated without LEO or with nanoemulsions or emulsions containing 0.1 % (v/v) LEO showed a higher microbial growth during storage time. Other authors (Olivas, Mattinson, & Barbosa-Cánovas, 2007; Wong, Tillin, Hudson, & Pavlath, 1994) observed a decreased respiration in coated fresh-cut fruits, suggesting that the diffusion of the headspace O_2 to the tissue was inhibited by the high O_2 resistance of the edible coating, thus slowing down the plant metabolism. The effect of the droplet size of LEO incorporated in edible coatings on the respiration of fresh-cut apples was not significant. Nevertheless, it could be observed a slightly higher O_2 consumption and CO_2 production in those apple slices coated with nanoemulsions containing 0.5 or 0.1 % (v/v) LEO compared to the non-nano form. This fact might be possibly be related to the high susceptibility of LEO nanoemulsions to be volatilized due to the increased surface area, thus leading to a decreased effective concentration of EOs in the edible coating.

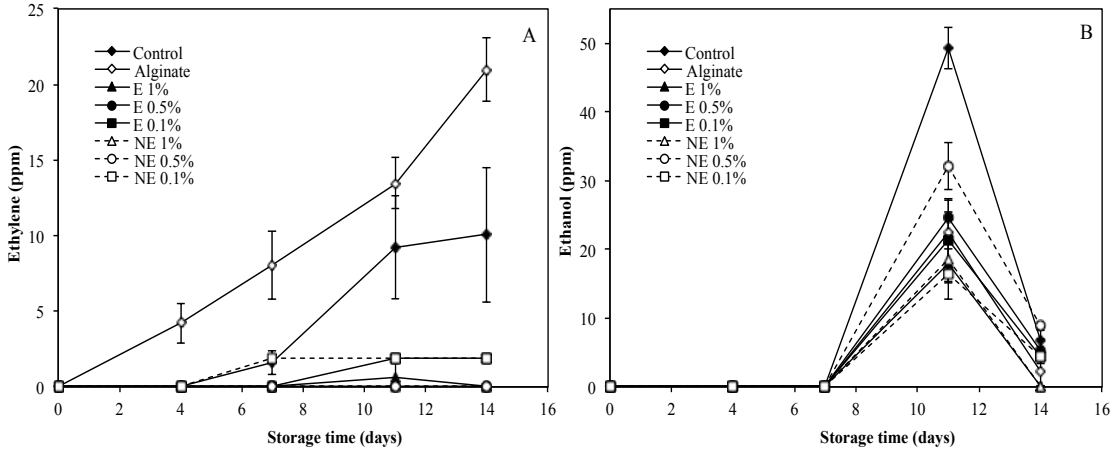

Figure 6 Ethylene (A) and Ethanol (B) headspace gas concentration (ppm) in trays containing uncoated or coated (with emulsions or nanoemulsions) apple pieces during storage at 4ºC. Data shown are the means ± standard deviation. Control: uncoated; Alginate: coated without lemongrass essential oil; E: emulsion; NE: nanoemulsion

Consistently with the results for O_2 consumption and CO_2 production, the ethylene concentration (Figure 6 A) on the headspace was greater in those fresh-cut apples coated only with sodium alginate in comparison with those uncoated. A concentration of LEO of 0.1% incorporated in edible coatings was sufficient to inhibit the ethylene production during storage time, regardless the droplet size of the emulsion. Ethylene is a secondary metabolite, which is related with aerobic metabolism of plant tissue (R. C. Soliva-Fortuny & Martín-Belloso, 2003). In contrast, ethanol is a secondary metabolite

that is produced during fermentative reactions in anaerobic conditions (Fonseca et al., 2002). An ethanol production peak was observed at day 11 of storage time (Figure 6 B), which was significantly higher in uncoated samples. Coated apple pieces exhibited a lower concentration of ethanol on the headspace, regardless of the formulation of the coating. Similarly to our results, Rojas-Graü and co-workers (Rojas-Graü et al., 2007) also observed a depletion on the ethylene and ethanol production in fresh-cut *Fuji* apples by incorporating EOs in alginate-based edible coatings.

3.3.2 Color

The concentration of LEO incorporated in edible coatings significantly affected the color (L and h parameters) of fresh-cut *Fuji* apples during refrigerated storage (Figure 7). The L and h values of uncoated or coated apple slices with sodium alginate remained practically constant during 15 days of cold storage. However, a gradual decay of the L and h values of apple pieces coated with emulsions or nanoemulsions containing LEO was observed through storage time. Such decrease was more noticeable when an oil concentration of 0.5 or 1 % (v/v) was added.

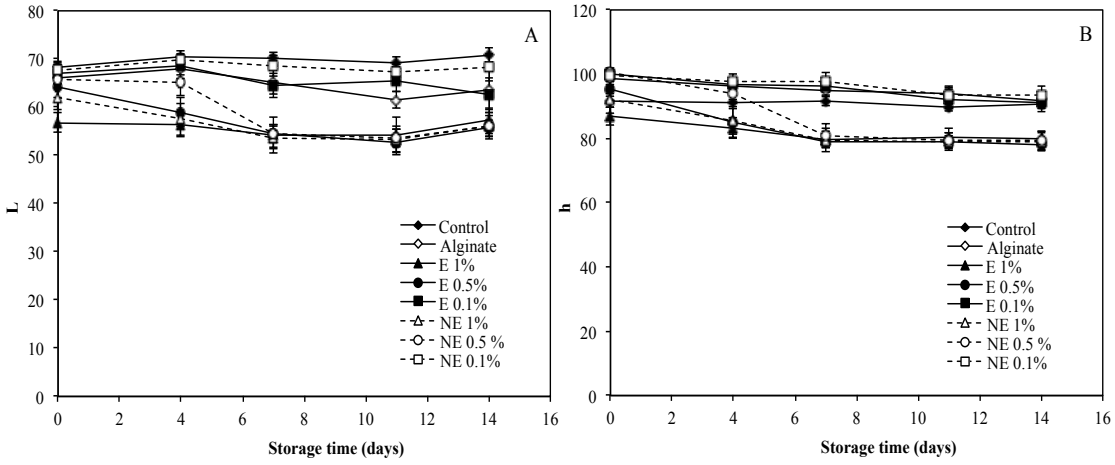

Figure 7 Changes in the color parameters, L (A) and h (B), of fresh-cut *Fuji* apples uncoated or coated with emulsions or nanoemulsions during storage time. Data shown are the means ± standard deviation. Control: uncoated; Alginate: coated without lemongrass essential oil; E: emulsion; NE: nanoemulsion

Browning of fresh-cut apples is basically indicated by a decrease in the lightness of fruit tissue (R. C. Soliva-Fortuny, Grigelmo-Miguel, Odriozola-Serrano, Gorinstein, & Martín-Belloso, 2001) and also an increase in a* (redness) values (McHugh & Senesi, 2000) that implies a decrease in the hue value. Color degradation in white flesh fruits,

such as apples, is mainly due to enzymatic browning. After mechanical stress due to minimal processing, polyphenol oxidase (PPO) is liberated from plant cells and interacts with polyphenols and oxygen, thus leading to the formation of brownish compounds (Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994; Toivonen & Brummell, 2008). Previously, the induction of browning reactions in fresh-cut apples coated with solutions containing EOs was also reported (Raybaudi-Massilia, Rojas-Graü, et al., 2008; Rojas-Graü et al., 2007). The incorporation of EOs in edible coatings might induce the browning of cut apple surface by two mechanisms: (i) phenolic compounds from EOs might be substrate themselves for PPO activity; and (ii) an increase in the permeability of plant cell membrane due to volatile compounds might cause a higher leakage of PPO and polyphenols from the cell cytoplasm. Even though the effect of the initial emulsion droplet size coating-forming solutions did not significantly determine the color of fresh-cut apples, a slightly lower browning was observed in those samples coated with nanoemulsions in comparison with conventional emulsions. The L value was higher during the first 5 days of storage in fresh-cut apples coated with nanoemulsions containing LEO at 0.5 and 1% (v/v) regarding the samples coated with conventional emulsions at the same LEO concentration. In the case of coatings with 0.1 % (v/v) LEO, this trend was maintained during 15 days of storage.

3.3.3 Firmness

Figure 8 shows the results of the flesh firmness (N) of coated and uncoated fresh-cut *Fuji* apples during cold storage. Neither the edible coatings nor the LEO concentration and its particle size had a significant influence on the firmness of apple slices. Moreover, the firmness of fresh-cut apples was maintained practically constant during storage time regardless the coating applied, with values ranging from 11.95 and 9.14. On the contrary, the softening of fresh-cut apples after applying edible coatings with EOs was reported previously (Raybaudi-Massilia, Rojas-Graü, et al., 2008; Rojas-Graü et al., 2007). They attributed the loss of firmness to the low pH of film forming solutions, which might cause the acid hydrolysis of pectic acid in fruit cells (Ponting, Jackson, & Watters, 1971). Also it has been suggested that such degradation of the texture might be caused by the penetration of the EOs on the cell tissue of the fruit, producing structural changes (Sánchez-González et al., 2011). On the other hand, it has been stated that the use of calcium salts to induce the cross-linking of sodium alginate may enhance the texture of minimally processed fruit by bonding to pectins and

preventing cell wall degradation (Lamikanra, 2002; Olivas et al., 2007; Poovaiah, Glenn, & Reddy, 1988). The differences between the results observed in the present work and previously reported data may rely on the high initial firmness values of the apple pieces as well as the concentration and type of EO incorporated to edible coatings or the intrinsic varietal firmness characteristics (B. C. Soliva-Fortuny, Oms-Oliu, & Martín-Belloso, 2002).

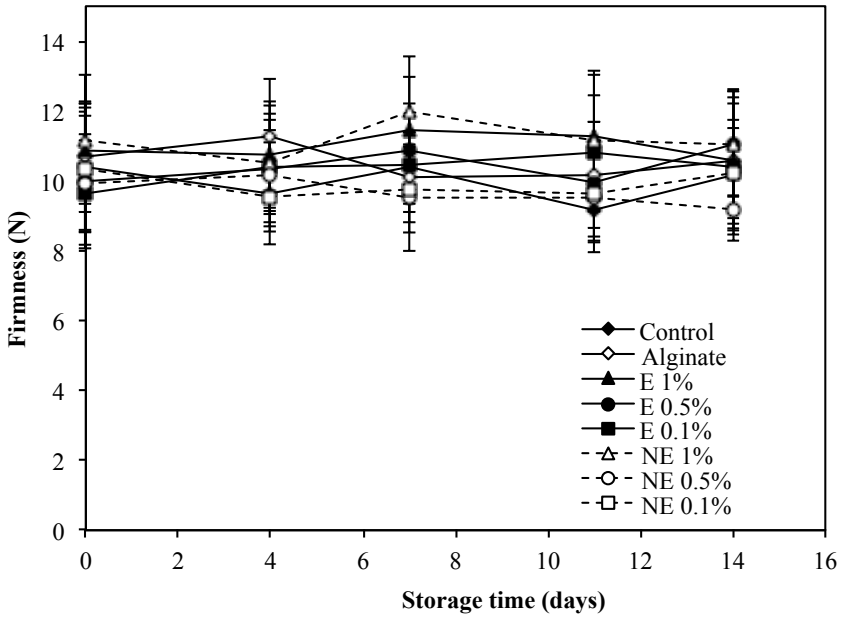

Figure 8 Firmness (N) of fresh-cut *Fuji* apples uncoated or coated with emulsions or nanoemulsions during storage time. Data shown are the means ± standard deviation. Control: uncoated; Alginate: coated without lemongrass essential oil; E: emulsion; NE: nanoemulsion

4 Conclusions

The results obtained in the present work point the potential advantages of using nanoemulsions over conventional emulsions as edible coatings to improve the safety and quality of fresh-cut fruits. Experimental data suggest that nanoemulsion-based edible coatings containing 0.1% (v/v) LEO presented higher *E. coli* inactivation as well as a slower psychrophilic bacteria growth on fresh-cut apples during storage time compared to the edible coatings with conventional emulsions at the same concentration. On the other hand, the respiration of fresh-cut apples was lower at increasing concentration of LEO in the edible coatings, without observing a significant influence of the emulsion droplet size. Edible coatings with emulsions or nanoemulsions

containing high concentrations of LEO caused surface browning on fresh-cut apples, whereas they did not have any substantial effect on the firmness of apple slices. Thus, the current work opens new perspectives to apply nanoemulsions as edible coatings to assure safety and quality of fresh-cut products, but more research is needed in order to proof the significance of these results in other food products.

Acknowledgements

This study was supported by the Ministerio de Ciencia e Innovación (Spain) throughout the project AGL2009-11475. Laura Salvia-Trujillo thanks the Ministry of Science and Education (Spain) for the predoctoral grant. Prof. Olga Martín-Belloso thanks the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia 2008 Award.

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PUBLICATIONS

Chapter VII

Influence of particle size on lipid digestion and β-carotene bio-accessibility in emulsions and nanoemulsions

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Food Chemistry 141 (2), 1472-1480

Abstract

The interest in incorporating carotenoids, such as β-carotene, into foods and beverages is growing due to their potential health benefits. However, the poor water-solubility and low bioavailability of carotenoids is currently a challenge to their incorporation into many foods. The aim of this work was to study the influence of particle size on lipid digestion and β-carotene bioaccessibility using corn oil-in-water emulsions with different initial droplet diameters: large (d_{43} ≈ 23 μm); medium (d_{43} ≈ 0.4 μm); and small (d_{43} ≈ 0.2 μm). There was a progressive increase in the mean particle size of all the emulsions as they passed through a simulated gastrointestinal tract (GIT) consisting of mouth, stomach, and small intestine phases, which was attributed to droplet coalescence, flocculation, and digestion. The electrical charge on all the lipid particles became highly negative after passage through the GIT due to accumulation of anionic bile salts, phospholipids, and free fatty acids at their surfaces. The rate and extent of lipid digestion increased with decreasing mean droplet diameter (small ≈ medium >> large), which was attributed to the increase in lipid surface area exposed to pancreatic lipase with decreasing droplet size. There was also an appreciable increase in β-carotene bioaccessibility with decreasing droplet diameter (small > medium > large). These results provide useful information for designing emulsion-based delivery systems for lipophilic bioactive compounds for food and pharmaceutical uses.

Keywords

β-carotene; nanoemulsions; emulsions; droplet size, digestibility; bioaccessibility.

Table of contents

1 Introduction

Carotenoids occur naturally in many plants and bacteria where they play an important role in photosynthesis and photo-protection reactions (Mayne, 1996). β-carotene is one of the major carotenoids present in the diet that has pro-vitamin A activity, and has also being associated with a decreased risk of various chronic human diseases (Johnson, 2002). Due to their potential health benefits, the incorporation of carotenoids into functional foods or dietary supplements is a major interest of both consumers and the food industry. However, there are challenges associated with incorporating them into many commercial foods and beverages due to their low water-solubility, high melting point, and poor chemical instability (Boon, McClements, Weiss & Decker, 2010), as well as their low bioavailability (Dimitrov et al., 1988).

The absorption of ingested carotenoids by the human body is a complex process that depends on many factors, including the nature of the original food matrix, the type of food processing steps involved, and the concentration, structure and conformation of the carotenoids and lipids consumed (Castenmiller & West, 1998). A number of physicochemical and physiological processes may influence the absorption of dietary carotenoids. Carotenoids may have to be released from the food matrix by mechanical, chemical, and enzymatic processes occurring in the mouth, stomach and small intestine (Van Het Hof, West, Weststrate & Hautvast, 2000). The released carotenoids may be incorporated into fat droplets formed by co-ingested lipids within the mouth and stomach. Alternatively, carotenoids may already be dissolved or dispersed within fat droplets within the original food prior to consumption (Faulks & Southon, 2005). Dietary lipids, such as triacylglycerols (TAG), undergo digestion within the stomach and small intestine due to the presence of gastric and pancreatic lipases leading to the formation of free fatty acids (FFA) and monoacylglycerols (MAG). These surface-active lipid digestion products interact with endogenous surface-active lipids (bile salts and phospholipids) to form mixed micelles, vesicles and other colloidal structures (Yonekura & Nagao, 2007; Kossena, Boyd, Porter & Charman, 2003). These colloidal structures are capable of solubilizing lipophilic bioactive components (such as β-carotene) and transporting them to the epithelium cells where they are absorbed. The lipid digestion stage has therefore been identified as a critical step in ensuring good absorption of ingested carotenoids (Furr & Clark, 1997).

The term “bioaccessibility” has been defined as the fraction of an ingested compound that is solubilized within intestinal fluids in a form that is available for absorption (Benito & Miller, 1998). In the case of lipophilic bioactive components, this form is usually considered to be solubilization within the mixed micelles formed during lipid digestion. Knowledge of the bioaccessibility of a bioactive compound is particularly important for predicting the nutritional efficiency of food formulations and is widely used *in vitro* studies (Fernández-García, Carvajal-Lérida & Pérez-Gálvez, 2009).

One means of controlling the oral bioaccessibility of lipophilic bioactive ingredients, such as β-carotene, is to encapsulate them within colloidal delivery systems (Das Neves, Kobayashi, & Nakajima, 2012; McClements & Li, 2012). Nanoemulsions (d < 200 nm) and emulsions (d > 200 nm) have been proposed as particularly effective colloidal delivery systems for encapsulating and delivering dietary bioactive compounds (McClements & Rao, 2011). These systems consist of small emulsifier-coated lipid particles dispersed within an aqueous medium. Nanoemulsions have been proposed to have several advantages over conventional emulsions as colloidal delivery systems due to their smaller particle size: higher optical clarity; improved physical stability; and, novel rheological properties (Mason, Wilking, Meleson, Chang & Graves, 2006, McClements, 2011). In addition, studies in the pharmaceutical area suggest that the oral bioavailability of encapsulated lipophilic compounds is increased when the size of the particles in colloidal delivery systems is reduced into the nano-sized range (Acosta, 2009). However, there is little empirical evidence for this claim for many food-grade delivery systems (Dalgleish 2006; McClements and Xiao, 2010).

The purpose of the current study was therefore to assess the influence of initial fat droplet size on the digestion of lipids and the bioaccessibility of β-carotene encapsulated within emulsions and nanoemulsions. As part of this study we characterized changes in the physicochemical properties and structure of the fat droplets as they passed through the various stages of a simulated gastrointestinal tract so as to provide a more mechanistic understanding of the influence of droplet characteristics on their potential biological fate.

2 Material and methods

2.1 Materials

β-carotene, Tween 20, lipase (from porcine pancreas) and bile extract (porcine) were purchased from Sigma Aldrich (St. Louis, MO). Corn oil was purchased from a local supermarket. The rest of the chemicals used were of analytical grade. All samples were prepared using ultra pure water obtained from a Mili-Q filtration system.

2.2 Methods

2.2.1 Emulsion and nanoemulsion formation

An oil phase was prepared by dispersing β-carotene (0.5% w/w) in corn oil by sonicating (1 min) and mild heating (< 50 ºC for 5 min) to ensure complete dissolution. An aqueous phase was prepared containing 10.0 mM phosphate buffer and 0.01% (w/w) sodium azide as a preservative (pH 7.0). A "large" emulsion was prepared by mixing oil phase (4% w/w corn oil containing β-carotene), surfactant (1.5% w/w Tween 20), and aqueous phase (94.5% w/w buffer solution) with a high-speed blender (10,000 rpm for 2 min). "Small" and "medium" emulsions were obtained by passing the large emulsion three times through a high-pressure microfluidizer (Model 101, Microfluidics, Newton, MA) working at 4 Kpsi or 5 times at 9 Kpsi, respectively (Li, Hu & McClements, 2011).

2.2.2 Simulated gastrointestinal tract model

An *in vitro* gastrointestinal tract (GIT) model consisting of mouth, gastric and intestinal phases was used to simulate the biological fate of ingested samples:

Mouth phase: Simulated saliva fluid (SSF), containing mucin and various salts, was prepared according to a previous study (Sarkar, Goh & Singh, 2009). A 5 mL-aliquot of the emulsions were mixed with 5 mL of SSF, so that the final mixture contained 2% (w/w) oil. The pH of the mixture was adjusted to 6.8 and it was incubated at 37 ºC for 10 min with continuous agitation at 100 rpm.

Gastric phase: Simulated gastric fluid (SGF) was prepared using a method reported previously (Sarkar, Goh, Singh & Singh, 2009) by dissolving 2 g of NaCl and 7 mL of HCl (37%) in 1 L of water and adjusting the pH to 1.2 using 1.0 M HCl. The "bolus" sample from the mouth phase was mixed with SGF at a 50:50 volume ratio so that the

final mixture contained 1% (w/w) oil. The pH of the sample was adjusted to 2.5 using NaOH (1M) and incubated at 37º C for 2 hours with continuous agitation at 100 rpm.

Small intestinal phase: A pH-stat (Metrohm, 147 USA Inc.) device was used to simulate the conditions in the small intestinal phase of the gastrointestinal tract (McClements & Li, 2010). An aliquot of 30 mL of the sample was placed in a temperature-controlled (37 ºC) chamber and the pH was set at 7.0 using NaOH solution. Then, 4 mL of bile extract (46.87 mg/mL) and 1 mL of calcium chloride (110 mg/mL) solutions dissolved in phosphate buffer were added to the sample and the pH was adjusted to 7.0 if necessary. Afterwards, 2.5 mL of freshly prepared lipase suspension (24 mg/mL) dissolved in phosphate buffer was incorporated into the mixture. The pH of the mixture was monitored and the volume of 0.25 M NaOH (mL) necessary to neutralize the free fatty acids (FFA) released from the lipid digestion (*i.e.,* to keep pH at 7.0) was recorded during 2 h. The amount of free fatty acids released was calculated from the titration curves as described previously (Li & Mcclements, 2010).

2.2.3 Physical stability

The particle size, size distribution, and charge (ζ-potential) were measured before and during the *in vitro* digestion process. The particle size distribution was measured using static light scattering ((Mastersizer 2000, Malvern Instruments Ltd, Worcestershire, UK). Samples were diluted in 10.0 mM phosphate buffer and stirred in the dispersion unit with a speed of 1250 rpm. The particle size was reported as either the surface-weighted mean diameter (d_{43}) or the volume-weighted mean diameter (d_{32}) (McClements, 2005).

