



Lipid oxidation in high fat omega-3 delivery emulsions

Yesiltas, Betül

Publication date:
2019

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):
Yesiltas, B. (2019). *Lipid oxidation in high fat omega-3 delivery emulsions*. Technical University of Denmark.

General rights

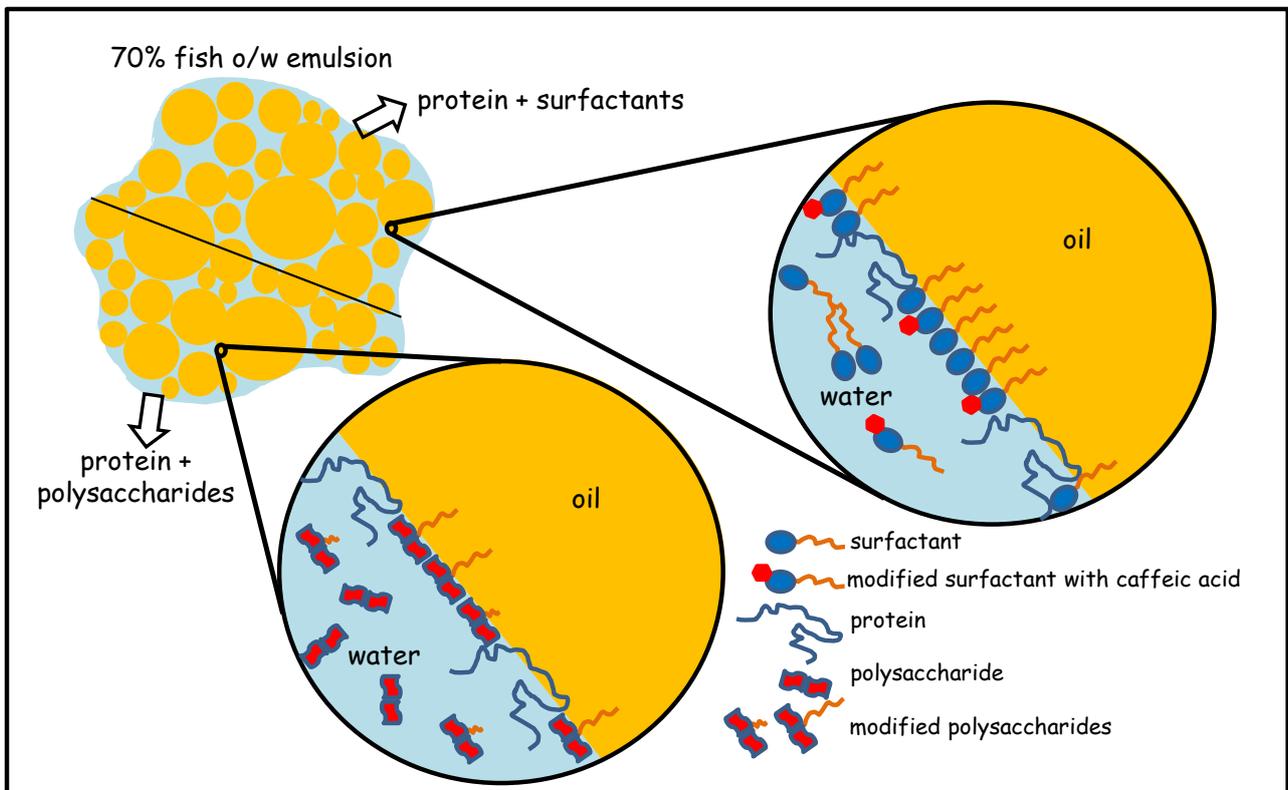
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Lipid oxidation in high fat omega-3 delivery emulsions

Betül Yeşiltaş
 PhD thesis
 2019



LIPID OXIDATION IN HIGH FAT OMEGA-3 DELIVERY EMULSIONS

Ph.D. Thesis by Betül Yesiltas

National Food Institute

Technical University of Denmark

January 2019, Kgs. Lyngby

PREFACE

This Ph.D. project entitled “Physical and oxidative stability of high fat omega-3 delivery emulsions” was carried out at the National Food Institute (DTU Food), Technical University of Denmark (DTU) and was supervised by Professor Charlotte Jacobsen (main supervisor), Ann-Dorit M. Sørensen and Pedro J. García-Moreno from DTU Food. This PhD project started in December 2015 and continued until January 2019 without any interruptions.