The electrical charge (ζ-potential) of samples before and during the simulated GIT conditions was measured by phase-analysis light scattering (PALS) (Zetasizer NanoZS, Malvern Instruments Ltd, Worcestershire, UK) to determine the surface charge at the interface of the droplets. Samples were diluted with buffer solution (1:10) and placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles.

2.2.4 Confocal fluorescence microscopy

Confocal fluorescence microscopy images were taken to determine structural changes that occurred within different phases of the GIT model. Initial emulsions and samples taken from the GIT model were dyed with Nile red, a fat-soluble fluorescent dye that was previously dissolved at 0.1% (w/v) in ethanol. An air-cooled argon ion laser Model

IMA1010 BOS (Melles Griot, Carlsbad, CA) was used to excite Nile red at 488 nm. A Nikon Confocal Microscope (Nikon D-Eclipse C1 80i, Nikon, Melville, NY) with a 60× oil immersion objective lens was used to capture the confocal images. The resulting fluorescent spectra of Nile red were detected in the 515 nm channel equipped with a narrow pass filter (HQ 515/30 m) with a pinhole size of 150 μm. The images generated had a size of 512 × 512 pixels, with a pixel size of 414 nm, and a pixel dwell time of 61.44 μs. All images were taken and processed using the instrument software program (EZ- CS1 version 3.8, Nikon, Melville, NY).

2.2.5 β -carotene bioaccessibility

The bioaccessibility of β-carotene was determined after the samples had been subjected to the full simulated GIT model using the method described previously (Qian *et al.* 2012). An aliquot of raw digesta was centrifuged at 4,000 rpm for 40 min at 25 ºC (CL10 centrifuge, Thermo Scientific). The supernatant was collected and was assumed to be the "micelle" fraction in which the bioactive compound is solubilized. In some samples, a top layer of tiny droplets was observed of non-digested oil and it was removed from the micelle fraction. Aliquots of 5 mL of the raw digesta or the micelle fraction were mixed with 5 mL of chloroform, vortexed and centrifuged at 1750 rpm for 10 min at 25 ºC. The bottom layer containing the solubilized β-carotene was collected, while the top layer was mixed with an additional 5 mL of chloroform and the same procedure was repeated. The bottom chloroform layer was added to the previous one and analyzed spectrophotometrically (Ultraspec 3000 pro, GE Health Sciences, USA) at 450 nm. A cuvette containing pure chloroform was used as a reference cell.

The concentration of β-carotene extracted from a sample was determined from a calibration curve of absorbance *versus* β-carotene concentration in chloroform. The bioaccessibility was then calculated using the following equation:

$$Bioaccessibility = 100 \times \left(\frac{C_{Micelle}}{C_{RawDigesta}} \right) \qquad \text{Eq. (1)}$$

where $C_{micelle}$ and $C_{RawDigesta}$ are the concentration of β-carotene in the micelle fraction and in the overall sample (raw digesta) after the pH-stat experiment, respectively.

2.2.6 Statistical analysis

All experiments were assayed in duplicate, and results were expressed as the mean and the standard deviation. A statistical analysis software program (JMP 8, SAS Institute

Inc.) was used to perform the analysis of variance. The Student's t test was run to determine significant differences at a 5% significance level ($p < 0.05$).

3 Results and discussion

3.1 Influence of initial droplet size on microstructure

Initially, we monitored the particle size and microstructure of the initial samples and of samples after they had been exposed to different stages of the simulated GIT. Initially, the mean particle diameters of the large, medium, and small emulsions were d_{43} = 23, 0.38 and 0.21 μm, and d_{32} = 14, 0.19 and 0.12 μm, respectively, which can be attributed to the increasing severity of the disruptive forces applied to the samples during their preparation. A number of different definitions have been given for the term "nanoemulsion", with upper particle size limits of 100, 200 and 500 nm being proposed in the literature (McClements, 2012). In practice, the precise size limit used to distinguish nanoemulsions from emulsions is fairly arbitrary since there is no dramatic change in physicochemical properties at a particular size. Nevertheless, there are some more gradual changes in physicochemical properties around this size range, *e.g.,* optical clarity and physical stability (McClements, 2012). In this study, we considered the "small" and "medium" emulsions to be nanoemulsions due to their relatively small mean droplet diameters, whereas the "large" emulsions were considered to be conventional emulsions. In general, the small and medium emulsions showed fairly similar behavior to each other as they passed through the simulated GIT without statistically significant differences, whereas the large emulsions behaved somewhat differently. We therefore discussed the behavior of these two groups of emulsions separately.

3.1.1 Small and Medium Emulsions

For the small and medium emulsions, the mean particle diameter (d_{43}) determined by light scattering increased appreciably after exposure to the mouth and stomach phases, and then increased further after exposure to the small intestine phase (**Figure 1**). Examination of the full particle size distributions of these emulsions after exposure to the mouth, stomach, and intestinal phases indicated that a large fraction of the droplets had fairly similar sizes to the initial emulsions (suggesting they were relatively stable to aggregation), but that there was also a population of relatively large particles formed

(suggesting that these droplets were unstable to aggregation) (**Figures 2a and 2b**). Further insights into the change in the microstructure of the nanoemulsions were obtained using confocal fluorescence microscopy (**Figure 3**). The images of the initial samples clearly showed that the small and medium emulsions contained much finer droplets than the large emulsions. However, it was difficult to distinguish the individual droplets in the nanoemulsions because they are close to the limit of resolution of the optical microscope.

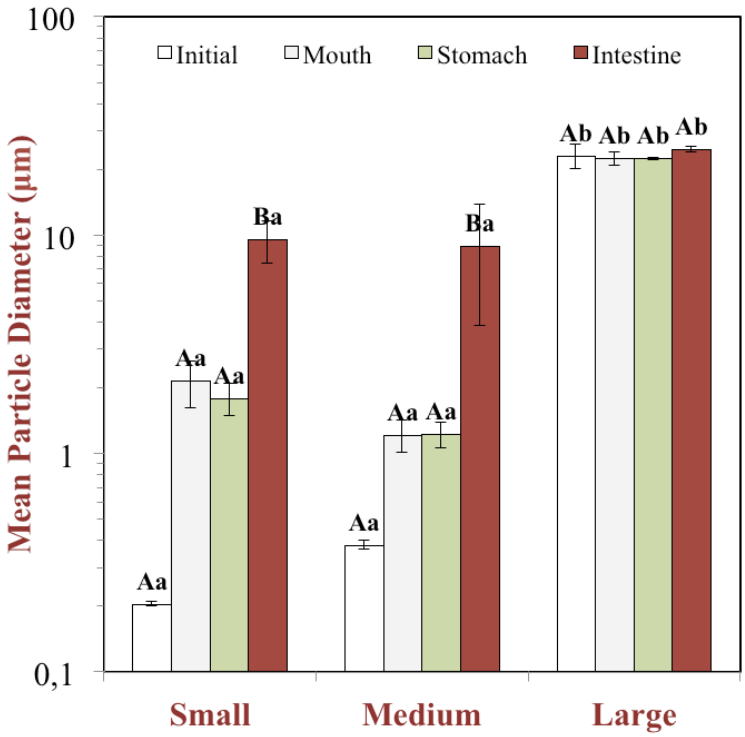

Figure 1. Influence of simulated gastrointestinal conditions on the mean droplet diameter (d_{43}) of corn oil-in-water emulsions stabilized by Tween 20. The emulsions initially had different droplet diameters: Small = 0.21 μm; Medium = 0.38 μm; Large = 23 μm. Different capital letters mean significant differences ($p<0.05$) on the droplet diameter of an emulsion between digestion phases. Different lowercase letters mean significant differences ($p<0.05$) on the droplet diameter between emulsion types (Small, Medium and Large) within the same digestion phase.

Large irregular clusters of lipid droplets were observed in the small and medium emulsions after exposure to the mouth and stomach conditions, which indicated that some droplet aggregation had occurred (**Figure 3**). Droplet aggregation within the mouth has previously been attributed to depletion and/or bridging flocculation caused by droplet interactions with mucin (Sarkar, Goh & Singh, 2009, Silletti, Vingerhoeds, Norde & van Aken, 2007, Vingerhoeds, Blijdenstein, Zoet & Van Aken, 2005).

Bridging flocculation occurs when a polymer (mucin) simultaneously binds to the surfaces of two or more droplets (Dickinson, 2003, McClements, 2005, Singh, Ye & Horne, 2009). On the other hand, depletion flocculation occurs due to the presence of sufficiently high levels of non-adsorbed polymer (mucin) molecules in the aqueous phase surrounding the droplets (Dickinson, 2003; Silletti et al, 2007).

Interestingly, there appeared to be some dissociation of these clusters when the emulsions moved from the mouth to the stomach stage (**Figure 3**). This result is in agreement with previous studies using small lipid droplets coated with a non-ionic surfactant, where dissociation of flocculated droplets was also reported after exposure to stomach conditions (Golding *et al* 2011). The dissociation of flocculated droplets within the stomach may have occurred for a number of reasons, such as sample dilution, changes in solution composition, and/or mechanical agitation (Dickinson, 2003; McClements 2005). Sample dilution weakens any depletion attraction between droplets (by lowering the non-adsorbed polymer concentration), and may also weaken bridging interactions (by promoting desorption of adsorbed polymers from droplet surfaces). Changes in solution pH and ionic strength alter the magnitude and range of any electrostatic interactions operating between the droplets, which would change their susceptibility to aggregation. Stirring samples for prolonged periods within the simulated stomach stage may have led to disruption of droplet clusters that were only held together by weak attractive forces. In the small intestine stage there appeared to be a fairly even distribution of lipid-rich particles throughout the confocal images (**Figure 3**). These particles are likely to be the digestion products generated by lipase-induced breakdown of triacylglycerol molecules, such as mixed micelles, vesicles and other colloidal structures, as well as any non-digested fat droplets. Malaki and co-workers (2011) have also reported the presence of some non-digested oil in β-carotene emulsions after lipase digestion.

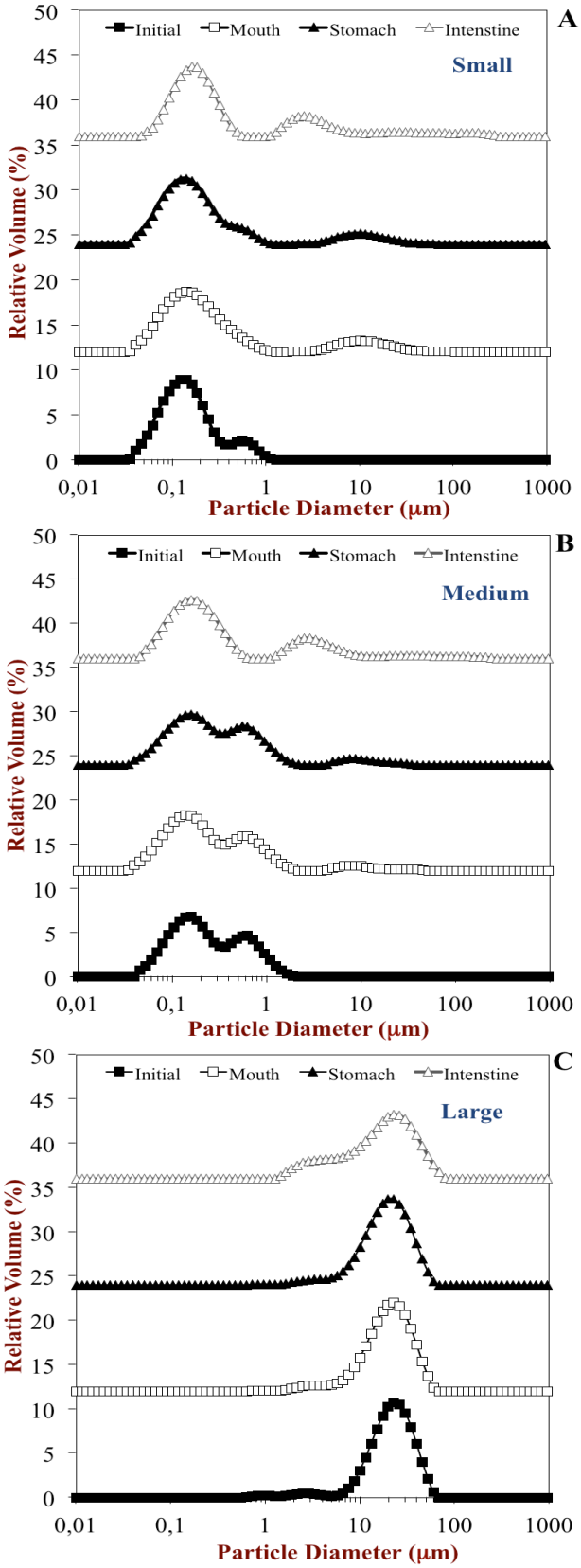

Figure 2. Influence of simulated gastrointestinal conditions on the particle size distributions of corn oil-in-water emulsions stabilized by Tween 20. The emulsions initially had different droplet diameters: (a) Small = 0.21 µm; (b) Medium = 0.38 µm; (c) Large = 23 µm.

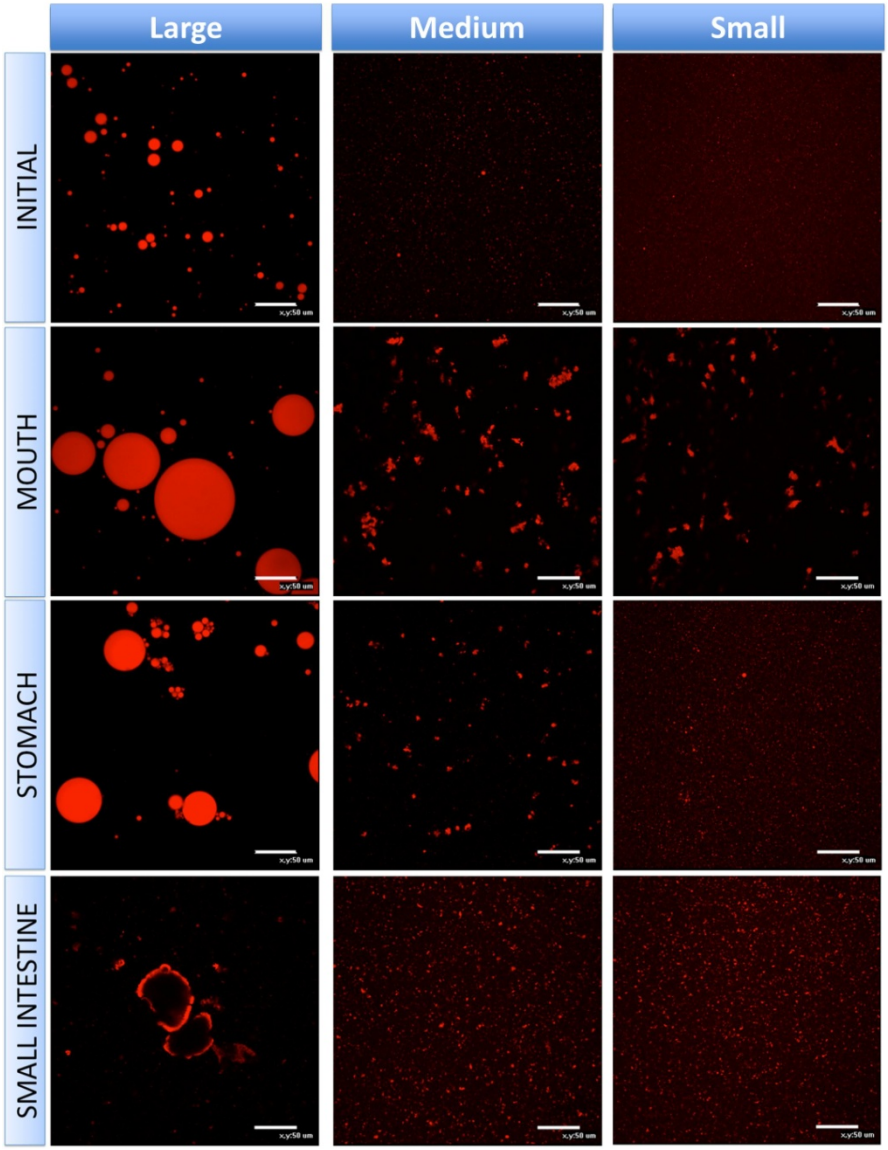

Figure 3. Influence of simulated gastrointestinal conditions on microstructure (determined by confocal fluorescence microscopy) of corn oil-in-water emulsions stabilized by Tween 20. The emulsions initially had different droplet diameters (as measured by light scattering): Small = 0.21 μm; Medium = 0.38 μm; Large = 23 μm. The scale bars represent a length of 50 μm, and the red regions represent lipids.

The change in the microstructures of the small and medium emulsions as they passed through the simulated GIT as observed by confocal microscopy were somewhat similar to those determined by light scattering, *i.e.,* the particles in the mouth, stomach and small intestine were considerably larger than those in the initial sample. However, there were also some important differences: the microscopy images suggested that the largest particles were present within the mouth (**Figure 3**), whereas the light scattering data suggested that the largest particles were present within the small intestine (**Figures 1 and 2**). This difference may have been due to artifacts associated with the analytical

methods used to determine sample microstructure. In particular, the light scattering instrument requires that samples to be highly diluted and stirred prior to analysis, which may have changed the particle size of the samples. For example, the clusters of fat droplets formed within the mouth stage may have broken down upon dilution and stirring in the stomach phase. This phenomenon highlights the importance of using a combination of different methods to determine the structural properties of complex emulsions.

3.1.2 Large Emulsions

For the large emulsions, we also observed quite different behavior of the samples in different regions of the GIT using light scattering and confocal microscopy. The light scattering measurements suggested that there was little change in mean particle diameter (**Figure 1**) or particle size distribution (**Figure 2**) in the various stages of the GIT: initial; mouth; stomach; intestine. However, the microscopy images showed that there was a large increase in droplet size going from the initial sample to the mouth, followed by an appreciable decrease in droplet size after incubation in the stomach (**Figure 3**). The microscopy images also showed that there were some large irregularly shaped particles formed in the small intestine after digestion of the large droplets. These particles appeared to have lipid-rich (bright red) outer regions and lipid-depleted (black) inner regions, and were very different from the structures observed in the small intestine after digestion of the nanoemulsions. These structures may have consisted of undigested oil or lipid digestion products. The differences in microstructure observed by light scattering and microscopy can again be attributed to artifacts associated with the analytical methods used. Dilution and stirring of the emulsions in the light scattering instrument may have promoted coalescence of smaller droplets and breakup of larger droplets so that a steady state droplet size distribution was reached where droplet coalescence and disruption mechanisms were balanced. In this case, the particle size distribution is mainly determined by the operating conditions of the light scattering instrument, rather than by the properties of the sample itself. This finding would suggest that it may be better to use microscopy methods, rather than light scattering, to obtain information about the microstructure of emulsified samples in simulated GIT conditions.

The appreciable increase in the size of the individual droplets observed by confocal microscopy after incubation of the large emulsions in the mouth stage suggests that they

were highly susceptible to coalescence under simulated oral conditions. Coalescence may have been promoted by droplet flocculation caused by depletion and/or bridging mechanisms in the mouth as discussed earlier. The subsequent decrease in droplet size observed in the stomach may have been due to the prolonged mechanical agitation that the samples received causing the disruption of the larger droplets. Indeed, *in vivo* measurements of the droplet size of phospholipid-stabilized emulsions found that the final mean droplet diameter in the human stomach was between 10 and 20 mm, irrespective of the initial droplet size (Armand et al., 1992, 1997). Ingested fat droplets larger than this size tended to be broken down within the stomach, whereas those smaller than this size tended to coalesce. This suggests that a steady state situation arose where the rate of droplet disruption was balanced by the rate of droplet coalescence, leading to a fairly constant droplet size distribution in the stomach. Conversely, studies using milk protein-stabilized fat droplets showed that the droplets remained small throughout the digestion process, suggesting that they were stable to coalescence (Michalski, Briard, Desage, & Geloen, 2005). Our results suggest that the very small fat droplets in the nanoemulsions were fairly resistant to extensive droplet coalescence and breakdown under mouth and stomach conditions, whereas the relatively large fat droplets in the conventional emulsions were highly susceptible to both droplet coalescence and breakdown.

3.2 Influence of initial droplet size on electrical characteristics

The electrical characteristics (ζ-potential) of the particles in the three different emulsions followed fairly similar trends after passage through the various regions of the simulated GIT (**Figure 4**), without statistically significant differences between them. The initial emulsions all had slightly negative surface charges, ranging between -4.8 and -6.3 mV. The non-ionic surfactant used to stabilize the emulsions (Tween 20) would not be expected to give any charge to the droplet surfaces. Nevertheless, studies have shown that fat droplets stabilized by non-ionic surfactants can have an appreciable negative charge, which has been attributed to preferential adsorption of hydroxyl ions (OH^-) from the aqueous phase or the presence of anionic impurities (such as free fatty acids) in the surfactant or oil used to prepare the emulsion (McClements, 2005).

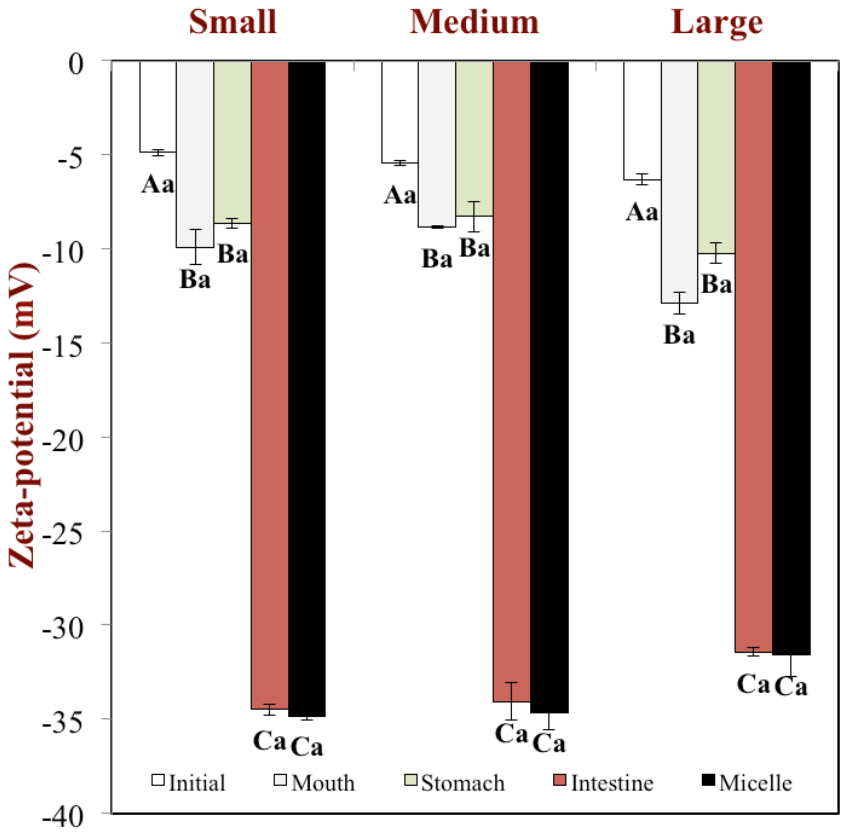

Figure 4. Influence of simulated gastrointestinal conditions on the particle charge (z-potential) of corn oil-in-water emulsions stabilized by Tween 20. The emulsions initially had different droplet diameters (Small, Medium, Large). Different capital letters mean significant differences ($p<0.05$) on the particle charge of an emulsion between digestion phases. Different lowercase letters mean significant differences ($p<0.05$) on the particle charge between emulsion types (Small, Medium and Large) within the same digestion phase.

The ζ-potential of the droplets became significantly more negative after exposure to the mouth and stomach phases (-8 to -12 mV), which may have been due to some adsorption of anionic species (such as mucin) to the droplet surfaces. In turn, the electrical charges on the particles remaining in the samples after the small intestine phase were significantly more negative (-31 to -35 mV). It is not entirely clear what the nature of the particles is that contribute to the z-potential signals in the small intestine. They could be undigested fat droplets or various types of colloidal structures formed by lipid digestion products. Either way, the high negative charge on these particles can be attributed to the presence of surface-active anionic species at their surfaces: (i) bile salts and phospholipids from small intestinal fluids: (ii) free fatty acids formed during lipid digestion (Reis, Holmberg, Watzke, Leser & Miller, 2009; Pouton & Porter, 2008; Singh, Ye & Horne, 2009). Other authors have also reported similar trends in the

electrical characteristics of the particles formed by the digestion of oil-in-water emulsions (Liu, Hou, Lei, Chang & Gao, 2012, Qian, Decker, Xiao & McClements, 2012).

3.3 Influence of initial droplet size on lipid digestion

The rate and extent of lipid digestion in the simulated small intestine was determined using the pH stat method for the three emulsions. The volume of NaOH titrated into the samples to maintain a constant pH (7.0) was measured as a function of digestion time, and then the fraction of free fatty acids released from the oil phase was calculated (**Figure 5**).

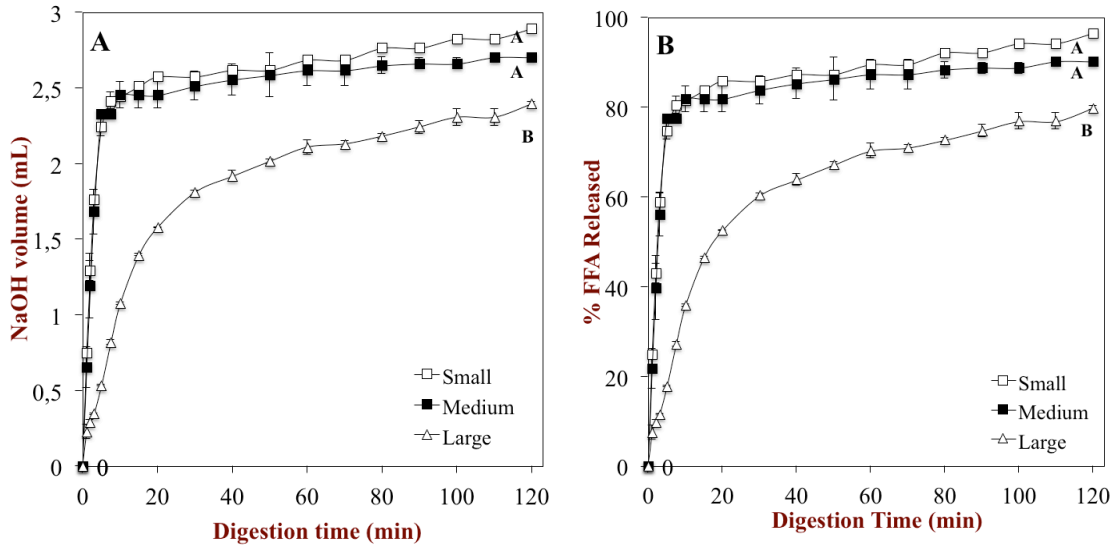

Figure 5. Influence of emulsion droplet diameter (Small, Medium, Large) on the NaOH (0.25 M) volume (mL) used during the pH-stat titration curves (120 min) to maintain the pH constant to 7 (A) and the calculated percentage of free fatty acids (FFA %) released (B). Different capital letters mean significant differences ($p<0.05$) on the digestibility of emulsions with different droplet diameters.

In general, there was a steep increase in the amount of FFAs released from the emulsions initially, followed by a more gradual increase at longer times, until a relatively constant final value was reached (**Figure 5**). However, there were statistically significant differences between the rate and extent of digestion depending on the initial droplet size. In the case of the nanoemulsions (small and medium emulsions), the initial rate of digestion was much faster, and the final amount of FFAs released after 2 hours of incubation was higher than for the conventional emulsions (large emulsions). Other researchers have also found that the amount of FFAs produced per unit time increases

as the droplet size decreases, which was attributed to changes in the surface area of oil exposed to the digestive enzymes (Golding & Wooster, 2010; Reis, Holmberg, Watzke, Leser & Miller, 2009; Li & McClements, 2010; Li, Hu & McClements, 2011).

More detailed information about the role of droplet size on the lipid digestion process can be obtained using a mathematical model developed recently (Li and McClements, 2010). It was shown that the percentage of free fatty acids released (F) from oil-in-water emulsions as a function of digestion time (t) can be modeled by the following equation:

$$\Phi = \phi_{\max}\left(1-\left(1+\frac{3kMt}{2d_0\rho_0}\right)^{-2}\right) \qquad \text{Eq.(2)}$$

Here, f_{max} is a measure of the total *extent* of lipid digestion (*i.e.,* the percentage of FFAs released by the end of digestion), k is the normalized digestion *rate* (*i.e.,* mmols of FFA released per unit droplet surface area per unit time), d_0 is the initial mean droplet diameter (d_{32}), r_0 is the oil droplet density (≈ 910 kg m^{-3} for corn oil), and M is the molar mass of the oil (≈ 0.800 kg mol^{-1} for corn oil). A pH-stat digestion profile can then be characterized in terms of f_{max} and k, which can be determined by finding the values that give the best fit between the experimental data and the mathematical model. This equation can also be used to calculate the "digestion time", which is the time (t_D) required for the FFAs released to increase to 50% (McClements and Li, 2011). The parameters determined for the three different sized emulsions using this approach are summarized in **Table 1**. The mean particle diameters used in the calculations were those of the emulsions after passage through the stomach phase.

Table 1. Parameters obtained by fitting the digestion model to the pH-stat data during the first 120 minute digestion period. It was assumed that the initial oil droplet diameter (d_{32}) was 14.1, 0.20 and 0.15 μm for the large, medium and small emulsions (after stomach), the molar mass of the oil (corn oil) was 800 g mol^{-1}, and the density of the oil was 910 kg m^{-3}.

Initial Droplet Size	Digestion extent F_{max} (%)	Digestion Rate k (mmols s^{-1} m^{-2})	Digestion Time t_D (min)
Small	92.7	0.35	2.2
Medium	88.6	0.56	1.9
Large	81.1	5.39	13.3

The mathematical analysis of the digestion curves provides some important information about the influence of initial droplet size on the rate and extent of droplet digestion. The total extent of digestion increased with decreasing droplet diameter, which can be

attributed to the fact that the overall oil-water surface area increased, and therefore there was more oil exposed to the lipase. Interestingly, the normalized digestion rate (*k*) actually decreased with decreasing droplet size, which means that the amount of FFAs produced per unit surface area per unit time decreased. A possible reason for this phenomenon is that the total concentration of lipase was the same in all of the emulsions, and therefore the amount of lipase per unit surface area decreased as the droplet size decreased. A similar phenomenon was reported in recent studies of the influence of droplet size on the digestion of β -lactoglobulin coated lipid droplets (Li and McClements, 2010).

A number of recent studies have also shown that other factors may also contribute to the overall dependence of digestion rate on initial droplet size, including the concentration of free surfactant remaining in the aqueous phase (since this can inhibit lipase absorption) and changes in interfacial structure with droplet size (since this can affect the ability of lipase to reach the oil phase) (Li and McClements, 2011; Ahmed, Li, McClements & Xiao, 2012, Troncoso, Aguilera & McClements, 2012).

3.4 Influence of initial droplet size on β-carotene bioaccessibility

Finally, we measured the influence of initial droplet size on the solubilization of β-carotene in the micelle phase generated at the end of the lipid digestion process (**Figure 6**). The bioaccessibility of the β-carotene was found to significantly decrease with increasing initial droplet diameter, falling from around 59% for the small emulsion to 34% for the large emulsion. A comparison of β-carotene bioaccessibility with the total amount of free fatty acids produced at the end of the lipid digestion process showed that the bioaccessibility increased as the amount of FFAs released increased (**Figure 7**). This result supports the hypothesis that the mixed micelles formed from the products of triacylglycerol digestion (FFAs and MAGs) play a crucial role in determining the total amount of β-carotene that can be incorporated into the micelle phase, as has also been observed by other workers (Malaki Nik, Wright & Corredig, 2011, Nik, Langmaid & Wright, 2012). The decrease in β-carotene bioaccessibility with increasing initial droplet size may therefore be attributed to two phenomena: (i) there was more undigested oil present, which could have retained the β-carotene; (ii) there were less mixed micelles present to solubilize the β-carotene.

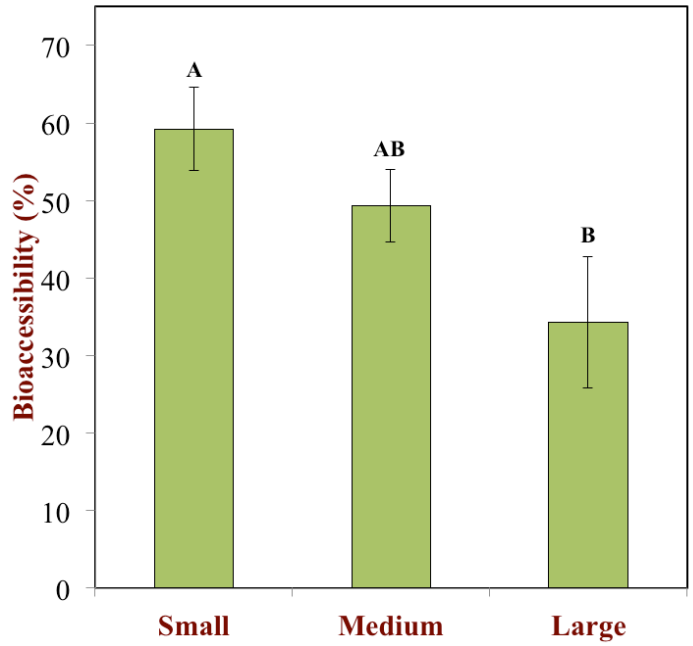

Figure 6. Influence of emulsion droplet diameter (Small, Medium, Large) on the bioaccessibility (%) of β-carotene after *in vitro* digestion. Different capital letters mean significant differences ($p<0.05$) on the digestibility of emulsions with different droplet diameters.

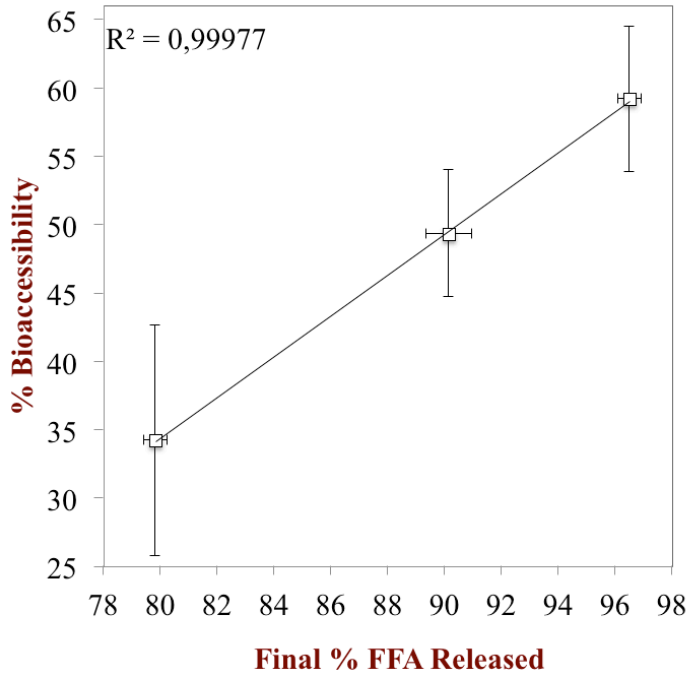

Figure 7. Correlation between the amount (%) of free fatty acids released after the *in vitro* digestion of emulsions with different droplet diameters (Small, Medium and Large) and the bioaccessibility (%) of β-carotene.

The absorption of dietary carotenoids from foods involves several steps, starting with the release of carotenoids from the food matrix, followed by incorporation into lipid droplets under gastric emulsions, and then solubilization within mixed micelles under intestinal conditions (Yonekura & Nagao, 2007). For this reason, β-carotene encapsulated within emulsified lipid phases tends to be more bioaccessible than β-carotene present within natural fruits and vegetables (Van Het Hof, West, Weststrate & Hautvast, 2000). Our results also suggest that the size of the fat droplets containing the β-carotene plays an important role in determining their bioaccessibility: decreasing the droplet size, increases the bioavailability. A recent study on soybean oil-in-water emulsions stabilized by decaglycerol monolaurate also reported that the bioaccessibility of β-carotene increased with decreasing droplet size after *in vitro* digestion (Wang *et al* 2012). The same authors found that bile and lipase played an important role in increasing the bioaccessibility of β-carotene encapsulated within emulsions containing relatively large droplets (d > 600 nm), but was less important for β-carotene in nanoemulsions with very small sizes (d < 70 nm). Presumably, the very small droplets in the nanoemulsions remained dispersed in the micelle phase after centrifugation. It should also be noted that various other factors also contribute to the overall bioaccessibility of carotenoids, such as oil type and concentration, since these factors influence the solubilization capacity of the micelle phase (Castenmiller & West, 1998; Cheng, Decker, Xiao and McClements, 2012).

The electrical characteristics of the particles in the mixed micelle system were fairly similar to those in the total digesta collected after the small intestinal phase (**Figure 5**). The high negative charge on these particles can be attributed to the dominance of anionic molecules within the colloidal particles in the mixed micelle phase, *i.e.,* free fatty acids, bile salts, and phospholipids.

4 Conclusions

The present work has shown the importance of the initial size of the fat droplets in oil-in-water emulsions on their potential biological fate within a simulated gastrointestinal tract (GIT). In general, there was an increase in particle size after exposure to mouth, stomach, and small intestine conditions for all the emulsions, which can be attributed to flocculation, coalescence, and digestion phenomenon. In addition, the particles in the digesta formed after incubation of the emulsions within the GIT model were all highly

negatively charged, which can be attributed to the presence of anionic bile salts, phospholipids, and free fatty acids. Nevertheless, there were appreciable differences in the behavior of emulsions with different droplet sizes. In particular, the emulsions containing the largest droplets appeared to be highly susceptible to droplet coalescence and disruption within different regions of the GIT, whereas those containing smaller droplets appeared to be more prone to flocculation. In addition, lipid digestion rate (expressed as FFAs released per unit time) increased with decreasing droplet size, but the normalized digestion rate (expressed as FFAs released per unit *surface area* per unit time) actually decreased. This effect was attributed to the fact that the amount of lipase present per unit surface area actually decreased with decreasing droplet size. Finally, β-carotene bioaccessibility decreased appreciably as the initial droplet size increased, which may be related to the fact that there were more undigested oil and less lipid digestion products present for large droplets. These results provide valuable information about the influence of emulsion characteristics on the bioaccessibility of lipophilic compounds, which can be used to optimize the formulation of effective delivery systems for bioactive lipids.

Acknowledgments

This material is based upon work supported by the Massachusetts Agricultural Experiment Station, a USDA/NRI/AFRI Grant, and a USDA/NSF/NIH Grant. Laura Salvia-Trujillo thanks the Ministry of Science and Education (Spain) for the pre-doctoral grant. Prof. Olga Martín-Belloso thanks the Institució Catalana de Recerca i Estudis Avançats (ICREA) for an Academia 2008 Award.

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PUBLICATIONS

Chapter VIII

Modulating β-carotene bio-accessibility by controlling oil composition and concentration in edible nanoemulsions

Laura Salvia-Trujillo, Chang Qian, Olga Martín-Belloso, D. Julian McClements

Food Chemistry 139 (1-4), 878-884

Abstract

Diets rich in carotenoids have been correlated to a reduced risk of developing certain types of chronic diseases, but these bioactive components have low intestinal absorption due to their hydrophobic nature. The aim of this work was to study the effect of carrier oil composition (medium-chain triglyceride (MCT) to long-chain triglyceride (LCT) ratio) and total carrier oil concentration (1% or 4% w/w) on the physical stability, lipid digestibility, and bioaccessibility of β-carotene-loaded nanoemulsions using a simulated digestion process. Lipolysis led to an appreciable increase in the size and negative charge on the particles in the system. The total fraction of triacylglycerols converted to free fatty acids decreased as the percentage of LCT within the lipid phase increased, particularly for the nanoemulsions with higher fat contents. There was an increase in b-carotene bioaccessibility as the LCT within the lipid phase increased for low fat nanoemulsions, which was attributed to the increase solubilization capacity of mixed micelles formed by LCT. β-carotene bioaccessibility showed a complex relationship on LCT content for high fat nanoemulsions, due to the opposing effects of lipid digestion and micelle solubilization. These results may facilitate the optimization of delivery systems for lipophilic bioactive compounds for food or pharmaceutical applications.

Keywords

β-carotene, nanoemulsions, oil type, LCT, MCT, fat content, digestibility, bioaccessibility

Table of contents

1 Introduction

β-carotene is one of the major carotenoids present in fruit and vegetables that exhibits pro-vitamin A activity once ingested by humans (Johnson, 2002). It is recognized as playing an important role in the prevention of certain human diseases due to its antioxidant and non-antioxidant activities (Bendich & Olson, 1989, Rao & Rao, 2007). The ingestion of sufficient levels of β-carotene has been related to a reduced risk of developing certain types of cancer, cardiovascular diseases, photosensitivity diseases, and cataracts (Mayne, 1996). Therefore, there is a growing interest among consumers and the food industry to incorporate this compound into functional foods and beverages. The oral bioavailability of highly lipophilic compounds, such as carotenoids, plays a major role in determining their overall biological activity. Studies have shown that the absorption of ingested dietary carotenoids may be greatly improved when they are consumed in the presence of lipids (Maiani et al., 2009, Yeum & Russell, 2002). Indeed, improvements in the oral bioavailabilities of poorly water-soluble drugs when they are co-administered with lipid excipients are well-established in the pharmaceutical industry (Dahan & Hoffman, 2008, Hauss, 2007, Porter, Pouton, Cuine & Charman, 2008, Pouton & Porter, 2008). Knowledge of the physicochemical and physiological processes that occur within the gastrointestinal tract after ingestion of dietary lipids can be used to design effective delivery systems to improve the oral bioavailability of bioactive compounds in foods (McClements *et al* 2009, Singh *et al* 2009). In many emulsified foods, carotenoids are initially present within triglyceride droplets dispersed within an aqueous medium (Rigotti, 2007). As these foods pass through the digestive tract the triglyceride molecules are digested due to the actions of gastric and pancreatic lipases, leading to the formation of free fatty acids and monoacylglycerols. These lipid digestion products may remain at the fat droplet surfaces, or they may move into the surrounding aqueous phase and form colloidal structures with bile salts and phospholipids ("mixed micelles") capable of solubilizing lipophilic molecules. As a result of these processes, carotenoid molecules may move from the original oil droplets into the mixed micelle phase formed by digestion. After solubilization, carotenoids are transported to the intestinal cells, where they are absorbed and then packed into chylomicrons that are secreted into the lymphatic system (Furr & Clark, 1997, Yonekura & Nagao, 2007, Rigotti 2007).

The bioaccessibility and bioavailability of carotenoids depend on several factors, including the type and amount of carotenoids consumed, the composition and structure of the food matrix, and the nature of the processing conditions used (Castenmiller & West, 1998; Reboul et al 2006). Many studies have shown that the total amount of dietary fat consumed plays an important role in carotenoid absorption, with the bioavailability normally increasing with increasing fat content (Dimitrov et al., 1988, Jayarajan, Reddy & Mohanram, 1980, Prince, Frisoli, Goetschkes, Stringham & LaMuraglia, 1991). There is also strong evidence that the nature of the triacylglycerol molecules present in a food influences carotenoid bioavailability (Borel et al., 1998; Huo, Ferruzzi, Schwartz & Failla, 2007). Recent studies have shown that the shorter the fatty acid chains in triglyceride molecules, the lower the bioaccessibility of carotenoids (Huo, Ferruzzi, Schwartz & Failla, 2007).