This PhD project was part of a larger project called Mapping and Characterizing Lipid Oxidation in Emulsified Systems (MAPOX), which was funded by The Danish Council for Independent Research. MAPOX project was carried out in collaboration with Aarhus University (AU), Penn State University (PSU) and a company called inOmega3. The overall aim of MAPOX was to produce emulsion systems, which can provide effective delivery of omega-3 PUFAs with extra-high loading capacity (50-70% oil). Therefore, the developing of cutting-edge techniques for monitoring lipid oxidation in both time and space in emulsion systems, the design and synthesis of multifunctional emulsifiers and stabilizers, and further study and characterization of the oil-water interface properties of these molecules by advanced spectroscopic and microscopy techniques were carried out.

The aim of this Ph.D. project was to understand the mechanism behind lipid oxidation in complex emulsion systems as well as to develop both physically and oxidatively stable high fat (50-70%) omega-3 delivery fish oil-in-water emulsions. Furthermore, employment of tailor-made multifunctional emulsifiers in the developed physically and oxidatively stable high fat omega-3 delivery oil-in-water emulsions was studied. Finally, the enrichment of a real food system namely mayonnaise by incorporating these physically and oxidatively stable high fat omega-3 delivery emulsions was investigated.

During the Ph.D. project period, I have carried out a research stay at the Food Science Department, Pennsylvania State University, which was supported by a research grant from Otto Mønsted Foundation. I have attended several conferences for the dissemination of my Ph.D. studies as oral and poster presentations, some of them were supported with travel grants by the New York Academy of Sciences and Otto Mønsted Foundation.

Betül Yesiltas

7th of January, 2019

Kgs. Lyngby, Denmark

ACKNOWLEDGEMENTS

Completion of this Ph.D. study could not be possible without the support of numerous people, whom I would like to mention here and express my sincere appreciation.

First of all, I would like to thank to my main supervisor, Charlotte Jacobsen, who has always encouraged me during my Ph.D. study. I am so grateful to her for motivating me continuously, finding time whenever I needed her opinion and sharing her immense knowledge. Her presence and insights have not only contributed to my professional life, but also at a personal level, I have learnt so much while working with her. I could not have imagined a better mentor.

My co-supervisors, Ann-Dorit M. Sørensen and Pedro J. García-Moreno, I consider myself very fortunate to have them as a constant support throughout my Ph.D. study. Our fruitful discussions and their experience in the field have contributed to this work invaluable. Moreover, I would also like to thank them so much for always being there as a friend as well.

I would like to thank to our partners in the MAPOX project, Peter Ogilby, Zheng Guo, Chiranjib Banerjee, Sampson Anankanbil, Thomas Breitenbach for the collaboration and scientific discussions during our meetings. I would also like to express my sincere thanks to Matti Knaapila and Mika Torkkeli for their collaboration in the application of small angle X-ray/neutron scattering techniques as well as their patience for teaching me how to handle the data obtained from these techniques. Moreover, sincere thanks to my supervisors at Penn State University, John Coupland and Ryan Elias, my colleague, Wai Fun Leong, as well as very helpful staff and students in Food Science Department of Penn State for their constant support and providing me a great environment to do my research using electron paramagnetic resonance spectroscopy during my stay. I would also like to thank to Grethe Hyldig and Rie Sørensen for their skillful work for conducting the sensory analysis on mayonnaise samples.

Many thanks go to Lis Berner, Inge Holmberg, Thi Thu Trang Vu, Heidi Jahn and Riuyinosa Igbinovia for their technical assistance and skillful work in the lab as well as sparing no effort in helping me whenever I needed. Moreover, their presence always feels great. Thanks to my students and technician trainees, Ioanna Anagnostara, Alyssa Marie Soria Caindec, Sevda Ekici, and Yuka Omura Lund, it was a great pleasure to get the chance to work with them. Each of my colleagues in “Bioactives” group deserves my sincere thanks for creating a great working environment and the

feeling of a big family. I always regard myself very fortunate to get the chance to work with each of them.

Thanks to my former and current office mates in the ‘aquarium office’ for your friendship and good mood. Special thanks go to my dear friend Petra, I am so grateful to have her not only as a great colleague, but also as a lifelong friend. It is so soon we will make ‘börek’ again!

I would like to thank to my friends in Turkey, who were still a big part of my life during my Ph.D. journey. My forever friends Caner and Bilgenur, thank you for always being there for me! Even though we hardly get to spend time together, their presence and our memories are enough to warm my heart! Feyza and Sinem, my former colleagues at Unilever and forever friends, they hold a special place in my heart, I am so thankful for having them in my life and for their constant support since the beginning of my Ph.D. Thanks to my dear friends from my Bachelor’s degree who I studied Food Science together and kept in touch since then, especially Hatice, Abbasov, Ulas and other members of ‘apaci gida’ for our hilarious memories and friendship.

My former flatmate, Johannes, who is moving back soon, I am thankful for his forever good mood and continuous encouragement during my Ph.D.! My friends, who have been living in Denmark since we met during my Erasmus exchange, Maria, Fede, Manu and Dimitris, I would like to thank them for their heartwarming friendship! Thanks to Asli, for our never ending and long conversations about...let’s say ‘life’. Thanks to my dear friends, Eric, Ady, Michele, Tim, Bethany, Emily, and Irene who made my stay in State college a fun experience! My dear friends from dancing, especially, Alex, Allan, and Steen, who never give up inviting me to go to listen to music and dance at Charlie Scott’s. All the people I met while travelling during my Ph.D., who showed interest in what I am doing and encouraged me to continue... Thank you very much!

My ‘Golden Girls’, Gamze and Hande, who were always with me regardless of the physical distance between us. Even when I lived far away, we managed to be in touch almost every day! They will always have a special place in my heart.

Lastly, but maybe the most importantly, I would like to express special thanks to my family; my brother, Murat, my mum, Emine, and my dad, İsmail for continuously supporting me throughout my life, as well as always believing in me in all my decisions and future plans. Without their love, the whole journey would be incomplete. Thank you!

Betül Yesiltas

SUMMARY

Intake of long chain n-3 polyunsaturated fatty acids (LC n-3 PUFAs) is inadequate in most Western populations due to the low consumption of fish and other seafood products. These long chain omega-3 fatty acids such as EPA (eicosapentaenoic acid, C20:5n-3) and DHA (docosahexaenoic acid, C22:6n-3) have been reported to be beneficial to health. Therefore, strategies for enrichment of food with LC n-3 PUFAs have been investigated by food scientists in order to comply with the recommended daily intake for EPA and DHA.

LC n-3 PUFAs are highly prone to lipid oxidation in the presence of heat, metal ions and oxygen. Oxidation of LC n-3 PUFAs results in loss of nutritious fatty acids, undesired off-flavors, and rancid/ fishy taste. Oil-in-water delivery emulsion systems have been proposed and investigated as one of the strategies to protect these lipophilic bioactive compounds. Previous studies were mainly focused on low fat n-3 delivery oil-in-water emulsion, regardless of the great potential of the high fat n-3 delivery oil-in-water emulsion systems (e.g. for the enrichment of highly viscous foods).

The focus of this Ph.D. study was mainly on the impact of emulsion composition and combination of multifunctional emulsifiers on oil-water interfacial structure and emulsifier distribution, as well as their influence on physical and oxidative stability of high fat fish oil-in-water emulsions. The potential of X-ray and neutron techniques as well as electron paramagnetic resonance in providing useful information about the interface structure and partitioning of emulsifiers, respectively, has also been demonstrated in this study.

The fish oil content, total emulsifier content, and the ratio between emulsifiers (sodium caseinate, CAS, combined with sodium alginate, ALG, or diacetyl tartaric acid esters of mono- and diglycerides, DATEM, or phosphatidylcholine, PC) affected physical and oxidative stability of the high fat fish oil-in-water emulsions. Combinations of CAS and ALG provided high viscosity and creaming stability for the high fat emulsions, whereas combinations of CAS and DATEM or PC yielded emulsions with lower viscosity and creaming stability compared to ALG.