Medium-chain triglycerides (MCT) are being used as a fat source in several food and beverage products rather than long-chain triglycerides (LCT) because they may lower cholesterol secretion, cardiovascular disease risk, and weight gain (Bach, Ingenbleek & Frey, 1996, Marten, Pfeuffer & Schrezenmeir, 2006). However, the potential influence of the fatty acid chain length of co-ingested fats on the oral bioavailability of lipophilic bioactive molecules (such as carotenoids) may have some undesirable consequences. A greater understanding of the interaction between lipophilic bioactive compounds and co-administered carrier oils during pre-absorptive intraluminal processing may lead to a more rational selection of the optimum carrier lipids for incorporation into lipid-based delivery systems (Sek, Porter, Kaukonen & Charman, 2002).

Recently, the interest in developing lipid-based delivery systems to incorporate carotenoids into functional foods has increased (Boon, McClements, Weiss & Decker, 2010; Nik, Langmaid, & Wright, 2012; Nik, Corredig & Wright, 2011; Nik, Wright, & Corredig, 2011). Nanoemulsions are emerging as a strong alternative to conventional emulsions to protect and deliver lipophilic functional components since they present enhanced physical stability, improved optical clarity, and increased bioaccessibility (McClements, 2011; Nik et al., 2012). Indeed, recent studies indicated that nanoemulsions may improve the bioaccessibility of lipophilic bioactive compounds such as β-carotene (Wang, Liu, Mei, Nakajima & Yin, 2012; Nik et al., 2011a,b, 2012).

The purpose of the present work was to study the potential biological fate of β-carotene-loaded nanoemulsions using simulated gastrointestinal conditions (Li & McClements, 2010, Li, Hu & McClements, 2011). In particular, our object was to determine the

influence of carrier lipid concentration and type (MCT and LCT) on triglyceride digestibility and β-carotene bioaccessibility.

2 Material and methods

2.1 Materials

β-carotene, lipase (porcine pancreas, type II, L3126, Batch # 096K0747), bile extract and calcium chloride were purchased from Sigma-Aldrich (St. Louis, MO). Miglyol 812 (MCT) and corn oil (LCT) were purchased from SASOL (Houston, TX) and from a local supermarket, respectively. All aqueous solutions were prepared using purified water from a Mili-Q filtration system.

2.2 Methods

2.2.1 Nanoemulsion formation

An oil phase was prepared by dispersing 0.5% (w/w) β-carotene into carrier lipid (MCT or LCT) and then sonicating for 1 minute and applying mild heating (< 50ºC for 5 min) so that complete dissolution was achieved. An aqueous phase containing surfactant was prepared by dispersing 1.5% (w/w) Tween 20 in aqueous buffer solution (10.0 mM phosphate buffer, 0.01% (w/w) sodium azide, pH 7.0). A coarse emulsion was prepared by blending the oil and aqueous phases together using a high-shear mixer at 10000 rpm for 2 min. Nanoemulsions were then obtained by passing the coarse emulsion three-times through a microfluidizer (Model 101, Microfluidics, Newton, MA) working at 9,000 psi. For some experiments, nanoemulsions were prepared from carrier lipids that consisted of different mass ratios of MCT-to-LCT (w/w): 100:0, 75:25, 50:50, 25:75 or 0:100. Nanoemulsions with "low-fat" contents were prepared using 1% (w/w) oil phase and 99% (w/w) aqueous phase. Nanoemulsions with "high-fat" contents were prepared using 4% (w/w) oil phase and 96% (w/w) aqueous phase.

2.2.2 Nanoemulsion characterization

The mean particle charge (ζ-potential) and size (mean volume diameter) of initial nanoemulsions, raw digesta, and mixed micelles were measured.

The particle size distribution was measured by dynamic light scattering (DLS) (Zetasizer NanoZS, Malvern Instruments Ltd, Worcestershire, UK) at a wavelength of 633 nm and temperature of 25 ºC using a backscatter detector (173 º). Samples were

diluted prior to analysis with buffer solution (1:10) to avoid multiple scattering effects and to achieve an attenuation of the laser beam between 5 and 10.

The ζ-potential of the particles was measured by phase-analysis light scattering (Zetasizer NanoZS, Malvern Instruments Ltd, Worcestershire, UK). Samples were diluted with buffer solution (1:10) and placed in a capillary cell equipped with two electrodes to assess the electrophoretic mobility of the particles.

2.2.3 *In vitro* digestion

The digestion of the lipid droplets under simulated small intestinal conditions was monitored using a pH-stat device (Metrohm USA, Riverview, FL) (McClements & Li, 2010). An aliquot of 30 mL of the nanoemulsions was placed in a temperature-controlled (37 ºC) chamber and the pH was set at 7.0 using NaOH solution. Then, 4 mL of bile extract (46.87 mg/mL) and 1 mL of calcium chloride (110 mg/mL) solutions dissolved in phosphate buffer were added to the sample and the pH was adjusted to 7.0 if necessary. 2.5 mL of freshly prepared lipase suspension (24 mg/mL) dissolved in phosphate buffer was then added to the mixture. The pH of the mixture was monitored and the volume of NaOH (mL) necessary to neutralize the free fatty acids (FFA) released from lipid digestion so as to maintain the pH at 7.0 was recorded during 2 h. The concentration of NaOH used was 0.25 M and 1 M for the emulsions containing 1% (w/w) and 4% (w/w) of oil phase, respectively. The following equation was used to calculate the percentage of FFAs released during the digestion process, assuming that each molecule of triglyceride (TAG) generates two molecules of FFA when completely digested (Mu & Høy, 2004):

$$FFA(t) = 100 \times \left(\frac{V_{NaOH}(t) \cdot C_{NaOH} \cdot M_{Oil}}{2 \cdot m_{Oil}} \right)$$

where $V_{NaOH}(t)$ is the volume of NaOH solution required to neutralize the FFAs produced at digestion time t (L), C_{NaOH} is the molarity of the NaOH solution used to titrate the sample (mol L^{-1}), M_{oil} is the molecular weight of the oil (g mol^{-1}), and m_{oil} is the total mass of oil initially present in the incubation cell (g). The molecular weights of the corn oil and the MCT oil were taken to be 800 g mol^{-1} and 400 g mol^{-1}, respectively. The molecular weight of the oil phase in the mixed emulsions was calculated proportionally to the mass ratio of the two oils in the blend.

Blanks were carried out in the absence of oil in the samples and subtracted from the reported values.

2.2.4 Bioaccessibility determination

The bioaccessibility of β-carotene in the nanoemulsions was determined after the *in vitro* digestion process using a method described previously (Qian, Decker, Xiao & McClements, 2012). An aliquot of the raw digesta was centrifuged (CL10 centrifuge, Thermo Scientific) at 4000 rpm for 40 min at 25 ºC. The supernatant was collected and was taken to be the "micelle fraction" in which the bioactive compound was solubilized. In some samples, a top layer of tiny droplets of non-digested oil was observed and it was completely removed from the micelle fraction prior to analysis with the aid of a glass pipette. The micelle phase was then collected using another glass pipette. Aliquots of 5 mL of raw digesta or micelle fraction were mixed with 5 mL of chloroform, vortexed and centrifuged at 1750 rpm for 10 min at 25 ºC. The bottom layer, with the solubilized β-carotene, was collected while the top layer was mixed with 5 mL of chloroform and the same procedure was followed. The bottom chloroform layer was added to the previous one and analyzed spectrophotometrically (Ultrospec 3000 pro, GE Health Sciences, USA) at 450 nm. A cuvette containing pure chloroform was used as a reference cell.

The concentration of β-carotene extracted from a sample was determined from a previously prepared calibration curve of absorbance *versus* β-carotene concentration in chloroform. The bioaccessibility was calculated using the following equation:

$$Bioaccessibility = 100 \times \left(\frac{C_{Micelle}}{C_{RawDigesta}} \right)$$

where $C_{micelle}$ and $C_{RawDigesta}$ are the concentration of β-carotene in the micelle fraction and in the overall sample (raw digesta) after the pH-stat experiment, respectively.

2.2.5 Statistical analysis

All experiments were assayed in duplicate, and results were expressed as the mean and the standard deviation. JMP 8 Macintosh Package (SAS Institute Inc.) was used to perform the analysis of variance. The Student's t test was run to determine significant differences at a 5% significance level ($p < 0.05$).

3 Results and discussion

3.1 Influence of oil composition on particle characteristics

Initially, we examined the influence of oil phase composition on the physical properties of the lipid nanoparticles in the "low-fat" nanoemulsions. The mean volume diameter of the particles was measured in the initial nanoemulsions, in the overall digesta after lipid digestion, and in the micelle fraction collected from the digesta (**Figure 1)**. The mean diameter of the initial nanoemulsions increased from around 146 to 415 nm as the LCT content in the mixed oil phase (MCT:LCT) increased from 0 to 100% (w/w). The reason for this increase in droplet size can be attributed to changes in the dispersed phase viscosity of the nanoemulsions (McClements, 2005). The efficiency of droplet disruption within a high pressure homogenizer usually increases as the viscosity of the disperse phase decreases. LCT has an appreciably higher viscosity than MCT, and therefore droplet breakup during homogenization becomes less efficient as the LCT fraction increases, leading to larger droplets.

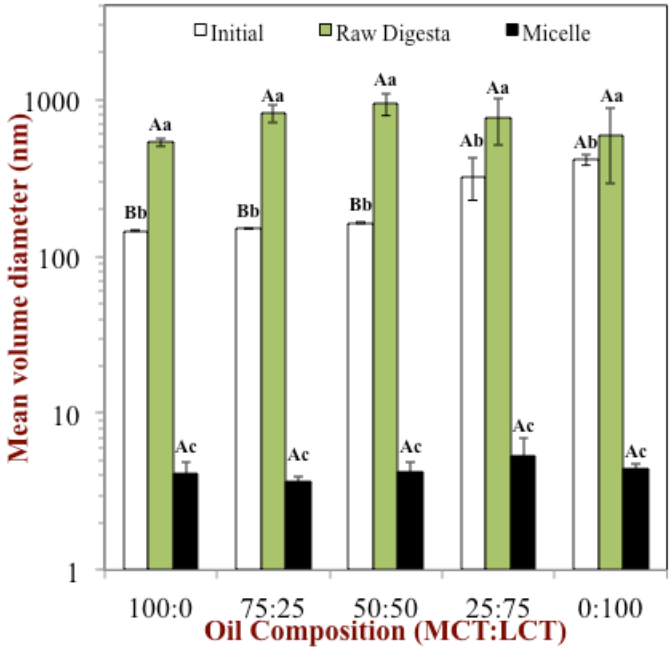

Figure 1. Volume mean diameter (nm) of initial nanoemulsions (white) with low fat content (1% w/w) formulated with different MCT:LCT ratio, after *in vitro* digestion (grey), and their respective micellar phase (black). Different capital letters mean significant differences ($p<0.05$) on the droplet diameter between emulsions with different MCT:LCT ratio within in the same digestion phase. Different lowercase letters mean significant differences ($p<0.05$) on the droplet diameter between digestion phases within the same MCT:LCT mixture.

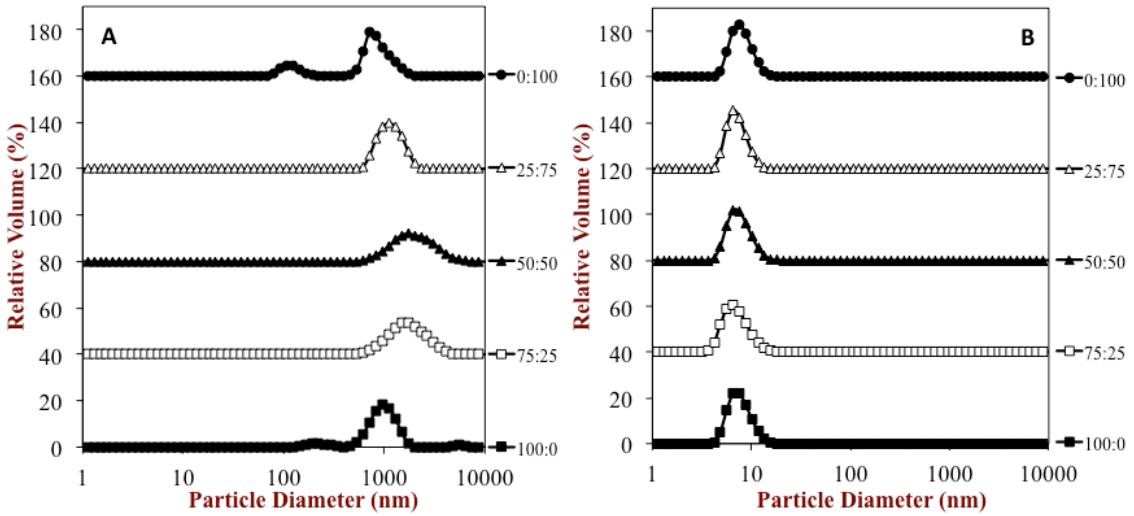

Figure 2. Effect of MCT:LCT ratio on the droplet size distribution of nanoemulsions with low fat content (1% w/w) after *in vitro* digestion (A) and of the micellar phase (B).

The mean particle size measured after the nanoemulsions had been subjected to *in vitro* digestion increased appreciably regardless of the initial oil phase composition (**Figure 1**). The droplet size distribution of the digesta samples was monomodal for all oil phase compositions with the exception of the system formulated with 100 % LCT, which was bimodal (**Figure 2a**). The increase in droplet size after simulated digestion conditions may be due to flocculation and/or coalescence phenomena depending on the nature of the system (Mun, Decker & McClements, 2007). These destabilization mechanisms may be promoted by changes in the composition and properties of the lipid droplet surfaces within the gastrointestinal tract, *e.g.* due to displacement or digestion of the original emulsifiers (Gargouri, Julien, Bois, Verger & Sarda, 1983, Singh, Ye & Horne, 2009). Moreover, the lipase activity at the oil droplet surface may also contribute to changes in interfacial properties leading to a decrease in coalescence stability (Reis, Holmberg, Watzke, Leser & Miller, 2009). It is known that complex association colloids, such as mixed micelles, vesicles and lamellar structures, are formed in the digestion medium as a result of the interactions of lipid digestion products, bile salts, phospholipids, and calcium (Kossena, Boyd, Porter & Charman, 2003). In this study, we found that the particles in the micelle phase had a monomodal distribution (**Figure 2b**) with a volume mean diameter < 10 nm (**Figure 1**), which suggests that they were predominantly mixed micelles. The particle size of the mixed micelles did not depend strongly on the initial oil phase composition (**Figures 1 and 2b**). Visually, the micelle fraction of all the samples was almost transparent, which can be attributed to weak light

scattering by the small particles present. Recent studies using curcumin-loaded nanoemulsions also found that the size of the particles in the micelle fraction (10-1000 nm) did not depend strongly on the initial oil phase composition (MCT or LCT) (Ahmed, Li, McClements & Xiao, 2012). On the other hand, other researchers have reported a slight increase in micelle size (from 3 to 7 nm) as the chain length of the fatty acids in the lipid phase increased (Christensen, Schultz, Mollgaard, Kristensen & Mullertz, 2004).

The ζ-potentials of the particles in the initial nanoemulsions, the overall digesta, and the micelle fraction were also measured (**Figure 3**). The negative charge of the initial nanoemulsions increased slightly with increasing LCT content in the oil phase, with the values going from -4.7 to -5.8 mV. Even though the initial nanoemulsions were stabilized by a non-ionic surfactant (Tween 20) it has been reported that they can give a negative charge to oil droplets, *e.g.,* due to preferential adsorption of hydroxyl ions from the aqueous phase or due to the presence of anionic impurities such as free fatty acids in the surfactant or oil phases (McClements, 2005). It is possible that the LCT contained more anionic impurities than the MCT, which meant that the droplets were slightly more negatively charged.

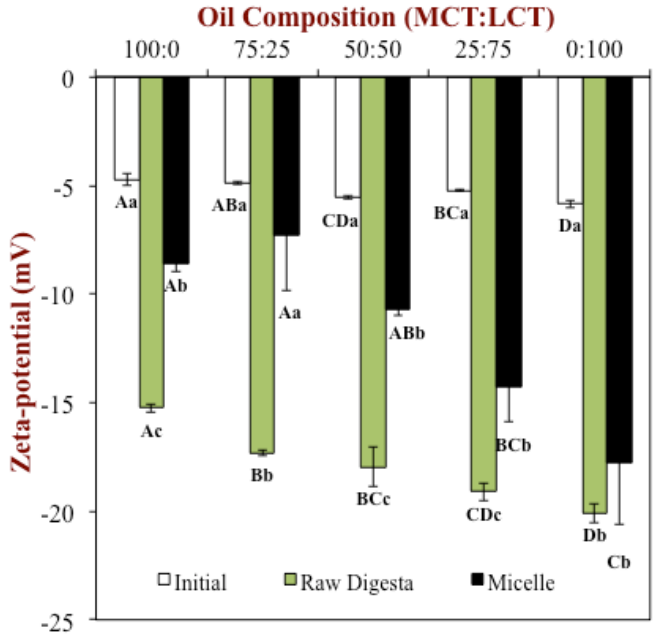

Figure 3. ζ-potential (mV) of initial nanoemulsions (white) with low fat content (1% w/w) formulated with different MCT:LCT ratio, after *in vitro* digestion (grey), and their respective micellar phase (black). Different capital letters mean significant differences ($p<0.05$) on the droplet diameter between emulsions with different MCT:LCT ratio within in the same digestion phase. Different lowercase letters mean significant differences ($p<0.05$) on the droplet diameter between digestion phases within the same MCT:LCT mixture.

The particles in the digesta (-15 and -20 mV) had an appreciably higher negative charge than those in the initial nanoemulsions (-4 and -5 mV). The magnitude of the negative charge on the particles in the digesta and in the micelle phase increased appreciably as the fraction of LCT in the initial oil phase increased (**Figure 3**). The higher negative charge on the particles in those samples containing a higher amount of LCT might be due to several reasons. When lipid droplets are exposed to simulated intestinal conditions anionic bile salts may displace the original surfactant molecules from their surfaces thereby altering their surface charge (Wickham, Garrood, Leney, Wilson & Fillery-Travis, 1998). In addition, the hydrolysis of triglycerides by pancreatic lipase at the surface of the lipid droplets, leads to the release of anionic free fatty acids, which also alters the surface charge (Singh, Ye & Horne, 2009; McClements et al., 2009). It is known that medium chain fatty acids produced during digestion of MCT are able to rapidly migrate into the surrounding aqueous phase whereas long chain fatty acids produced by LCT tend to accumulate at the oil-water interface (Pouton & Porter, 2008; Ahmed, Li, McClements & Xiao, 2012, Li, Hu & McClements, 2011). Consequently, more anionic fatty acids may have remained at the particle surfaces for the systems containing LCT than those containing MCT.

3.2 Influence of oil composition on triglyceride digestibility

In this section, we examined the influence of initial oil composition (MCT:LCT) on the rate and extent of triglyceride digestion in the nanoemulsions with a low fat content (1% w/w) using the pH-stat method (**Figure 4**). For all nanoemulsions studied, the amount of free fatty acids (FFA) liberated increased steeply during the first 40 minutes after adding pancreatic lipase to the digestion medium. The amount of FFAs released then increased more slowly after 40 min of digestion. The total amount of FFAs produced after 2 hours of digestion decreased as the fraction of LCT in the initial oil phase increased. For example, the total amount of FFA released decreased from 123 to 88% as the LCT concentration increased from 0 to 100% (**Figure 4**). There are a number of reasons that the final amount of FFA released was greater than 100% for the MCT systems. First, there might have been some conversion of monoacylglycerols into glycerol and FFAs (Carey, Small & Bliss, 1983), which was not taken into account in the calculations. Second, there may have been other components in the systems that were hydrolyzed and contributed to the pH stat measurements, such as phospholipids,

proteins, or surfactants in the digestion medium. It is known that medium chain fatty acids are able to migrate rapidly to the surrounding aqueous phase whereas long chain fatty acids tend to accumulate at the oil-water interface and therefore inhibit lipase activity, which would account for the observed decrease in digestibility at high LCT levels (Dahan & Hoffman, 2008, Sek, Porter, Kaukonen & Charman, 2002). Our results are in agreement with other recent studies that have reported a higher digestibility of MCT compared to LCT when they are incorporated in emulsions or nanoemulsions (Ahmed, Li, McClements & Xiao, 2012; Li, Hu & McClements, 2011; Li & McClements, 2010).

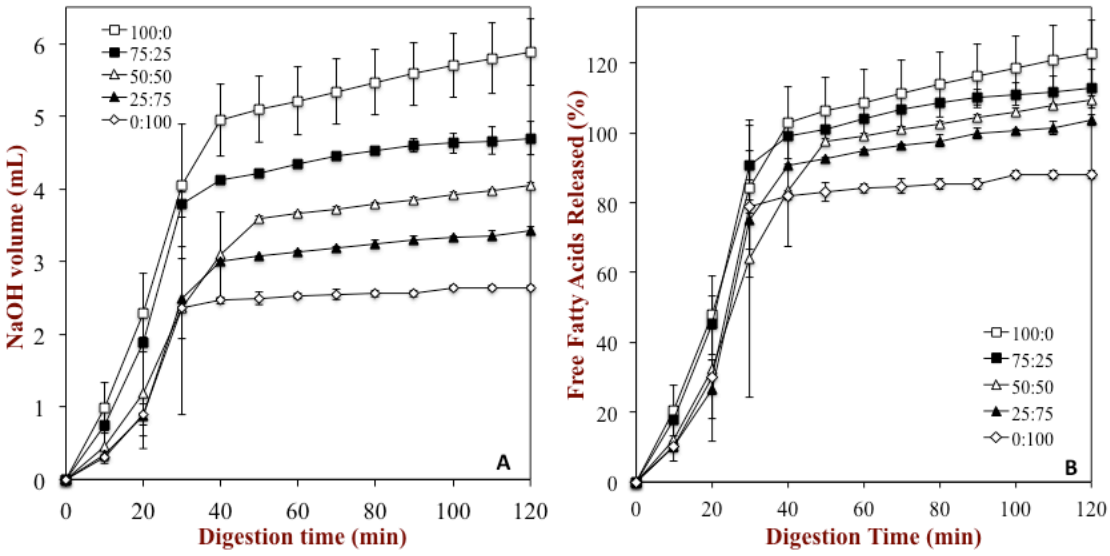

Figure 4. Influence of MCT:LCT ratio on the NaOH (0.25 M) volume (mL) used during the pH-stat titration curves (120 min) of nanoemulsions with low fat content (1% w/w) to maintain the pH constant to 7 (A) and the calculated percentage of free fatty acids (FFA %) released (B).