The effect of homogenizer type on physical and oxidative stability of high fat fish oil-in-water emulsions was investigated. Emulsions stabilized with CAS and DATEM or PC and produced with colloid mill resulted in higher viscosity, less creaming and smaller droplet sizes compared to emulsions produced with Stephan mixer. This indicated that emulsions produced with colloid mill provided better physical stability. However, emulsions produced with Stephan mixer had higher oxidative stability compared to those produced with colloid mill when evaluated by the formation of primary and secondary lipid oxidation products. This was mainly attributed to larger droplets and

creaming of emulsions produced with Stephan mixer yielding less contact between prooxidants and lipids.

Surface activity of a commonly used thickener, ALG, was enhanced by the modification with short and long alkyl chains. Combination of the short chain modified ALG and CAS improved both physical and oxidative stability of the high fat fish oil-in-water emulsions compared to emulsions stabilized with CAS. On the other hand, combined use of long chain modified ALG and CAS did not improve the oxidative stability of the high fat fish oil-in-water emulsion, even though physical stability was significantly improved resulting in smaller droplets and higher viscosity.

Antioxidant and surface activity of DATEM and PC were improved by the addition of caffeic acid to the glycerol backbone and by the attachment of alkyl chains at various lengths, which had different effects on physical and oxidative stability of high fat fish oil-in-water emulsions. Emulsions produced with modified DATEMs showed better oxidative stability compared to emulsion with commercial DATEM plus an equivalent amount of free caffeic acid, confirming the advantage of covalent attachment of caffeic acid to the emulsifier. Modified DATEM with C14 alkyl chain (DATEM C14) replaced more CAS from the interface in 70% fish oil-in-water emulsion compared to modified DATEM with C12 alkyl chain (DATEM C12). Furthermore, emulsions produced with DATEM C14 had significantly decreased amounts of primary and secondary oxidation products compared to emulsions containing DATEM C12. This was mainly attributed to the higher concentration of the antioxidant DATEM C14 compared to DATEM C12 at the oil-water interface. Emulsion stabilized with modified PC with C14 alkyl chain (PC C14) led to smaller droplets and higher viscosity, whereas modified PC with C16 alkyl chain (PC C16) had higher protein surface load, which resulted in a thicker interfacial layer. Modified PCs enhanced oxidative stability compared to emulsions with PC and free caffeic acid due to the attachment of caffeic acid, which brings the antioxidant in the vicinity of interface. PC C16 led to higher oxidative stability compared to PC C14, mainly explained by the thicker interfacial layer provided by PC C16.

Finally, incorporation of n-3 delivery 70% fish oil-in-water emulsions produced with modified DATEMs into mayonnaise was investigated. The physical stability of the mayonnaises enriched with delivery emulsions was low due to the pH adjustment between delivery emulsion and mayonnaise. However, mayonnaise containing high fat emulsions stabilized with combinations of CAS and modified DATEMs had higher oxidative stability compared to mayonnaises produced with added neat fish oil, indicating the feasibility of using high fat fish oil in water emulsions to enrich food systems such as mayonnaise.

RESUMÉ

Indtaget af langkædede n-3 flerumættede fedtsyrer (LC n-3 PUFA) er utilstrækkelig i de fleste befolkninger i den vestlige verden på grund af det lave forbrug af fisk og andre fiskeprodukter. Disse langkædede omega-3 fedtsyrer, især EPA (eicosapentaensyre, C20: 5n-3) og DHA (docosahexaensyre, C22: 6n-3) er blevet rapporteret at være gavnlige for helbredet. I litteraturen er rapporteret forskellige strategier til at berige fødevarer med LC n-3 PUFA mhp. at opnå det anbefalede daglige indtag af EPA og DHA.

LC n-3 PUFA er meget tilbøjelige til at oxidere i tilstedeværelse af varme, metalioner og ilt. Oxidering af LC n-3 PUFA resulterer i tab af de sunde fedtsyrer og en uønsket harsk / fiskeagtig smag. Olie-i-vand delivery emulsionssystemer er blevet foreslået og undersøgt som en af strategierne til beskyttelse af disse lipofile bioaktive forbindelser. Tidligere undersøgelser har primært fokuseret på lavfedt n-3-olie-i-vand-emulsioner, på trods af det store potentiale i olie-i-vand-emulsionssystemer med højt fedtindhold (f.eks. til berigelse af højviskøse fødevarer). Dette ph.d. studie har primært fokuseret på effekten af emulsionssammensætningen og forskellige kombinationer af multifunktionelle emulgatorer på olie-vand-grænsefladestruktur og emulgatorfordeling samt deres indflydelse på fysisk og oxidativ stabilitet af højfedtholdige fiskeolie-i-vand-emulsioner. Dette ph.d studie har også demonstreret potentialet i at anvende røntgen og neutron scattering teknikker samt elektron paramagnetisk resonans (EPR) til at opnå brugbar information om grænsefladestrukturer og fordeling af emulgatorer imellem de forskellige faser i emulsionen.

Det blev konstateret, at indholdet af fiskeolie, total emulgatorindhold og forholdet mellem emulgatorer (natriumkaseinat, CAS, i kombination med natriumalginat, ALG, eller diacetylvinysyreestere af mono- og diglycerider, DATEM, eller phosphatidylcholin, PC) påvirkede den fysiske og oxidative stabilitet af fiskeolie-i-vand emulsioner med højt fedtindhold. Kombinationen af CAS og ALG resulterede i emulsioner med høj viskositet og fysisk stabilitet, mens kombinationen af CAS og DATEM eller PC gav emulsioner med lavere viskositet og fysisk stabilitet sammenlignet med ALG.

Effekten af homogenisatorstype på fysisk og oxidativ stabilitet af højfedtholdige fiskeolie-i-vand-emulsioner blev undersøgt. Emulsioner stabiliseret med CAS og DATEM eller PC og produceret med kolloidmølle resulterede i højere viskositet, mindre grad af creaming og mindre dråbestørrelser sammenlignet med emulsioner fremstillet med Stephan-mixer. Dette indikerede, at emulsioner produceret med kolloidmølle resulterede i bedre fysisk stabilitet. Imidlertid havde emulsionerne fremstillet med Stephan-mixeren højere oxidativ stabilitet, end dem der blev fremstillet med kolloidmølle, når oxidationen blev evalueret ved måling af dannelsen af primære og sekundære

lipidoxiderationsprodukter. Dette skyldtes hovedsageligt større dråber og creaming i emulsionerne fremstillet med Stephan-mixeren, hvilket gav en mindre kontakt mellem prooxidanter og lipider.

Overfladeaktiviteten af et almindeligt anvendt fortykningsmiddel, ALG, blev forbedret ved modifikation med korte og lange alkylkæder. Kombinationen af den kortkædede modificerede ALG og CAS forbedrede både fysisk og oxidativ stabilitet af emulsionerne sammenlignet med emulsioner stabiliseret med CAS. På den anden side forbedrede kombineret brug af langkædede modificerede ALG og CAS ikke den oxidative stabilitet af emulsionerne, selvom den fysiske stabilitet blev signifikant forbedret, hvilket resulterede i mindre dråber og højere viskositet.

Antioxidant- og overfladeaktivitet af DATEM og PC blev forbedret ved påsætning af koffeinsyre på glycerol-skelettet og ved vedhæftning af alkylkæder af forskellige længder, som havde forskellige virkninger på den fysiske og oxidative stabilitet af højfedtholdige fiskeolie-i-vand-emulsioner. Emulsioner produceret med modificeret DATEM viste bedre oxidativ stabilitet sammenlignet med en emulsion, hvor der var anvendt kommerciel DATEM plus en ækvivalent mængde fri koffeinsyre. Disse resultater bekræfter fordelene ved kovalent binding af koffeinsyre til emulgatoren. Modificeret DATEM med C14 alkylkæde (DATEM C14) erstattede mere CAS på olie-vand grænsefladen i 70% fiskeolie-i-vand emulsioner sammenlignet med modificeret DATEM med C12 alkylkæde (DATEM C12). Desuden havde emulsioner fremstillet med DATEM C14 signifikant lavere indhold af primære og sekundære oxidationsprodukter efter lagring sammenlignet med emulsioner fremstillet med DATEM C12. Dette skyldes hovedsageligt den højere koncentration af den antioxidative DATEM C14 på olie-vand grænsefladen sammenlignet med DATEM C12. Emulsionen stabiliseret med modificeret PC med C14 alkylkæde (PC C14) havde mindre dråber og højere viskositet, mens modificeret PC med C16 alkylkæde (PC C16) havde højere proteinload på grænsefladen, hvilket resulterede i et tykkere grænsefladelag. Anvendelsen af modificeret PC førte til en forbedret oxidativ stabilitet af emulsionerne sammenlignet med emulsioner med PC og fri koffeinsyre på grund af den kovalente binding af koffeinsyre til PC molekylet, hvilket bringer antioxidant i nærheden af grænsefladen. PC C16 førte til lavere dannelse af primær og sekundære oxidationsprodukter end PC14, hvilket primært kunne forklares med, at PC16 førte til et tykkere grænsefladelag.