3.3 Influence of oil composition on β-carotene bioaccessibility

The influence of oil phase composition on β-carotene bioaccessibility in the low fat (1% w/w) nanoemulsions was determined by measuring the β-carotene concentrations in the micelle phase and in the overall digesta (**Figure 5**). As a comparison, the percentage of FFAs released by the end of the lipid digestion step is plotted on the same figure. A significant increase in β-carotene bioaccessibility was observed as the fraction of LCT in the initial oil phase increased. For example, the bioaccessibility of β-carotene increased from around 14 to 86 % as the LCT percentage in the oil phase increased from 0 to 100%.

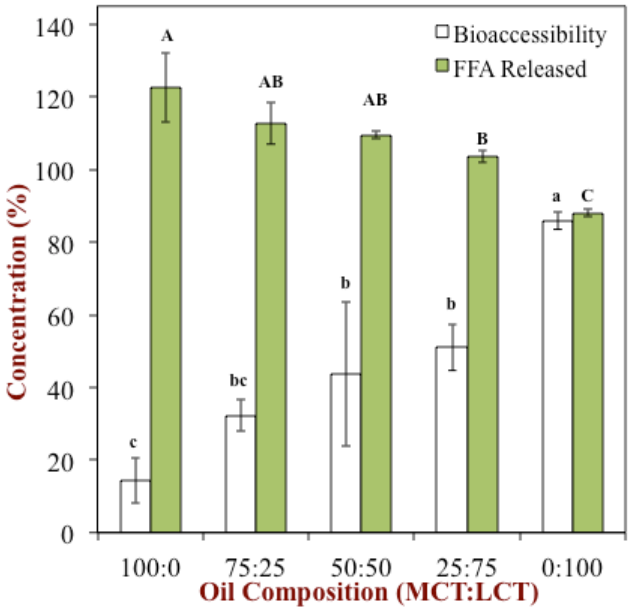

Figure 5. Effect of MCT:LCT ratio on the percentage of free fatty acid released (FFA %) after 120 min of digestion (grey) and on the bioaccessibility (%) of β-carotene incorporated in nanoemulsions (white) with low fat content (1% w/w). Different capital letters mean significant differences ($p<0.05$) on the percentage of free fatty acid release after 120 min. Different lowercase letters mean significant differences ($p<0.05$) on the bioaccessibility of β-carotene.

The absorption of dietary carotenoids in the human body has been reported to be similar to that of triglycerides (Rigotti, 2007). Carotenoids incorporated within oil droplets are transferred into mixed micelles formed by bile salts and lipolysis products after the triglycerides are digested (Yonekura & Nagao, 2007). Our study using nanoemulsion-based delivery systems therefore supports previous work that has shown that the presence of fat is essential for carotenoid solubilization and absorption (Furr & Clark, 1997), and that the amount of lipophilic compounds solubilized in mixed micelles tends to increase with increasing fatty acid chain length (Christensen, Schultz, Mollgaard, Kristensen & Mullertz, 2004). This latter effect may be related to the fact that the solubilization capacity of a mixed micelle depends on how easily a lipophilic compound can be incorporated into its structure (Laher & Barrowman, 1983). One would expect that relatively long chain non-polar molecules like β-carotene would be more easily accommodated into mixed micelles formed by long chain fatty acids than those formed by medium chain fatty acids. Having said this, our dynamic light scattering measurements suggested that the mixed micelles formed by digestion of different kinds of triglycerides were fairly similar in dimensions (**Figure 1**). This may have been due to limitations of the dynamic light scattering method when used to analyze a system that

contains various kinds of colloidal structures, such as mixed micelles, vesicles, and lamellar structures. Alternatively, the colloidal structures that solubilized the β-carotene may have been different in systems containing different types of oil phases. Studies with pharmaceutical preparations have shown that hydrophobic drugs administered with LCT are solubilized within mixed micelles after digestion, whereas those administered with MCT are trapped within vesicles (Kossena, Boyd, Porter & Charman, 2003). It is therefore possible that some or all of the relatively large vesicles in the digesta were removed during the centrifugation process, thereby reducing the amount of β-carotene measured in the micelle phase. Other recent studies in our laboratory have reported a lower bioaccessibility of lipophilic bioactive compounds encapsulated within MCT oils than in LCT oils (Ahmed, Li, McClements & Xiao, 2012, Qian, Decker, Xiao & McClements, 2012).

3.4 Effect of high fat content on digestion and bioaccessibility

Finally, we examined the influence of a relatively high initial fat content on triglyceride digestibility and β-carotene bioaccessibility. This was achieved by using nanoemulsions containing 4% (w/w) total oil phase in the digestion medium, rather than 1% (w/w). This study was carried out because the total amount of fat present in the human gastrointestinal tract may change depending on the type and amount of food consumed. In addition, this study also highlights the importance of using an appropriate fat level in *in vitro* digestion studies.

The overall changes in particle size and charge for the high-fat nanoemulsions were similar to those for the low-fat nanoemulsions (data not shown). The rate and extent of free fatty acid release was much more dependent on the initial oil phase composition for the systems containing high fat contents (**Figure 6**) than those containing low fat contents (**Figure 4**). For example, the final percentage of FFAs released after two hours of digestion decreased from 89 to 34% for the high fat system and from 123 to 88% for the low fat system as the LCT content in the oil phase increased from 0 to 100%. Overall, the fraction of FFAs released from the triglycerides was considerably lower for the high-fat nanoemulsions than the low-fat ones, which has also been reported by other authors (Li, Hu & McClements, 2011). This phenomenon might occur due to several reasons associated with the fact that the composition of the simulated small intestinal fluids was kept constant as the fat content was increased. First, the ratio of lipase-to-

triglyceride in the digestion medium decreases as the total fat content increased, and so the enzyme activity of the lipase may have decreased (Li, Hu & McClements, 2011). Second, the ratio of bile salts-to-triglyceride digestion products decreased, and so there may not have been sufficient mixed micelles formed to solubilize all of the FFAs released from the high fat systems (Li, Hu & McClements, 2011). Third, the ratio of calcium-to-triglyceride digestion products also decreased and so there may not have been sufficient calcium ions present to precipitate long chain fatty acids generated at the droplet surfaces (Li, Hu & McClements, 2011). The removal of FFAs from lipid droplet surfaces during digestion by solubilization in bile salts or precipitation by calcium ions is known to play an important role in promoting full hydrolysis of triglycerides (Dahan & Hoffman, 2008, Pouton & Porter, 2008). It should be noted that even though the *fraction* of FFAs released was lower in the high fat systems, the absolute amount of FFAs released would be higher because of the higher initial concentration of triglycerides present.

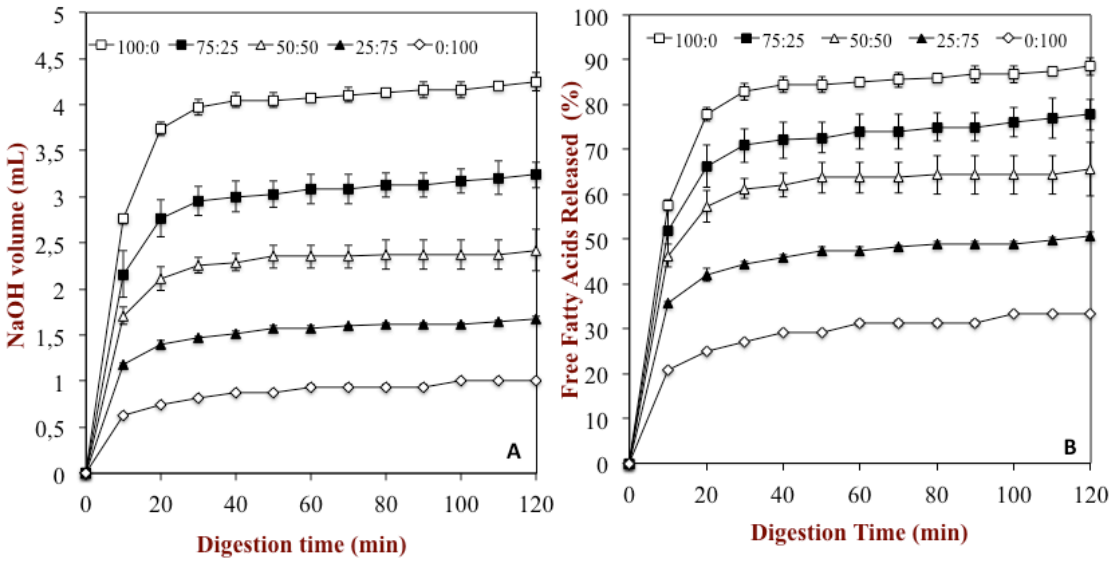

Figure 6. Influence of MCT:LCT ratio on the NaOH (1 M) volume (mL) used during the pH-stat titration curves (120 min) of nanoemulsions with a high fat content (4% w/w) to maintain the pH constant to 7 (A) and the calculated percentage of free fatty acids (FFA %) released (B).

The influence of oil phase composition on β-carotene bioaccessibility and the final amount of FFAs released after digestion is compared in **Figure 7** for the high fat systems. The behavior of the high fat system was quite different from that of the low fat system (**Figure 5**). The total amount of FFAs released decreased appreciably with

increasing LCT in the lipid phase. As mentioned earlier, this effect may have been due to the lack of sufficient amounts of bile salts and calcium ions in the simulated small intestinal fluids to solubilize and precipitate the long chain FFAs generated at the droplet surfaces during lipid digestion. For the pure MCT system, the bioaccessibility of the carotenoid was relatively high (84%) in the high fat system (**Figure 7**) whereas it was relatively low (14%) in the low fat system (**Figure 5**). This effect may be attributed to the fact that a greater absolute amount of mixed micelles and other association colloids capable of solubilizing β-carotene were formed after the high fat systems were digested. However this cannot be the only reason since the bioaccessibility increased six-fold (from 14 to 84%) when the total initial fat content increased four-fold (from 1 to 4%). There may therefore have also been a change in the structure of the association colloids formed in the micelle phase after digestion of the high fat systems, which altered their solubilization capacities.

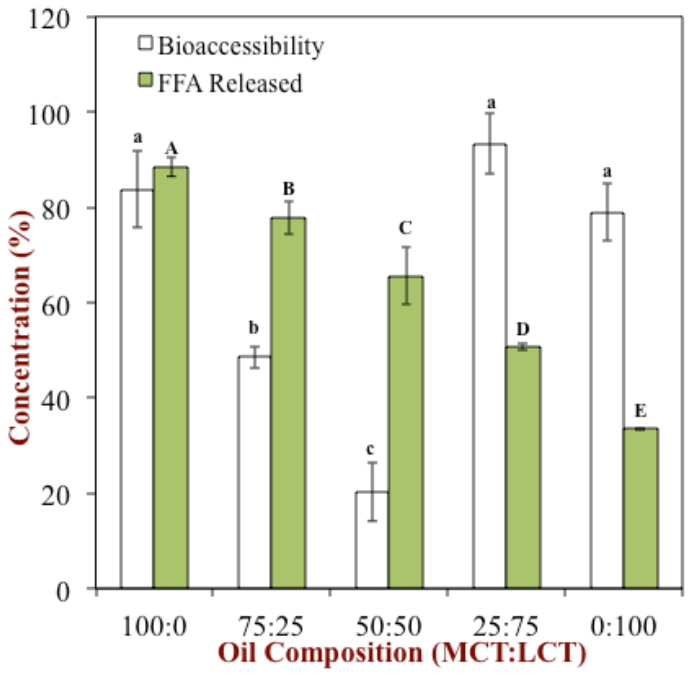

Figure 7. Influence of MCT:LCT ratio on the percentage of free fatty acid released (FFA %) after 120 min of digestion (grey) and on the bioaccessibility (%) of β-carotene incorporated in nanoemulsions (white) with a high fat content (4% w/w). Different capital letters mean significant differences ($p<0.05$) on the percentage of free fatty acid release after 120 min. Different lowercase letters mean significant differences ($p<0.05$) on the bioaccessibility of β-carotene.

In the high fat systems, β-carotene bioaccessibility decreased as the LCT content in the oil phase increased from 0 to 50%, but then it increased when the LCT content was increased from 50 to 100% (**Figure 7**). This behavior differs from the progressive

increase in β-carotene bioaccessibility observed in the low fat systems with increasing LCT content (**Figure 5**). We attribute the relatively complex behavior observed in the high fat systems to a number of competing physicochemical phenomena. First, the total amount of non-digested triglycerides in the system increases as the LCT content increases, which may inhibit the incorporation of β-carotene into mixed micelles because carotenoids were trapped within the non-digested oil droplets (Nik et al, 2011a). This effect would lead to a decrease in bioaccessibility with increasing LCT content, and may account for the decrease observed between 0 and 50% LCT (**Figure 7**). Second, the solubilization capacity of the mixed micelles increases as the LCT content within them increases (Section 3.3). This effect would lead to an increase in bioaccessibility with increasing LCT content, and may account for the increase observed between 50 and 100% LCT (**Figure 7**). The fact that the β-carotene bioaccessibility was relatively high (≈79%) in the pure LCT system containing a high fat content, even though an appreciable amount of the triglycerides had not been digested (≈ 67%) suggests that carotenoid molecules were able to move from the non-digested oil phase and into the micelle phase. Presumably, this occurred because of the relatively high absolute concentration and solubilization capacity of the mixed micelles formed at high LCT levels.

These experiments highlight the importance of using appropriate levels of fat in simulated digestion studies, and also highlight the potential importance of non-digested fat on the bioavailability of lipophilic substances. However, it should be noted that in practice, the human body can respond to higher fat contents by slowing down the emptying of the stomach, or by secreting more bile salts and lipase into the small intestine, and therefore these results should be treated with some reservations.

4 Conclusions

We have shown that a bioactive carotenoid (β-carotene) can successfully be incorporated into food-grade nanoemulsions with different oil phase compositions. Nanoemulsions containing different ratios of MCT and LCT in the oil phase behaved fairly similarly in a simulated digestion model: there was an appreciable increase in mean size and negative charge of the particles after digestion. Nevertheless, there were some differences in the physical characteristics of the particles produced by digestion depending on initial oil composition. The negative charge of the particles in the overall

digesta and in the mixed micelle phase increased as the LCT content increased, which was attributed to the accumulation of long-chain fatty acids at the particle surfaces.

The overall extent of triglyceride hydrolysis after digestion was also influenced by oil composition, decreasing with increasing LCT in the lipid phase for both low-fat (1%) and high-fat (4%) nanoemulsions. The influence of oil composition on β-carotene bioaccessibility depended on the initial total fat content. For low-fat nanoemulsions, the bioaccessibility progressively increased with increasing LCT content, which was attributed to the greater solubilization capacity of mixed micelles containing long chain fatty acids. For high-fat nanoemulsions, the bioaccessibility decreased and then increased with increasing LCT content, which was attributed to changes in the amount of non-digested oil present, as well as in the solubilization capacity of the micelle phase.

Overall, this study provides valuable information that can be used to design nutraceutical delivery systems that optimize the bioaccessibility of lipophilic compounds. For example, a high β-carotene bioaccessibility can be achieved either by using low-fat LCT nanoemulsions or high-fat MCT nanoemulsions. The bioaccessibility in mixed oil nanoemulsions appears to be highly dependent on oil composition. Further studies would be useful to better understand the various physicochemical mechanisms that determine triglyceride digestion and the release of bioactive compounds form mixed oil systems.

Acknowledgments

This material is based upon work supported by the Cooperative State Research, Extension, Education Service, United State Department of Agriculture, Massachusetts Agricultural Experiment Station and a United States Department of Agriculture, CREES, NRI and AFRI Grants. Laura Salvia-Trujillo thanks the Ministry of Science and Education (Spain) for the predoctoral grant. Prof. Olga Martín-Belloso thanks the Institució Catalana de Recerca i Estudis Avançats (ICREA) for the Academia 2008 Award.

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RESULTS AND DISCUSSION

Results discussion

Table of contents

1 Antimicrobial nanoemulsions

The use of natural essential oils (EOs) as antimicrobials is recently becoming popular due to consumers demand of foods free from synthetic additives. The incorporation of EOs in nanoemulsions as delivery systems in foods is arising as a promising alternative due to the beneficial properties associated to their small droplet size. In the current work, the factors affecting the physicochemical properties and antimicrobial activity of EO-loaded nanoemulsions were studied, focusing on the influence of the fabrication method (ultrasounds or microfluidization), the concentration of each component and the EO type. Eventually, nanoemulsion-based edible coatings were used as a preservative treatment to maintain the quality of fresh-cut Fuji apples.

1.1 Factors affecting nanoemulsions physicochemical characteristics

1.1.1 Formation method and processing conditions

Ultrasounds

First, the effect of ultrasound processing conditions, being sonication amplitude (μm) and time (s), on the physicochemical properties of lemongrass essential oil (LEO) nanoemulsions was assessed.

On the one hand, the average droplet size of LEO-alginate nanoemulsions decreased gradually at increasing sonication amplitude and time. At the mildest processing conditions (30 μm for 30 s) nanoemulsions were already achieved with a mean droplet diameter of 34.95 ± 8.55 nm. However, the droplet size distribution was not uniform, indicating the presence of droplets bigger than 1000 nm. Increasing the sonication amplitude up to 100 μm and treatment time to 30 s decreased the average droplet diameter to 10.67 ± 1.80 nm, but bigger droplets out of the nano range were still present. It was necessary to apply an ultrasound treatment of 100 μm for 180 s to obtain monomodal size distribution with an average droplet diameter of 4.31 ± 0.18 nm. Confirming our results, other authors observed a decrease in the droplet size of nanoemulsions obtained at higher sonication amplitudes and times (Jafari, He, & Bhandari, 2007; Mahdi Jafari, He, & Bhandari, 2006). Nonetheless, in agreement with our results, it has been described that high amplitudes and treatment times are required for droplet size reduction during emulsification processes in high-viscous media

(Canselier, Delmas, Wilhelm, & Abismaïl, 2002). On the other hand, the ζ-potential of nanoemulsions significantly decreased at increasing sonication time regardless the ultrasonic wave amplitude. The droplet electrical charge of the coarse emulsion was -18.0 ± 2.9 mV, whereas it decreased up to -55.8 ± 6.4 mV after sonication at 100 μm for 180 s. The negative electrical charge of lipid droplets is given by the anionic nature of the sodium alginate molecules dissolved in the aqueous phase. In agreement with our results, other authors (Pongsawatmanit, Harnsilawat, & McClements, 2006) observed a strong droplet charge of oil in water emulsions stabilized with sodium alginate, showing a ζ-potential up to -69 mV. It is believed that some biopolymers can adsorb at the oil-water interface depending on interactions and competition adsorption between previously adsorbed species such as small molecule surfactants (Dickinson, 2003, 2009). The lower ζ-potential observed in nanoemulsions compared with the coarse emulsion could be due to (i) the breakage of sodium alginate molecules during sonication and therefore an increase of ionisable sites in the biopolymer chain, or to (ii) an induced adsorption of sodium alginate molecules in the oil droplets surface caused by the oil droplet disruption. In this sense, it can be considered that nanoemulsions are more stable than the coarse emulsion, as the stronger electrical charge prevents droplet aggregation or coalescence phenomena due to the dominance of repulsive forces between droplets (Heurtault, Saulnier, Pech, Proust, & Benoit, 2003).

The viscosity of nanoemulsions was significantly affected by the ultrasound processing parameters, since it decreased gradually when the sonication time and ultrasound amplitude increased. The viscosity of coarse emulsion before being sonicated was 30.8 ± 0.9 mPa.s. After applying ultrasound treatments for 180 s at amplitude of 30, 60 or 100 μm, the viscosity of the nanoemulsions was 12.45 ± 0.77, 11.05 ± 0.21 or 10.03 ± 0.52 mPa.s, respectively. The relationship between oil droplet size or ζ-potential and emulsion viscosity was studied but a conclusive correlation could not be established. A positive correlation (R^2 of 0.72) was observed between the oil droplet size and nanoemulsion viscosity, as the lower the droplet size of nanoemulsions, the lower the viscosity. However, oppositely to these results, other authors (Chanamai & McClements, 2000; Cortés-Muñoz, Chevalier-Lucia, & Dumay, 2009; Derkach, 2009; Pal, 2011) reported a negative correlation between oil droplet size and emulsion viscosity, observing a lower viscosity in emulsions with a higher droplet size. This behavior was attributed to an increase in the effective volume fraction in emulsions with small droplet size due to the contribution of the interfacial layer surrounding oil droplets

where droplets cannot come into contact. However, this effect was mainly observed in high concentrated nanoemulsions, with oil content above 20%. In the case of less concentrated emulsions, the main factor determining the overall viscosity is the continuous phase. Also, oppositely to the observed results, an increase in the emulsion viscosity would be expected due to an increase in the droplet charge, caused by the electroviscous effect (Hiemenz & Rajagopalan, 1997). In the present work, the thickening properties of sodium alginate are thought to govern the rheology of nanoemulsions, thus the viscosity loss after sonication was attributed to a damage to the biopolymer molecules, as it has also been observed by other authors (Camino, Pérez, & Pilosof, 2009).

Regarding the whiteness index (WI) of nanoemulsions, it decreased with increasing the sonication amplitude and time. The WI of the primary emulsion was 46.78 ± 0.16, and diminished up to a minimum of 27.86 ± 0.33 in the nanoemulsion sonicated at 100 μm for 180 s. Nanoemulsions were translucent to the naked eye after being sonicated for 60 s, regardless the sonication amplitude. A strong correlation was observed between nanoemulsions droplet size and whiteness index. This fact was attributed to the weak light scattering of oil droplets when they are in the nano-range (McClements, 2002).

Microfluidization

The formation of LEO nanoemulsions by microfluidization was studied in terms of the influence of processing pressure (MPa) and cycles on the nanoemulsion droplet size, ζ-potential, viscosity and whiteness index.

The average droplet size of nanoemulsions decreased with increasing the processing pressure and number of cycles. The droplet size of the coarse emulsion was 1410 ± 366 nm, and it was drastically reduced up to 53 ± 5, 46 ± 7 and 23 ± 2 nm after being passed 1 cycle through the microfluidization system working at 50, 100 or 150 MPa, respectively. Nanoemulsions with a minimum droplet size of around 7 nm and a narrow size distribution were obtained after passing the samples 3 cycles at 150 MPa. It was observed that passing the nanoemulsion more than 3 cycles did not lead to a significant reduction of the average droplet size, as nanoemulsions treated at 150 MPa for 10 cycles showed a droplet size of around 6 nm. Other authors (Qian & McClements, 2011) also reported a drastic reduction of the oil droplet size in corn oil nanoemulsions passed once through the microfluidizer regardless the pressure applied, but further cycles had a fair effect. This behavior was previously named as “saturation oil droplet size”, which

corresponds to the limit when shearing the emulsion at the same pressure does not yield any additional rupturing or change in the size distribution (Meleson, Graves, & Mason, 2004). The microfluidization technique efficiency is limited, since the oil droplets nearer to the walls of the interaction chamber are submitted to a smaller shear flow, thus further passes though the interaction are needed to obtain an homogeneous nanoemulsion. Recently, several papers have been published dealing with the production of nanoemulsions incorporating EOs by high-pressure homogenization techniques, and results are in agreement with the ones observed in the present work (Donsì, Annunziata, Vincensi, & Ferrari, 2011; Rao & McClements, 2011).

After passing the coarse emulsion through the microfluidization system a statistically significant reduction of the droplet ζ-potential was observed regardless the number of cycles or the pressure applied, which meant an increase on the electrical net charge of lemongrass oil droplets. Whereas the ζ-potential of coarse emulsion was -17.61 ± 2.89 mV, it decreased up to a minimum of -51.95 mV. Similarly to the trend observed after sonication of nanoemulsion, the negative droplet charge of oil droplets was attributed to adsorbed anionic sodium alginate molecules to the oil-water interface. Even though alginates, as well as most food hydrocolloids, do not possess hydrophobic moieties (Dickinson, 2003, 2009), it has recently been observed that can present surface activity under certain conditions (Garti & Leser, 2001; Yang, Jiang, He, & Xia, 2012). The mechanical stress during microfluidization can lead to break up of sodium alginates molecules thus increasing the number of molecules potentially adsorbed on the oil-water interface. This fact could explain the ζ-potential strengthening observed in microfluidized nanoemulsions.

Similarly to the trend observed with ultrasonication, the viscosity of nanoemulsions significantly decreased with increasing the microfluidization pressure and number of pressure cycles. The viscosity of the coarse emulsion was 35.65 ± 2.33 mPa.s and was decreased up to 16.85 ± 0.21 mPa.s after a single pass though the interaction chamber at 150 MPa. Further increasing the number of cycles up to 10 at a pressure of 150 MPa diminished the nanoemulsion viscosity to 8.25 ± 0.30 mPa.s. In this sense, the drop of viscosity in nanoemulsions was attributed to changes in the molecular structure of sodium alginate during microfluidization. Other authors observed a viscosity decrease in aqueous biopolymer solutions after high-pressure homogenization due to molecular weight loss, thus decreasing their thickening properties (Harte & Venegas, 2010; Lagoueyte & Paquin, 1998). This effect has also been recently reported in emulsions

with biopolymeric aqueous phases treated with high-pressure homogenization (Bonilla, Atarés, Vargas, & Chiralt, 2012; Floury, Desrumaux, Axelos, & Legrand, 2003; Floury, Desrumaux, & Lardières, 2000). It is believed that the mechanical stress during microfluidization has an effect on the conformation of biopolymer molecules, thus inducing irreversible molecule degradation (Lagoueyte & Paquin, 1998).

The behavior of nanoemulsions WI after microfluidization was similar to the one observed after ultrasonication. The WI of nanoemulsions decreased gradually at increasing the microfluidization pressure and cycles. The WI of the coarse emulsion was 54.83 ± 0.18 and it diminished up to 42.79 ± 0.04, 36.65 ± 0.08 and 35.71 ± 0.16 in nanoemulsions processed for 3 cycles at 50, 100 and 150 MPa, respectively. Passing nanoemulsions through the interaction chamber additional cycles did not have any further significant effect on the WI of nanoemulsions. In this case, the decrease of WI was also related with the small droplet size exhibited by nanoemulsions, due to weak light scattering. Other authors have also obtained visually transparent nanoemulsions by microfluidization (Wooster, Golding, & Sanguansri, 2008).

1.1.2 Concentration of the different components

The influence of LEO, surfactant and sodium alginate on the formation of nanoemulsions in terms of droplet size, ζ-potential, viscosity and color, was evaluated by means of pseudo-ternary phase experimental design adapted to a response surface methodology. Mixtures were prepared by mixing the ingredients with high-speed blender and a subsequent treatment with microfluidization (3 cycles at 150 MPa).

The average droplet size of emulsions ranged between 2 and 1300 nm depending on the concentration of LEO, tween 80 and sodium alginate. The LEO and tween 80 concentrations were the most significant factors influencing the oil droplet size of nanoemulsions. In this sense, mixtures with concentrations of LEO and tween 80 lower than 0.6 and higher than 0.9 % (v/v), respectively, led to nanoemulsions with particle sizes smaller than 100 nm. Increasing the concentration of LEO and decreasing the amount of surfactant resulted in emulsions with droplet sizes higher than 500 nm. Therefore the presence of a sufficient amount of surfactant to cover the oil-water interfacial area is of crucial importance for the formation of nanoemulsions, that is greater in nanoemulsions compared with coarse emulsions (Kralova & Sjöblom, 2009). Nevertheless, the concentration of alginate had a less pronounced but significant influence on the droplet size of the obtained nanoemulsions. Mixtures with absence of a

surfactant (tween 80) and a concentration of sodium alginate dissolved in the aqueous phase higher than 1.5 % (w/v) exhibited droplet sizes between 200 and 300 nm, thus evidencing the role of sodium alginate as emulsifier in the formation of nanoemulsions. When nanoemulsions are produced by microfluidization, the viscosity of the continuous phase may affect the droplet size throughout an increased droplet fragmentation due to an increased disruptive shear stress, or a decreased droplet re-coalescence caused by a decreased droplet collision frequency (Qian & McClements, 2011).

The ζ-potential of oil droplets with different formulations varied significantly according to their composition, ranging between 0 and -98.65 mV. The sodium alginate concentration was the most significant factor influencing the droplet electrical charge. Sodium alginate is an anionic molecule in aqueous solution explained by its low dissociation constant at a wide range of pH (Tønnesen & Karlsen, 2002). In this sense, slightly increasing the concentration of sodium alginate beyond 0.1 % (w/v) led to oil droplets with ζ-potential around -30 mV. Further increase of the sodium alginate concentration led to a decrease in the droplet charge up to -90 mV. Nevertheless, despite being a non-ionic molecule, the presence of tween 80 in the absence of sodium alginate led to droplets slightly negatively charged. However, other authors also described the anionic nature of small molecule surfactants under certain conditions (Hsu & Nacu, 2003). In the present work a significant interaction was observed between the surfactant and the sodium alginate concentrations regarding the droplet ζ-potential. In this sense, at constant concentration of alginate, the addition of a moderate amount of tween 80 increases the ζ-potential of the mixture. Nevertheless, this behaviour was surfactant concentration-dependent. At low surfactant concentration, there might be not enough surfactant molecules to cover the oil-water interface, thus inducing sodium alginate adsorption and in turn leading to a more negative electrical charge (Goddard, 2002). At high surfactant concentration, the addition of sodium alginate led to a more moderate drop of the ζ-potential. This fact could be explained by the following reasoning: (i) the surfactant molecules adsorbed at the oil/water interface might repel polymer molecules (De Gennes, 1990); (ii) the hydrophilic sites of polymer molecules might bind surfactant molecules thus lowering the adsorption of sodium alginate to the oil surface (Neumann, Schmitt, & Iamazaki, 2003).

The viscosity of emulsions and nanoemulsions was mainly governed by the concentration of sodium alginate in the aqueous phase. As expected, the higher the sodium alginate concentration, the higher the viscosity was due to its thickening

properties. Emulsions containing a concentration of sodium alginate below 1% (w/v) exhibited a viscosity below 15 mPa.s. However, increasing the concentration of sodium alginate in the aqueous phase from 1% (w/v) to 2% (w/v) caused a remarkable increment of the viscosity. Regarding the color of mixtures, it was observed a significant interaction between the oil and surfactant concentrations. The WI of blends ranged between 25.48 and 72.20 depending on the formulation. At low oil and high surfactant concentrations the WI of emulsions showed the lowest values. Emulsion color is given as a consequence of the scattering of light colliding with droplets. It is described that the light scattering of oil droplets depends on the refractive index of continuous and dispersed phase, oil concentration and also droplet size (D J McClements, 2002). In this sense, it could be assumed that the influence of oil and surfactant concentration determined the emulsion visual appearance due to their direct effect on the droplet size of emulsions and nanoemulsions. Similarly to our results, Rao & McClements (Rao & McClements, 2012) also reported an increase on the turbidity of lemon oil nanoemulsion when increasing the oil content or decreasing the tween 80 concentration.

1.1.3 Essential oil type

Nanoemulsions containing different EOs (lemongrass, clove, tea tree, thyme, geranium, marjoram, palmarosa, rosewood, sage and mint) were prepared in order to study the influence of the EO type on their physicochemical properties. Moreover, the characteristics of nanoemulsions containing different EOs were compared with their respective conventional emulsions.

Strong differences among emulsion droplet size and size distribution were observed depending on the EO used in the nanoemulsion formulation. Microfluidized lemongrass oil-loaded nanoemulsions exhibited a dramatic reduction of their average droplet size up to 5.49 ± 0.28 nm, in comparison with the values of the coarse emulsion, which averaged 1121.43 ± 228.93 nm. Oppositely, coarse emulsions incorporating palmarosa and rosewood EOs exhibited average droplet sizes already in the nano-scale range (32.91 ± 6.92 or 13.82 ± 0.48 nm, respectively) before microfluidization. In both cases, microfluidization had a fair effect on the droplet size and droplet size distribution. Regarding the rest of EOs (clove, tea tree, thyme, geranium, marjoram, sage or mint), the average droplet size of coarse emulsions was also in the nano-scale range, with values between 13.16 ± 0.92 and 86.49 ± 4.31 nm. However, major intensity peaks of

particles of around 1000 nm were observed in the droplet size distribution as well as intensity peaks of particles of around 10 nm, that might correspond to surfactant micelles. This evidenced the wide range of droplet sizes in these coarse emulsions. The impact of the microfluidization treatment on the droplet size distribution of clove, tea tree, thyme, geranium, marjoram, sage or mint EOs emulsions was more pronounced compared than in the case of palmarosa and rosewood EOs. In this sense, there was a clear disruption of droplets bigger than 100 nm, as it was seen in the size distribution. Besides the EO type, the final nanoemulsion droplet size is a complex interplay between processing conditions and the adsorption of surface active molecules (Wooster et al., 2008). In the case of EOs, their ability to form nanoemulsions depends on their molecular characteristics, such as molecular weight, polarity and conformation (Djekic & Primorac, 2008). Recently, other authors reported that EOs with a higher concentration of polar compounds might reduce the interfacial tension and facilitate the droplet disruption during microfluidization (Ziani, Fang, & McClements, 2012). In general, plant EOs are constituted of low molecular weight compounds and present low viscosity values, which favors the formation of naneomulsions (Sell, 2010).

As observed in previous experiments, microfluidization significantly decreased the droplet charge of nanoemulsions compared with the respective coarse emulsions. In fact, most of the nanoemulsions had ζ-potential values more negative than -30 mV, with the exception of palmarosa-loaded nanoemulsions (-20.9 mV). This behavior was related to the breakdown of sodium alginate molecules, thus increasing the number of molecules to be potentially adsorbed on the oil-water interface. However, the EO type significantly influenced the ζ-potential of coarse emulsions or nanoemulsions. For instance, coarse emulsions containing thyme, palmarosa, sage or mint exhibited a ζ-potential of -12, -11, -13 or -10 mV, respectively. The ζ-potential decreased after microfluidization, but was still not as negative as in the case of clove, tea tree or marjoram microfluidized nanoemulsions. Recently, other authors (Bonilla et al., 2012) stated that differences observed in the ζ-potential of emulsions and nanoemulsions formulated with different EOs might be attributed to differences in the dissociation degree and number of ionisable compounds of oils themselves. Moreover, other authors (Terjung, Löffler, Gibis, Hinrichs, & Weiss, 2012) recently observed that carvacrol and eugenol are preferentially located in the oil-water interfaces of nanoemulsions. Therefore, it can be thought that the ζ-potential of oil nanopdroplets is determined by the location of EOs within the lipid nanoparticle, their ionisable groups in the molecular

structure, and therefore their interplay with the ionic surface-active compounds in the aqueous phase.

The viscosity of coarse emulsions ranged between 22.95 and 36.05 mPa.s depending on the type of EO used in the formulation. Mint EO led to more viscous coarse emulsions, whereas thyme EO rendered the lowest viscosity values. It is known that the characteristics of the dispersed phase have a direct impact on the rheological properties of emulsions (McClements, 2005). Similarly to our results, Bonilla and co-workers (2012) found that thyme oil-loaded emulsions stabilized with chitosan showed lower viscosity values in comparison with emulsions containing basil EO. They attributed these variations to differences in the dissociation degree and number of ionisable compounds of the different EO, since biopolymeric molecules would be adsorbed at the oil droplet surface and lead to changes in the effective thickening concentration in the aqueous phase. However, as observed in previous experiments, a dramatic reduction of emulsion viscosity was observed after microfluidization up to values around 10 MPa.s, without noticing differences between the EOs used in the formulation.

The type of EO used in the formulation significantly influenced the WI of coarse emulsions and nanoemulsions. Coarse emulsions containing lemongrass, thyme, geranium or palmarosa EOs presented higher WI, with values of 47.9, 49.6, 57.8 or 51.3, respectively. On the contrary, coarse emulsions formulated with clove, tea tree or marjoram EOs exhibited the lowest WI, with values of 24.4, 29.9 or 27.8, respectively. In general, microfluidization significantly affected the WI of mixtures generating nanoemulsions with lower WI. Nonetheless, the extent of WI decrease after microfluidization depended on the EO type used in the formulation. For instance, nanoemulsions incorporating lemongrass, sage or geranium EOs showed a drastic decrease in their WI from 47.9, 36.0 and 57.8 up to 32.2, 27.7 and 49.6, respectively. Nevertheless, emulsions incorporating thyme EO exhibited an opposite behaviour, as the WI of microfluidized nanoemulsions was higher than that of the coarse emulsion. In this case, the emulsion WI was related with the emulsion droplet size. Large particles scatter the light more intensely than smaller ones, which causes an increase in the lightness, opacity and WI of emulsions (McClements, 2011). In fact, only tea tree, geranium and marjoram oil nanoemulsions became completely transparent after microfluidization, which is consistent with the light scattering measurements showing that these nanoemulsions possessed the lowest average droplet size and narrowest size distribution.

1.2 Factors affecting nanoemulsion antimicrobial properties

1.2.1 Formation method and processing conditions

Ultrasounds

Nanoemulsions with LEO were produced by ultrasounds at different amplitude and treatment time and their antimicrobial activity was assessed through the inactivation kinetics of *Escherichia coli* during 30 min. A blank control was carried out putting in contact *E. coli* with distilled water to assure the survival of the microorganism during 30 min. The survival fraction of the inoculated *E. coli* population decreased gradually with contact time, regardless the amplitude or the sonication time used to prepare the nanoemulsions. In this sense, the *E. coli* population decreased 0.66, 2.25 and 5.85 log-units after 5, 15 and 30 min of contact time with the coarse emulsion. The sonication amplitude and time significantly influenced the antimicrobial activity of nanoemulsions. The higher the ultrasound amplitude and the treatment time, the lower the antimicrobial activity of nanoemulsions compared with the coarse emulsion. In this regard, the nanoemulsion sonicated at 30 μm for 180 s reduced the *E. coli* population 4.2 log-units after 30 min whereas that treated at 100 μm for 180 only achieved 0.3 log reductions. A Weibull equation was successfully fitted to experimental data of the inactivation kinetics of sonicated nanoemulsions, presenting a concavity downwards ($\alpha > 1$). This implies a slower inactivation of microbial cells with nanoemulsions at early contact times compared with the coarse emulsion. The results observed evidence a deleterious effect of ultrasounds on the volatile compounds of LEO, responsible of its antimicrobial activity. This fact was attributed to (i) the volatilization of aromatic compounds due to the temperature increase during sonication (Sell, 2010), and (ii) the oxidation of aromatic compounds during cavitation phenomena (Riesz, Berdahl, & Christman, 1985). In fact, this phenomenon was corroborated by other authors, who reported lipid oxidation during sonication of nanoemulsions (Chemat, Grondin, Sing, & Smadja, 2004).

Microfluidization

The influence of microfluidization processing conditions on the subsequent antimicrobial properties of the obtained nanoemulsions was studied by determining the inactivation kinetics of *Escherichia coli*. Oppositely to the response observed in the case

of nanoemulsions produced by ultrasounds, microfluidized nanoemulsion showed an enhanced inactivation of *E. coli* compared with the coarse emulsion. Increasing the cycles through the interaction chamber significantly increased the bactericidal properties of nanoemulsions. Coarse emulsion led to 5.85 log reductions of *E. coli* population after 30 min of contact time whereas nanoemulsions (1 cycle at 150 MPa) reduced the microbial population 7.07 log-units after 30 min of contact time. Inactivation curves were modeled with a Weibull equation, observing that the more cycles through the microfluidizer the lower β value, which implies a higher antimicrobial potential. Oppositely to the fact observed with ultrasounds, the inactivation kinetics of *E. coli* in contact with microfluidized nanoemulsions exhibited a concavity upwards ($\alpha < 1$), depicting a faster inactivation of the microorganism at early contact time. The enhanced antimicrobial potential of nanoemulsions observed in the present work was related to the high active surface area due to the reduced droplet size of nanoemulsions. Microfluidization was found to be a more gentle technology to produce nanoemulsions with heat-sensitive compounds, since it is able to rapidly cool down the temperature increase associated to high pressure processing. In agreement with our results, other authors observed the same effect in nanoemulsions containing antimicrobial phenolic compounds, evidencing that a smaller droplet size favors the inactivation of microbial cells (Donsì, Annunziata, Sessa, & Ferrari, 2011). Nevertheless, other authors reported a lower antimicrobial activity of carvacrol or eugenol nanoemulsions compared to the respective conventional emulsion, attributing this effect to a sequestering of antimicrobials in emulsion interfaces (Terjung et al., 2012).

1.2.2 Concentration of the different components

As expected, the concentration of LEO was the ingredient with the greatest effect on the antimicrobial activity of emulsions and nanoemulsions. In this sense, blends with a LEO concentration lower than 0.06 % (v/v) did not reduce the *E. coli* inoculum after 30 min of contact time. On the contrary, a maximum reduction of 7.37 log-units was observed after 30 min of contact with formulations containing a concentration of LEO above 1 % (v/v). Nevertheless, significant interaction was observed between the LEO concentration and the other ingredients in the formulation. The addition of a surface-active compound, such as tween 80, or a texturizing agent, such as sodium alginate, significantly enhanced the bactericidal action of LEO. This fact could be related with

the reduced droplet size achieved in the presence of stabilizing agents, namely tween 80 and sodium alginate. Recent research papers also state the enhanced antimicrobial activity of nanoemulsions compared with conventional emulsions (Donsì, Annunziata, Sessa, et al., 2011; Ziani, Chang, McLandsborough, & McClements, 2011). Nonetheless, the contribution of stabilizing agents to the homogeneity and dispersion of active compounds and, in turn, to the functionality has recently pointed out by other authors. Liang and co-workers (2012) observed an increased and prolonged antimicrobial activity against *Listeria monocytogenes* or *Staphylococcus aureus* of nanoemulsions containing peppermint EO and stabilized with starch in comparison with the bulk peppermint EO.

1.2.3 Essential oil type

The type of EO used in the formulation of coarse emulsions and nanoemulsions mainly governed the antimicrobial activity against *E. coli*. Coarse emulsions containing palmarosa, lemongrass, clove or thyme showed the highest antimicrobial activity, reducing 4.10, 3.98, 3.52 or 2.36 log-units respectively of the *E. coli* inoculum after 30 min of contact time. On the contrary, coarse emulsions containing tea tree, geranium, marjoram, rosewood, sage or mint EOs presented less than 1 log reduction of *E. coli* after 30 min of contact time. It is known that the antimicrobial activity of each EO is due to their phenolic compounds and it largely depends on their concentration as well as on their chemical structure (Dorman & Deans, 2000). Those EOs containing carvacrol and thymol terpenes, such as thyme EO, are reported to have a strong bactericidal action (Lambert, Skandamis, Coote, & Nychas, 2001). Nevertheless, eugenol and citral, the major components of clove and lemongrass or rosewood EO, respectively, have been found to inactivate a broad spectrum of microorganisms (Friedman, Henika, Levin, & Mandrell, 2004). Other aromatic compounds such as linalool (tea tree), pinene (mint), geraniol (geranium), or borneol (sage), exhibit a lower inhibitory influence against bacteria (Zachariah & Leela, 2006).