Til slut blev inkorporering af n-3 delivery fiskeolie-i-vand emulsioner - produceret med modificeret DATEM – i mayonnaise undersøgt. Den fysiske stabilitet af mayonnaiser beriget med delivery emulsioner var lav pga. forskelle i pH mellem delivery emulsioner og mayonnaise. Ikke desto mindre havde mayonnaise indeholdende højfedtholdige emulsioner stabiliseret med en kombination af CAS og modificeret DATEM højere oxidativ stabilitet sammenlignet med mayonnaise produceret med kun fiskeolie, hvilket indikerer, at der er fordele ved at bruge højfedtholdige fiskeolie-i-vand emulsioner til at berige fødevarer såsom mayonnaise.

LIST OF PUBLICATIONS

- Paper I **Yesiltas, B.,** García-Moreno, P. J., Sørensen, A. M., Jacobsen, C., **2017.** Physical and oxidative stability of high fat fish oil-in-water emulsions stabilized with combinations of sodium caseinate and sodium alginate. *European Journal of Lipid Science and Technology*, *119*, 1600484.
- Paper II **Yesiltas, B.,** García-Moreno, P. J., Sørensen, A. M., Akoh, C. C., Jacobsen, C., **2019.** Physical and oxidative stability of high fat fish oil-in-water emulsions stabilized with sodium caseinate and phosphatidylcholine as emulsifiers. *Food Chemistry*, *276*, 110–118.
- Paper III **Yesiltas, B.,** Torkkeli, M., Almasy, L., Dudás, Z., Dalgliesh, R., Wacha, A. F., García-Moreno, P. J., Sørensen, A-D. M., Jacobsen, C., Knaapila, M., **2018.** Interfacial structure of 70% fish oil-in-water emulsions stabilized with combinations of sodium caseinate and phosphatidylcholine. Submitted to *Colloids And Surfaces B: Biointerfaces*.
- Paper IV **Yesiltas, B.,** Sørensen, A. M., García-Moreno, P. J., Anankanbil, S., Guo, Z., Jacobsen, C., **2018.** Combination of sodium caseinate and succinylated alginate improved stability of high fat fish oil-in-water emulsions. *Food Chemistry*, *255*, 290–299.
- Paper V **Yesiltas, B.,** García-Moreno, P. J., Sørensen, A. M., Anankanbil, S., Guo, Z., Jacobsen, C., **2018.** Effects of modified DATEMs with different alkyl chain lengths on improving oxidative and physical stability of 70% fish oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, *66*, 12512-12520.
- Paper VI **Yesiltas, B.,** Sørensen, A. M., García-Moreno, P. J., Anankanbil, S., Guo, Z., Jacobsen, C., **2018.** Modified phosphatidylcholine with different alkyl chain length and covalently attached caffeic acid affects the physical and oxidative stability of omega-3 delivery 70% oil-in-water emulsions. Submitted to *Food Chemistry*.

LIST OF ATTENDED CONFERENCES*Oral presentations***American Oil Chemists' Society Annual Meeting, Orlando, Florida, USA**

Yesiltas, B., Sørensen, A. M., García-Moreno, P. J., Anankanbil, S., Guo, Z., Jacobsen, C. Oxidative and physical stability of fish oil-in-water emulsions stabilized with sodium caseinate and modified alginate. 2017, April 30-May 3.