Moreover, the reduction of droplet size after microfluidization significantly enhanced the antimicrobial properties of nanoemulsions. However, this pattern was only observed in those nanoemulsions exhibiting a stronger antimicrobial activity. Nanomeulsions containing lemongrass and clove EOs exhibited faster inactivation kinetics of the microbial population, as observed in the survival fraction of *E. coli* at early contact

times. The shape of the fitted equation for lemongrass or clove oil nanoemulsions shows a concavity upwards in the inactivation curve whereas the respective coarse emulsions show a concavity upwards, indicating a faster inactivation of the microorganism at early contact times. It is generally assumed that nanoencapsulated lipophilic antimicrobials would be able to penetrate more easily though microbial membranes thus leading to an improved bactericidal action. In agreement with the results of the present work, recent research papers report an enhancement on the antimicrobial activity of nanoemulsions containing flavor oils such as thyme oil (Chang, McLandsborough, & McClements, 2012), peppermint oil (Liang et al., 2012), limonene (Donsì, Annunziata, Sessa, et al., 2011) or eugenol and cinnamaldehyde (Gomes, Moreira, & Castell-Perez, 2011). Nevertheless, other authors also pointed out that the electrical charge of lipid nanoparticles might exert a crucial role determining the bactericidal action of nanoemulsions (Ziani et al., 2011). Nevetheless, the results obtained in the present work indicate that the bactericidal effect of nanoemulsions largely depends on the EO type, and that the enhancement of their antimicrobial properties by reducing the emulsion droplet size is limited to certain EOs.

1.3 Nanoemulsions as antimicrobial edible coatings in fresh-cut Fuji apples

Nanoemulsion-based edible coatings containing LEO at different concentrations (0.1, 0.5 or 1 % v/v) were applied to fresh-cut *Fuji* apples. In this sense, the effect of edible coatings on the safety and quality parameters of fresh-cut *Fuji* apples during cold storage was assessed. Blank batches of uncoated or coated without LEO apple pieces were prepared. Moreover, the influence of edible coatings formed from conventional emulsions compared with nanoemulsions was assessed in order to establish a possible enhancement of LEO antimicrobial activity.

1.3.1 Microbial inhibition on fresh-cut Fuji apples

1.3.1.1 Escherichia coli inactivation

To assess the bactericidal effect of nanoemulsion-based edible coatings, *E. coli* was inoculated on apple pieces at a concentration of 10^6 CFU/g being subsequently coated. *E.coli* counts were reduced by 0.8 log-units just after applying the sodium alginate coating without LEO. As expected, the LEO concentration in edible coatings had a significant effect on their bactericidal activity against *E. coli*. In fresh-cut apples coated

with solutions with 0.5% or 1% (v/v) of LEO the *E. coli* population was inactivated up to undetectable levels (2 log units) practically immediately after coating, and remained undetected during 2 weeks of refrigerated storage. On the other hand, edible coatings with a LEO concentration of 0.1% (v/v) exhibited a significantly different behavior depending on the initial droplet size of the emulsion. In this sense, nanoemulsion-based edible coatings exhibited a faster and enhanced inactivation of *E. coli* on apple slices during storage time in comparison with coatings formed from conventional emulsions. Nanoemulsion-based edible coatings with 0.1% (v/v) LEO reduced the *E. coli* inoculum until undetectable levels after 11 days of storage time, whereas apple pieces coated with conventional emulsions with the same concentration of LEO still presented 3.3 log units of *E. coli* after 14 days. As it has also been observed in previous experiments, the reduction of emulsion droplet size would allow antimicrobial compounds to penetrate faster in the bacterial cell (Weiss, Takhistov, & McClements, 2006), thus explaining the behavior observed. Despite the increasing number of scientific publications dealing with nanoemulsions with active compounds, there is limited published information about the application of antimicrobial nanoemulsions in food products. In this sense, Donsí and co-workers (2011) applied antimicrobial nanoemulsions containing a mixture of terpenes to enhance orange or pear juice shelf life, observing that the effect was dose-dependent. Therefore, there is a need of more scientific evidence to proof the potential advantages of antimicrobial nanoemulsions once incorporated to real food systems.

1.3.1.2 Microbial growth

The growth of natural microbial population of molds and yeasts and psychrophilic bacteria of uncoated and coated fresh-cut *Fuji* apples was determined during refrigerated storage. Microbial counts in uncoated apple pieces increased gradually during 14 days of storage, reaching 5.2 and 5.5 log CFU/g for molds and yeasts and bacteria respectively. As expected, the concentration of LEO in edible coatings significantly affected the spoilage of fresh-cut apples. Coatings with a concentration of LEO of 1% or 0.5% (v/v) completely inhibited the growth of natural spoilage microorganisms during 2 weeks of refrigerated storage, regardless the emulsion droplet size of the coating-forming solution. Moreover, edible coatings with 0.1% (v/v) LEO slowed down the microbial growth in comparison with control samples, but did not completely inhibit the microbial growth during the storage period. In this case, even though the emulsion droplet size did not have a categorical effect on the antimicrobial

activity of the edible coatings a slightly lower microbial growth was observed in fresh-cut apples coated with nanoemulsion compared with conventional emulsions. For instance, apple pieces coated with a nanoemulsion with 0.1% (v/v) LEO showed a final bacterial concentration of 4.6 log CFU/g, whereas with conventional emulsion coating with the same LEO concentration exhibited 5.4 log CFU/g. Recent papers evidenced the shelf life extension of fresh-cut apples (Raybaudi-Massilia, Rojas-Graü, Mosqueda-Melgar, & Martín-Belloso, 2008; Rojas-Graü et al., 2007), melon (Raybaudi-Massilia, Mosqueda-Melgar, & Martín-Belloso, 2008) or grapes (Sanchez-Gonzalez et al., 2011) by applying edible coatings with EOs. However, as far as we know there are no reported data about the incorporation of nanoemulsions of EOs as edible coating to food products.

1.3.2 Quality of coated fresh-cut Fuji apples

The quality of uncoated and coated fresh-cut *Fuji* apples during refrigerated storage was assessed in terms of the tray headspace gas composition (O_2, CO_2, ethylene and ethanol), fresh-cut fruit color and texture.

1.3.2.1 Headspace gas composition

Edible coatings formed from emulsions or nanoemulsions with LEO at 0.5 (v/v) or 1% (v/v) significantly decreased the respiration of fresh-cut apples compared with the uncoated apple pieces, diminishing the O_2 consumption and CO_2 production. The decrease in the respiratory activity of coated fresh-cut apples due to EOs presence was also described by other authors (Raybaudi-Massilia, Rojas-Graü, et al., 2008; Rojas-Graü et al., 2007), being attributed to a major resistance of the edible coating to gas diffusion resulting from the lipophilic nature of EOs (Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2011). However, apple pieces coated without LEO or coated with nanoemulsions or conventional emulsions with 0.1% (v/v) LEO exhibited an increased respiration (higher O_2 consumption and CO_2 production) during storage time in comparison with the uncoated ones. Peeling and cutting operations of fruits cause a physiological stress on plant tissues, thus triggering the respiration metabolism (Soliva-Fortuny & Martín-Belloso, 2003). Aerobic respiration in plant tissues consists of oxidative breakdown of organic reserves of carbohydrates, lipids and organic acids to simpler molecules, including CO_2 and water, with release of energy (Fonseca, Oliveira, & Brecht, 2002). Coated apple pieces with sodium alginate might

have had higher carbohydrate accessibility from the biopolymer molecules thus exhibiting an increased respiratory activity. This trend was also observed in fresh-cut apples coated with emulsions or nanoemulsions containing 0.1% (v/v) LEO. This fact might also be related to the contribution of microbial respiration in fresh-cut apples on the headspace gas composition, since apple pieces coated without LEO or with nanoemulsions or emulsions at 0.1 % (v/v) LEO showed a higher microbial growth during storage time. The effect of the emulsion droplet size of edible coating on the respiration of fresh-cut apples was not significant. Nevertheless, fresh-cut apples coated with nanoemulsions containing 0.5 or 0.1 % (v/v) LEO showed a slightly higher O_2 consumption and CO_2 production compared with fresh-cut apples coated with conventional emulsions containing the same LEO concentration. This fact might be possibly related to the high susceptibility of LEO nanoemulsions to be volatilized due to the increased surface area, thus leading to a decreased effective concentration of EOs in the edible coating. On the other hand, ethylene is a secondary metabolite, which is related with aerobic metabolism of plant tissue (Soliva-Fortuny & Martín-Belloso, 2003). Consistently to the respiration results, in-package ethylene concentration for fresh-cut apples coated only with sodium alginate was greater than for those uncoated. However, edible coatings with 0.1 % (v/v) LEO or higher were able to inhibit the ethylene production during storage time regardless the droplet size of the initial emulsion. In contrast, ethanol is a secondary metabolite that is produced during fermentative reactions in anaerobic conditions (Fonseca et al., 2002). An ethanol production peak was observed at day 11 of storage time, which was significantly higher in uncoated samples. Coated apple pieces exhibited a lower concentration of ethanol on the headspace, regardless of the formulation of the edible coating.

1.3.2.2 Color

Browning of apple pieces is basically indicated by a decrease in the lightness of fruit tissue (Soliva-Fortuny, Grigelmo-Miguel, Odriozola-Serrano, Gorinstein, & Martín-Belloso, 2001) and also an increase in a* (redness) values (McHugh & Senesi, 2000) that implies a decrease in the hue value. The concentration of LEO in edible coatings significantly affected the color (L and h parameters) of fresh-cut *Fuji* apples during refrigerated storage. The L and h values of uncoated or coated apple pieces with sodium alginate remained practically constant during 15 days of cold storage. However, a gradual decay of both parameters was observed during storage time in apple cylinders

coated with LEO emulsions or nanoemulsions. Such decrease was more noticeable when LEO concentration of 0.5 or 1 % (v/v) was used. Browning in white flesh fruits is mainly due to enzymatic reactions, since after mechanical stress, polyphenol oxidase (PPO) is liberated from plant cells and interacts with polyphenols and oxygen, thus leading to the formation of brownish compounds (Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994; Toivonen & Brummell, 2008). The induction of browning reactions in fresh-cut apples coated with solutions containing EOs was also reported previously by other authors (Raybaudi-Massilia, Rojas-Graü, et al., 2008; Rojas-Graü et al., 2007). The incorporation of EOs in edible coatings might induce the browning of cut apple surface by two mechanisms: (i) phenolic compounds from EOs might be substrate themselves for PPO activity; and (ii) an increase in the permeability of plant cell membrane due to volatile compounds might cause a higher leakage of PPO and polyphenols from the cell cytoplasm. The LEO emulsion droplet size in edible coatings did not have a significant effect on the browning of fruit flesh. However, a less pronounced browning was observed in those fresh-cut apples coated with LEO nanoemulsions in comparison with conventional emulsions. The L value was higher during the first 5 days of storage in apples coated with nanoemulsions at 0.5 and 1% (v/v) LEO regarding the apple pieces coated with conventional emulsions at the same LEO concentration. In the case of coatings with 0.1 % (v/v) LEO, this trend was maintained during 15 days of storage.

1.3.2.3 Firmness

Neither the edible coatings nor the LEO concentration and its particle size had a significant influence on the firmness of apple slices. Moreover, the firmness of fresh-cut apples was maintained practically constant during storage time regardless the coating applied, with values ranging from 11.95 and 9.14 N. Oppositely to the results obtained in the present work, other authors reported the softening of fresh-cut apples after applying edible coatings with EOs (Raybaudi-Massilia, Rojas-Graü, et al., 2008; Rojas-Graü et al., 2007). They attributed the loss of firmness to the low pH of film forming solutions, which might cause the acid hydrolysis of pectic acid in fruit cells (Ponting, Jackson, & Watters, 1971). Also it has been suggested that such degradation of the texture might be caused by the penetration of the EOs on the cell tissue of the fruit, producing structural changes (Sánchez-González et al., 2011). However, the use of calcium salts to induce the cross-linking of sodium alginate and therefore forming the

edible coating on the fruit surface may in turn enhance the texture of minimally processed fruit by bonding to pectins and preventing cell wall degradation (Lamikanra, 2002; Olivas, Mattinson, & Barbosa-Cánovas, 2007; Poovaiah, Glenn, & Reddy, 1988). The differences between the results observed in the present work and previously reported data may rely on the high initial firmness values of the apple pieces as well as the concentration and type of EO incorporated to edible coatings or the intrinsic varietal firmness characteristics (Soliva-Fortuny, Oms-Oliu, & Martín-Belloso, 2002).

2 Bioactive nanoemulsions

The fortification of food products with lipophilic bioactive compounds, such as β-carotene, is still a challenge due to their poor absorption in the gastrointestinal tract (GIT). The delivery of bioactive compounds in nanoemulsion-based delivery systems might be a good strategy to favor the digestibility and therefore bioaccessibility of such ingredients in the human gut. However, little is known about the factors affecting the biological fate of nanoemulsions in the GIT. Therefore, the aim of the present work was to study the influence of the emulsion droplet size as well as the lipid carrier type and concentration on the physicochemical properties of the initial nanoemulsion and on the oil digestibility and β-carotene bioaccessibility after simulated *in vitro* digestion conditions.

2.1 Factors affecting nanoemulsions biological fate

2.1.1 Emulsion droplet size

As discussed in previous sections, the particle size of nanoemulsion-based delivery systems might have a direct impact on the functionality of lipophilic active ingredients. The digestion of fat emulsions implies the lipolysis of lipid droplets by the action of lipase that is adsorbed at the oil-water interfaces (Reis, Holmberg, Watzke, Leser, & Miller, 2009). An increase in the droplet active surface might favor the digestion of lipid carrier and may enhance the adsorption of lipophilic active compounds incorporated in edible nanoemulsions. Thus, in this section the effect of emulsion droplet size on the biological fate of β-carotene-loaded lipid particles was studied. For this purpose, three delivery systems with different initial emulsion droplet diameters, being large ($d_{43} \approx 23$ μm); medium ($d_{43} \approx 0.4$ μm); and small ($d_{43} \approx 0.2$ μm), were

prepared. Changes of the emulsion physicochemical properties were evaluated during the *in vitro* digestion steps (mouth, stomach and small intestine) as well as the oil digestibility and β-carotene bioaccessibility.

2.1.1.1 Changes during in vitro digestion

In general, the small and medium emulsions showed a similar behavior to each other as they passed through the simulated GIT without statistically significant differences, whereas the large emulsions behaved differently. Therefore small and medium emulsions were discussed together and separately from large emulsion.

The small and medium emulsions exhibited an increase in the mean particle diameter up to values between 1 and 2 μm after exposure to the mouth and stomach phases, and then increased up to values around 10 μm after exposure to the small intestine phase. In this sense, the droplet size distribution indicated that a large fraction of droplets after mouth, stomach or small intestine had fairly similar sizes to the initial emulsions but intensity peaks corresponding to bigger particles were observed. Confocal images displayed large irregular clusters of lipid droplets in the small and medium emulsions after exposure to the mouth and stomach conditions, indicating droplet aggregation. Droplet aggregation within the mouth has previously been attributed to depletion and/or bridging flocculation caused by droplet interactions with mucin (Sarkar, Goh, & Singh, 2009; Silletti, Vingerhoeds, Norde, & van Aken, 2007; Vingerhoeds, Blijdenstein, Zoet, & Van Aken, 2005). However, there appeared to be some dissociation of these clusters when the emulsions moved from the mouth to the stomach stage. Other authors also reported dissociation of flocculated small lipid droplets coated with a non-ionic surfactant after exposure to stomach conditions (Golding et al., 2011), which might be attributed to a dilution effect and/or mechanical agitation (Dickinson, 2003; McClements, 2005). Confocal images of the small intestine stage showed a fairly even distribution of lipid-rich particles, which were identified as digestion products such as mixed micelles, vesicles and other colloidal structures as well as non-digested fat droplets. Other authors also reported the presence of some non-digested oil in β-carotene emulsions after lipase digestion (Malaki Nik, Wright, & Corredig, 2011). Comparing the light scattering measurements and the confocal images of small and medium emulsions it can be noticed that light scattering suggested that the biggest particles are in the small intestine phase whereas confocal images exhibit the largest particles in the mouth. This fact was attributed to artifacts associated with the analytical

methods used to determine the sample microstructure. For instance, light scattering measurements imply sample dilution and stirring prior to analysis, which might change the particle size of the samples. This evidences the importance of using several analytical techniques to characterize lipid nanoparticles.

In the case of large emulsion, the light scattering measurements indicated little changes in particle diameter or particle size distribution in the various stages of *in vitro* digestion, showing mean particle sizes between 23 and 24 μm. As occurred in the case of small and medium emulsions, confocal images revealed a different behavior from the one observed by light scattering. In this sense, the confocal images evidenced a large increase in droplet size after mouth stage simulation, followed by an appreciable decrease after gastric conditions. As discussed earlier, the increase in the droplet size after simulated oral conditions might me due to droplet flocculation caused by depletion and/or bridging mechanisms and subsequent droplet coalescence. The decrease of droplet size after stomach conditions was also attributed to mechanical agitation, which might have caused the disruption of the larger droplets. Furthermore, some large irregularly shaped particles formed after small intestine conditions of large emulsion could be observed in confocal images. These particles appeared to have lipid-rich (bright red) outer regions and lipid-depleted (black) inner regions, which were identified as undigested oil or lipid digestion products.

The electrical droplet charge of initial emulsions ranged between -4.8 and -6.3 mV, without statistically significant differences between small, medium or large emulsions. Despite the non-ionic nature of tween 20, as observed in the case of tween 80 used in the formulation of antimicrobial nnaoemulsions, it confers an appreciable negative charge to oil droplets. This was attributed to preferential adsorption of hydroxyl ions (OH-) from the aqueous phase or the presence of anionic impurities (such as free fatty acids) in the surfactant or oil used to prepare the emulsion (McClements, 2005). The ζ-potential of the droplets became significantly more negative after exposure to the mouth and stomach phases (-8 to -12 mV), which may have been due to some adsorption of anionic species (such as mucin) to the droplet surfaces. In turn, the electrical charges on the particles remaining in the samples after the small intestine phase were significantly more negative (-31 to -35 mV). The high negative charge after small intestine simulation might be attributed to the adsorption of anionic species at their surfaces such as bile salts, phospholipids or free fatty acids released during digestion (Pouton & Porter, 2008; Reis et al., 2009; Singh, Ye, & Horne, 2009). Recently published works

report a similar pattern of the droplet electrical charge after digestion of oil-in-water emulsions (Liu, Hou, Lei, Chang, & Gao, 2012; Qian, Decker, Xiao, & McClements, 2012).

2.1.1.2 Oil digestibility

In general, there was a pronounced increase in the amount of free fatty acids (FFAs) released from the emulsions initially, followed by a more gradual increase at longer times, until a relatively constant final value was reached. However, there were statistically significant differences between the rate and extent of digestion depending on the initial droplet size. In the case of emulsions with smaller droplet size (small and medium emulsions), the initial rate of digestion was much faster, and the final amount of FFAs released after 2 hours of incubation was higher than for the large emulsions. In this regard, in small and medium emulsions the FFAs release after 2 hours of digestion was 96% and 90% respectively, whereas in large emulsions it dropped to 80%. In this sense, the total extent of digestion increased with decreasing droplet diameter, which can be attributed to the fact that the overall oil-water surface area increased, and therefore there was more oil exposed to the lipase activity. Other researchers have also found that the amount of FFAs produced per unit time increases as the droplet size decreases, which was attributed to changes in the surface area of oil exposed to the digestive enzymes (Golding & Wooster, 2010; Li, Hu, & McClements, 2011; Li & Mcclements, 2010; Reis et al., 2009).

2.1.1.3 β-carotene bioaccessibility

The bioaccessibility of the β-carotene was found to significantly decrease with increasing droplet diameter of initial emulsions. In this sense, the β-carotene bioaccessibility in small and medium emulsions was 59% and 49% respectively, whereas it dropped up to 34% after *in vitro* digestion of large emulsions. A direct relationship was found between the β-carotene bioaccessibility and the total amount of free fatty acids released at the end of the lipid digestion. In this sense, the β-carotene bioaccessibility increased as the amount of FFAs released increased. This result supports the hypothesis that the mixed micelles formed from the products of triacylglycerol digestion play a crucial role in determining the total amount of β-carotene that can be incorporated into the micelle phase, as has also been observed by other authors (Malaki Nik et al., 2011; Nik, Langmaid, & Wright, 2012). The decrease

in β-carotene bioaccessibility with increasing initial droplet size may therefore be attributed to two phenomena: (i) there was more undigested oil present, which could have retained the β-carotene; (ii) there were less mixed micelles present to solubilize the β-carotene. Thus, from these results, it can be stated that nanoemulsions with sub-micron lipid nanoparticles are able to favor the bio-accessibility of lipid active ingredients.

2.1.2 Lipid carrier composition

The absorption of lipophilic active compound is very much related with the digestion of lipids in the GIT. Therefore the lipid carrier composition, specially the length of the fatty acids of triglycerides, might have a direct impact on the biological fate of β-carotene-loaded nanoemulsions. For that purpose, mixtures of medium chain triglyceride oil (MCT) and long chain triglyceride oil (LCT, being corn oil) at different ratios (100:0, 75:25, 50:50, 25:75 or 0:100 MCT-to-LCT) were used to form nanoemulsions by microfluidization (3 cycles at 63 MPa). The concentration of oil in nanoemulsion formulation in this set of experiments was maintained at 1% (w/w). The biological fate of β-carotene-loaded nanoemulsions in terms of their physicochemical parameters, digestibility and β-carotene bioaccessibility was studied under simulated digestion conditions. In this set of experiments, only the small intestine step was simulated, since major changes of nanoemulsion characteristics and lipid nanoparticles digestion occur in this phase, being negligible the influence of the mouth and gastric phases.

2.1.2.1 Changes during in vitro digestion

The mean volume diameter (d_{43}) and ζ-potential of the particles was measured in the initial nanoemulsions, in the overall digesta after lipid digestion, and in the micelle fraction collected from the digesta. The mean diameter of the initial nanoemulsions increased from around 146 to 415 nm as the LCT content in the mixed oil phase (MCT:LCT) increased from 0 to 100% (w/w). The reason for this increase in droplet size might be attributed to changes in the dispersed phase viscosity of the nanoemulsions (McClements, 2005). As it has been discussed in previous sections, the efficiency of droplet disruption within a high pressure homogenizer usually increases as the viscosity of the disperse phase decreases. LCT has an appreciably higher viscosity

than MCT, and therefore droplet breakup during homogenization becomes less efficient as the LCT fraction increases, leading to larger droplets. The mean particle size of nanoemulsions measured after the *in vitro* digestion increased appreciably regardless of the initial oil phase composition, up to values as high as 944 nm. Such increase might be attributed to flocculation and/or coalescence phenomena depending on the nature of the system (Mun, Decker, & McClements, 2007). These destabilization mechanisms may be promoted by changes in the composition and properties of the lipid droplet surfaces within the GIT due to displacement or digestion of the original emulsifiers (Gargouri, Julien, Bois, Verger, & Sarda, 1983). Moreover, complex association colloids, such as mixed micelles, vesicles and lamellar structures, are formed in the digestion medium as a result of the interactions of lipid digestion products, bile salts, phospholipids, and calcium (Kossena, Boyd, Porter, & Charman, 2003). In the present study, particles in the micelle phase had a monomodal size distribution with volume mean diameter below 10 nm, regardless the initial oil phase composition. In agreement with the present results, recent studies using curcumin-loaded nanoemulsions also showed that the size of the particles in the micelle fraction (10-1000 nm) did not depend strongly on the initial oil phase composition (MCT or LCT) (Ahmed, Li, McClements, & Xiao, 2012).

The ζ-potential of the particles in the initial lipid particles, after *in vitro* digestion and the micelle fraction were also measured. The charge of the initial nanoemulsions was negative and decreased slightly with increasing LCT content in the oil phase, with values going from -4.7 to -5.8 mV. As discussed in the case of antimicrobial nanoemulsions, the negative electrical charge exhibited by nanoemulsions stabilized by a non-ionic surfactant (Tween 20) might be attributed to preferential adsorption of hydroxyl ions from the aqueous phase or due to the presence of anionic impurities such as free fatty acids in the surfactant or oil phases (McClements, 2005). This suggests that LCT contained more anionic impurities than the MCT, which meant that the droplets were slightly more negatively charged. The particles in the digesta had an appreciably higher negative charge than those in the initial nanoemulsions, with values ranging from -15 to -20 mV. Likewise, the magnitude of the negative charge on the particles in the digesta and in the micelle phase increased appreciably as the fraction of LCT in the initial oil phase increased. This fact might be explained by the lipase activity during digestion. In this sense, the hydrolysis of triglycerides by pancreatic lipase at the surface of the lipid droplets, leads to the release of anionic free fatty acids, which also alters the

surface charge (McClements, Decker, Park, & Weiss, 2009; Singh et al., 2009). It is known that medium chain fatty acids produced during digestion of MCT are able to rapidly migrate into the surrounding aqueous phase whereas long chain fatty acids produced by LCT tend to accumulate at the oil-water interface (Ahmed et al., 2012; Li et al., 2011; Pouton & Porter, 2008). Consequently, more anionic fatty acids may have remained at the particle surfaces for the systems containing LCT than those containing MCT.

2.1.2.2 Oil digestibility

In this section, the influence of initial oil composition (MCT:LCT) on the rate and extent of triglyceride digestion in the nanoemulsions is discussed. For all nanoemulsions studied, the amount of FFAs liberated increased steeply during the first 40 minutes after adding pancreatic lipase to the digestion medium. The amount of FFAs released then increased more slowly after 40 min of digestion. The total amount of FFAs produced after 2 hours of digestion decreased as the fraction of LCT in the initial oil phase increased. For example, the total amount of FFA released decreased from 123 to 88% as the LCT concentration increased from 0 to 100%. The release of FFA above 100 % for the MCT nanoemulsions might be explained by several reasons. On the one hand, there might have been some conversion of monoacylglycerols into glycerol and FFAs (Carey, Small, & Bliss, 1983), which was not taken into account in the calculations. On the other hand, there may have been other components in the system, such as phospholipids, proteins, or surfactants in the digestion medium, that were hydrolyzed and contributed to the pH stat measurements. The medium chain FFAs are known to migrate more rapidly to the surrounding aqueous phase than long chain FFAs, which tend to accumulate to the oil-water interface and therefore inhibit the lipase activity (Dahan & Hoffman, 2008; Sek, Porter, Kaukonen, & Charman, 2002). This hypothesis is also supported by other studies that have reported a higher digestibility of MCT compared to LCT when they are incorporated in emulsions or nanoemulsions (Ahmed et al., 2012; Li et al., 2011; Li & Mcclements, 2010).

2.1.2.3 β-carotene bioaccessibility

A significant increase in β-carotene bioaccessibility was observed as the fraction of LCT in the initial oil phase increased. For example, the bioaccessibility of β-carotene

increased from around 14 to 86 % as the LCT percentage in the oil phase increased from 0 to 100%. It is known that after the digestion of triglycerides, the carotenoids incorporated within oil droplets are transferred into mixed micelles formed by bile salts and lipolysis products (Yonekura & Nagao, 2007). The results obtained in the current work support previous studies that have shown that the presence of fat is essential for carotenoid solubilization and absorption (Furr & Clark, 1997), and that the amount of lipophilic compounds solubilized in mixed micelles tends to increase with increasing fatty acid chain length (Christensen, Schultz, Mollgaard, Kristensen, & Mullertz, 2004). This latter effect may be related to the fact that the solubilization capacity of a mixed micelle depends on how easily a lipophilic compound can be incorporated into its structure (Laher & Barrowman, 1983). Therefore, it might be expected that the longer the fatty acid chain that forms the mixed micelles is, the easier the lipophilic active compound can be accommodated in the mixed micelles. Nonetheless, the size of the micelle fraction of nanoemulsions with different initial oil phase composition was fairly similar. This fact might have been due to limitation of the dynamic light scattering technique and to precipitation of bigger colloidal structures during centrifugation to obtain the micellar phase. Other recent studies have reported a lower bioaccessibility of lipophilic bioactive compounds encapsulated within MCT oils than in LCT oils (Ahmed et al., 2012; Qian et al., 2012).

2.1.3 Lipid carrier concentration

It is known that the absorption of carotenoids in the GIT is related with the amount of fat ingested (Furr & Clark, 1997). Therefore, in order to study the effect of a high fat content on the biological fate of β-carotene-loaded nanoemulsions, the same experimental design described in the previous section was repeated increasing the oil concentration up to 4% (w/w). The emulsion droplet size and ζ-potential of lipid nanoparticles was measured in initial nanoemulsions with different MCT and LCT ratios as well as after the *in vitro* digestion and in the micelle fraction. The overall changes in particle size and charge for the high-fat nanoemulsions (4% w/w oil) were similar to those for the low-fat nanoemulsions (1% w/w oil). Therefore these have not been further discussed in this section. However, the oil concentration had a significant influence on the behavior exhibited during *in vitro* digestion conditions having a direct impact on the oil digestibility and on the β-carotene bioaccessibility.

2.1.3.1 Oil digestibility

The rate and extent of FFAs release was much more dependent on the initial oil phase composition for the systems containing high fat concentrations (4% w/w) than those containing low fat concentrations (1% w/w). In this sense, the final percentage of FFAs released after two hours of digestion decreased from 89 to 34% for the high fat system and from 123 to 88% for the low fat system as the LCT content in the oil phase increased from 0 to 100%. Overall, the fraction of FFAs released from the triglycerides was considerably lower for the high-fat nanoemulsions than the low-fat ones. This fact has also been observed by other authors, who reported a slower and up to a lower extent lipolysis in emulsions with an increased oil content (Li et al., 2011). This trend might be explained by the fact that the composition of small intestinal fluids was kept constant as the fat content of nanoemulsion systems was increased to 4% (w/w). The lipolysis reaction depends on a complex interplay of several factors. On one hand, the ratio of lipase-to-triglyceride in the digestion medium decreases as the total fat content increased, and so the enzyme activity of the lipase may have decreased (Li et al., 2011). On the other hand, the removal of FFAs from lipid droplet surfaces during digestion by solubilization in bile salts or precipitation by calcium ions is known to play an important role in promoting full hydrolysis of triglycerides (Dahan & Hoffman, 2008; Pouton & Porter, 2008). Thus, there may not have been sufficient mixed micelles or calcium ions to solubilize or precipitate the FFA release from the high fat systems. Nevertheless, it should be considered that, despite the lower triglyceride percentage release in the high-fat nanoemulsions compared to the low-fat nanoemulsions, the absolut amount of FFAs released was greater because of the higher initial concentration of triglycerides present. This fact was indicated by the higher concentration of NaOH needed to maintain the pH constant during the *in vitro* digestion in the pH-stat.

2.1.3.2 β-carotene bioaccessibility

The initial fat concentration in nanoemulsions had a significant effect on the β-carotene bioaccessibility after *in vitro* digestion. In this regard, the behavior of the high fat system was quite different from that of the low fat system. The β-carotene bioaccessibility after digestion of high-fat nanoemulsions with pure MCT in the oil phase was of 87%, whereas it was of 14 % in the low-fat system. This effect may be attributed to the fact that a greater absolute amount of FFAs were released and therefore

more mixed micelles and other association colloids capable of solubilizing β-carotene were formed after the digestion of high-fat systems compared with the low-fat systems. Nevertheless, high-fat content nanoemulsions with pure LCT in the oil phase had a similar β-carotene bioaccessibility compared to those shown by low-fat nanoemulsions, which was 79% and 85% respectively. In the high fat systems, β-carotene bioaccessibility decreased as the LCT content in the oil phase increased from 0 to 50%, but then it increased when the LCT content was increased from 50 to 100%. This behavior differs from the progressive increase in β-carotene bioaccessibility observed in the low fat systems with increasing LCT content. The complex pattern observed might be explained by several competing phenomena. On the one hand, the decrease of β-carotene bioaccessibility when LCT content was increased up to 50% might be due to a significant amount of β-carotene entrapped within the non-digested oil droplets, thus leading to a decrease on the solubilized β-carotene in the mixed micelles (Nik et al., 2011). On the other hand, the increase in β-carotene bioaccessibility when LCT content was further increased up to 100% was attributed to the high global amount of FFAs available after digestion. Despite the low oil digestibility for nanoemulsions with high-fat content and pure LCT oil phase (33%), it can be suggested that carotenoid molecules were able to move from the non-digested oil phase and into the micelle phase.

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CONCLUSIONS

CONCLUSIONS

Based on the results observed in the present research work and from their interpretation, the following statements can be concluded:

Antimicrobial nanoemulsions

- Ultrasounds are a feasible technology to produce essential oil-loaded nanoemulsions with droplet sizes in the nano-scale. The emulsion droplet size decreased at increasing sonication amplitude and time, up to values below 10 nm after sonication at 100 μm for 180 s. However, the antimicrobial activity of the nanoemulsions decreased gradually with the increase of sonication amplitude and time, causing the total loss of bactericidal action after processing at 100 μm for 180 s.

- Microfluidization processing parameters, being pressure and number of cycles, significantly influence the nanoemulsion droplet size and antimicrobial activity. The droplet size of nanoemulsions was reduced up to values around 10 nm with a microfluidization treatment conducted 150 MPa and 3 cycles. Moreover, the antimicrobial properties of microfluidized nanoemulsions were improved compared with the conventional emulsions at the same essential oil concentration (1% v/v), showing a faster and higher microbial inactivation.

- The concentration of each individual component in the mixture determines the final physicochemical properties of the emulsions or nanoemulsions. The droplet size of the mixture was mainly determined by the oil and surfactant concentration, since the formation of nanoemulsions with droplet sizes below 100 nm was achieved with a lemongrass oil concentration lower than 0.6 % (v/v) and surfactant concentration higher than 0.9 % (Tween 80, v/v). Nevertheless, the addition of sodium alginate as a thickening agent in the mixture contributed to stabilize nano-sized droplets.

- The antimicrobial activity of nanoemulsions is determined by the essential oil type used in the formulation rather than by their droplet size. Despite all essential oils (lemongrass, clove, tea tree, thyme, geranium, majoram, palmarosa, rosewood, sage or mint) were able to form nanoemulsions with droplet sizes smaller than 20 nm after microfluidization (150 MPa, 3 cycles), only lemongrass or clove essential oil-

loaded nanoemulsions showed an enhanced antimicrobial activity compared with the conventional emulsions.

- The application of nanoemulsion-based edible coatings containing lemongrass essential oil at low concentration (0.1 % v/v) can better control the microbial growth and the quality decay of fresh-cut Fuji apples, as well as show a higher bacterial inactivation compared with edible coatings formed from conventional emulsions with the same essential oil concentration.

Bioactive nanoemulsions

- The emulsion droplet size has an important role in the behavior of nanoemulsions loaded with β-carotene in the gastrointestinal tract. Nanoemulsions showed a higher degree of triglyceride digestibility due to their higher active surface area exposed to lipase activity compared with emulsions with larger droplet size, which resulted in an enhanced β-carotene *in vitro* bio-accessibility.

- The overall extent of triglyceride lipolysis after digestion is influenced by oil composition, diminishing with increasing the long chain triglycerides and with decreasing the medium chain triglycerides in the lipid phase, regardless the oil concentration of nanoemulsions.

- For low-fat nanoemulsions (1 % oil, w/w), the β-carotene *in vitro* bio-accessibility is increased with increasing long chain triglycerides content in the lipid phase, which can be attributed to a greater solubilization capacity of mixed micelles containing long chain fatty acids. Increasing the concentration of oil up to 4 % (w/w) was effective to enhance the *in vitro* bio-accessibility β-carotene contained in nanoemulsions with predominance of medium chain triglycerides in the lipid phase.

CONCLUSIONES

En base a los resultados observados en el presente proyecto y de su interpretación, se deducen las siguientes conclusiones:

Nanoemulsiones antimicrobianas:

- Los ultrasonidos son una tecnología efectiva para la obtención de nanoemulsiones conteniendo aceites esenciales con un tamaño de partícula comprendido en el rango nanométrico. El tamaño de partícula disminuyó hasta valores inferiores a 10 nm al incrementar la amplitud de onda y el tiempo de sonicación a 100 μm durante 180 s. Sin embargo, la actividad antimicrobiana de las nanoemulsiones disminuyó gradualmente al incrementar la amplitud de onda y el tiempo de sonicación, alcanzándose la pérdida total de dicha actividad tras un tratamiento a 100 μm durante 180 s.

- Los parámetros de microfluidización, presión y número de ciclos, tienen una influencia significativamente en el tamaño de partícula y actividad antimicrobiana de las nanoemulsiones. El tamaño de partícula disminuyó hasta valores de alrededor de 10 nm tras un tratamiento de 3 ciclos de microfluidización a 150 MPa. Además, las propiedades antimicrobianas de las nanoemulsiones obtenidas mediante microfluidización mejoraron en comparación con las de emulsiones convencionales con la misma concentración de aceite esencial (1% v/v), mostrando una mayor y más rápida inactivación microbiana.

- La composición de las nanoemulsiones presenta un efecto determinante sobre sus propiedades físico-químicas. La concentración de aceite y de surfactante afectó significativamente al tamaño de partícula. De este modo, se obtuvieron nanoemulsiones con tamaños de partícula inferiores a 100 nm con una concentración de aceite esencial inferior a 0,6% (v/v) y de surfactante superior a 0,9% (Tween 80, v/v). No obstante la presencia de alginato de sodio como agente texturizante contribuyó a la estabilización de las nanoemulsiones.

- El tipo de aceite esencial utilizado en la formulación y, en menor medida, el tamaño de partícula de las nanoemulsiones, pueden determinar su actividad antimicrobiana. A pesar de que todos los aceites esenciales evaluados (de citronela, clavo, árbol de

té, tomillo, geranio, mejorana, palmarosa, palo de rosa, salvia o menta) formaron nanoemulsiones con tamaños de partícula inferiores a los 20 nm después de un tratamiento de microfluidización (150 MPa, 3 ciclos), solamente las nanoemulsiones conteniendo aceite esencial de citronela o clavo presentaron una mejora en la actividad antimicrobiana en comparación con las respectivas emulsiones convencionales.

- La aplicación de recubrimientos comestibles formados a partir de nanoemulsiones de aceite esencial de citronela a baja concentración (0,1% v/v) permite controlar de manera más efectiva la pérdida de calidad en manzana Fuji fresca cortada, además de causar una mayor inactivación bacteriana en comparación con la aplicación de recubrimientos comestibles formados a partir de emulsiones convencionales conteniendo la misma concentración de aceite esencial.

Nanoemulsiones bioactivas:

- El tamaño de partícula tiene un papel importante en el comportamiento de las nanoemulsiones conteniendo β-caroteno a lo largo de la diferentes etapas simulando las fases digestivas. Se observó una mayor digestibilidad de los triglicéridos en las nanoemulsiones debido a su mayor superficie activa expuesta a la actividad de la lipasa en relación con las emulsiones con mayor tamaño de partícula, lo cual se tradujo en un aumento en la bioaccesibilidad *in vitro* del β-caroteno.

- La composición de la fase lipídica puede influenciar el grado de digestibilidad de los triglicéridos, disminuyendo al incrementar la proporción de triglicéridos de cadena larga y al disminuir la proporción de triglicéridos de cadena media en la fase lipídica, con independencia de la concentración de aceite en las nanoemulsiones.

- En el caso de las nanoemulsiones con bajo contenido en aceite (1% w/w), la bioaccesibilidad *in vitro* del β-caroteno puede incrementar al aumentar la proporción de triglicéridos de cadena larga en la fase lipídica, lo cual puedo ser atribuido a una mayor capacidad de solubilización del β-caroteno en las micelas formadas por ácidos grasos de cadena larga. El aumento de la concentración de aceite en la formulación de las nanoemulsiones hasta un 4% (w/w) mejoró la bioaccesibilidad *in vitro* del β-caroteno contenido en nanoemulsiones con un mayor contenido de triglicéridos de cadena media en la fase lipídica.

CONCLUSIONS

D'acord amb els resultats observats en el present treball i de la seva interpretació, es poden deduir les següents conclusions:

Nanoemulsiones antimicrobianes:

- Els ultrasons són efectius per a la obtenció de nanoemulsions amb olis essencials amb una mida de partícula en el rang nanomètric. La mida de partícula va disminuir fins a valors inferiors a 10 nm al augmentar l'amplitud d'ona i el temps de sonicació fins a 100 μm durant 180 s. No obstant, l'activitat antimicrobiana de les nanoemulsions va disminuir al incrementar l'amplitud d'ona i el temps de sonicació, arribant a la pèrdua total de dita activitat després d'aplicar un tractament a 100 μm durant 180 s.

- Els paràmetres de microfluidització, pressió i el número de cicles, influencien de manera significativament la mida de partícula i l'activitat antimicrobiana de les nanoemulsions. La mida de partícula va disminuir fins a valors inferiors als 10 nm després d'un tractament de 3 cicles de microfluidització a 150 MPa. A més, les propietats antimicrobianes de les nanoemulsions obtingudes per microfluidització es van millorar en comparació amb les de emulsions convencionals amb la mateixa concentració d'oli essencial (1% v/v), ja que van presentar una major i més ràpida inactivació microbiana.

- La composició de les nanoemulsions presetnta un efecte determinant sobre les seves propietats fisicoquímiques. La concentració d'oli i de surfactant van afectar significativament la mida de partícula. Així, es van obtenir nanoemulsions amb una mida de partícula inferior als 100 nm amb una concentració d'oli essencial inferior a 0,6% (v/v) i de surfactant superior a 0,9% (Tween 80, v/v). No obstant, la presència d'alginat sòdic com agent texturitzant va contribuir positivament a l'estabilització de les nanoemulsions.

- L'activitat antimicrobiana de les nanoemulsions és determinada principalment pel tipus d'oli essencial utilitzat en la formulació i, en menor mesura, per la seva mida de partícula. Tot i que tots els olis essencials estudiats (de citronel·la, clau, arbre de té, farigola, gerani, majorana, palma-rosa, pal de rosa, sàlvia o menta) van formar

nanoemulsions amb una mida de partícula inferior a 20 nm després de ser tractades amb microfluidització (150 MPa, 3 cicles), només les nanoemulsions formulades amb oli essencial de citronel·la o clau van presentar una millora en l'activitat antimicrobiana en comparació amb les respectives emulsions convencionals.

- L'aplicació de recobriments comestibles formats a partir de nanoemulsions amb oli essencial de citronel·la a baixa concentració (0,1% v/v) permeten controlar de manera més efectiva la pèrdua de qualitat en poma Fuji fresca tallada, a més de causar una major inactivació bacteriana en comparació amb l'aplicació de recobriments comestibles formats a partir d'emulsions convencionals amb la mateixa concentració d'oli essencial.

Nanoemulsiones bioactives:

- La mida de partícula té un paper important en el comportament de les nanoemulsions amb β-carotè durant la simulació de les fases del tracte gastrointestinal. Es va observar una major digestibilitat dels triglicèrids en les nanomemulsions en comparació amb les emulsions convencionals, degut a la seva major superfície activa exposada a l'activitat de la lipasa, la qual cosa es va traduir en un augment en la bioaccessibilitat *in vitro* del β-carotè.

- La composició de la fase lipídica influencia de manera significativa el grau de digestibilitat dels triglicèrids, disminuint al incrementar la proporció de triglicèrids de cadena llarga i al reduir la de triglicèrids de cadena mitja en la fase lipídica, amb independència de la concentració d'oli en les nanoemulsions.

- En el cas de les nanoemulsions amb un baix contingut en oli (1% w/w), la bioaccessibilitat *in vitro* del β-carotè incrementa al augmentar la proporció de triglicèrids de cadena llarga en la fase lipídica, el qual es pot atribuir a una major capacitat de solubilització del β-carotè en les micel·les formades per àcids grassos de cadena llarga. L'augment de la concentració d'oli en la formulació de les nanoemulsions fins a un 4% (w/w) va millorar la bioaccessibilitat *in vitro* del β-carotè en les nanoemulsions amb un major contingut en triglicèrids de cadena mitja en la fase lipídica.