2nd International Symposium on Lipid Oxidation and Antioxidants, Graz, Austria

Yesiltas, B., García-Moreno, P. J., Sørensen, A. M., Anankanbil, S., Guo, Z., Jacobsen, C. Effects of modified DATEMs with different alkyl chain lengths on improving oxidative and physical stability of 70% fish oil-in-water emulsions. 2018, June 4-6.

Neutrons and Food 2018, Sydney, Australia

Yesiltas, B., Torkkeli, M., Almasy, L., Dudás, Z., Dalgliesh, R., Wacha, A. F., García-Moreno, P. J., Sørensen, A.-D. M., Jacobsen, C., Knaapila, M. Interface structure of 70% fish oil-in-water emulsions stabilized with combinations of sodium caseinate and phosphatidylcholine: use of small angle neutron and X-ray scattering techniques. 2018, Oct 16-19.

*Poster presentations***10th Central European Training School on Neutron Techniques, Budapest, Hungary**

Yesiltas, B., Almásy, L., García-Moreno, P. J., Sørensen, A. M., Jacobsen, C., Knaapila, M. Partitioning of sodium caseinate and phosphatidylcholine in high fat (70%) delivery fish oil-in-water emulsions - a candidate for small angle neutron scattering (SANS) studies. 2016, May 2-6.

1st International Symposium on Lipid Oxidation and Antioxidants, Euro Fed Lipid, Porto, Portugal (Best poster award)

Yesiltas, B., García-Moreno, P. J., Sørensen, A. M., Jacobsen, C. Lipid oxidation in high fat (50-70%) omega-3 delivery fish oil-in-water emulsions prepared with sodium caseinate and sodium alginate as emulsifiers. 2016, June 5-7.

American Oil Chemists' Society Annual Meeting, Orlando, Florida, USA

Yesiltas, B., García-Moreno, P. J., Sørensen, A.M., Jacobsen, C. Oxidative and physical stability of fish oil-in-water emulsions stabilized with sodium caseinate and DATEM. 2017, April 30-May 3.

“Journey through Science Day” at New York Academy of Sciences, New York City, New York, USA

Yesiltas, B., García-Moreno, P. J., Sørensen, A.M., Jacobsen, C. Oxidative and physical stability of fish oil-in-water emulsions stabilized with sodium caseinate and DATEM. 2017, September 18.

17th Food Colloids Conference : Application of Soft Matter Concepts, The University of Leeds, Leeds, UK

Yesiltas, B., Sørensen, A. M., García-Moreno, P. J., Anankanbil, S., Guo, Z., Jacobsen, C. Effects of modified DATEMs with different alkyl chain lengths on improving oxidative and physical stability of 70% fish oil-in-water emulsions. 2018, April 8-11.

American Oil Chemists' Society Annual Meeting, Minneapolis, Minnesota, USA

Yesiltas, B., Sørensen, A. M., García-Moreno, P. J., Jacobsen, C. Physical and oxidative stability of 50-70% fish oil-in-water emulsions stabilized with sodium caseinate and phosphatidylcholine. 2018, May 6-9.

The Nordic Lipid Forum, Ålesund, Norway

Yesiltas, B., García-Moreno, P. J., Sørensen, A. M., Anankanbil, S., Guo, Z., Jacobsen, C. Effects of modified DATEMs with different alkyl chain lengths on improving oxidative and physical stability of 70% fish oil-in-water emulsions. 2018, Oct 24-25.

AWARDS AND GRANTS*Grants*

Otto Mønsted Foundation for travelling to a conference “Neutrons and Food 5” in Sydney, Australia (DKK 7.500,00). 2018, Sep 21.

PepsiCo and the New York Academy of Sciences for attending the “Journey through Science Day” in New York City, New York, USA (\$500). 2017, Jul 19.

Otto Mønsted Foundation for travelling to a conference “American Oil Chemists' Society Annual Meeting” in Orlando, Florida, USA (DKK 5.555,00). 2017, Apr 12.

Otto Mønsted Foundation for external stay as a visiting scholar in Penn State University in State College, Pennsylvania, USA (DKK 38.480,00). 2017, Mar 15.

Budapest Neutron Center (BNC) for 2 days of beamtime for Small Angle Neutron Scattering beamline at BNC. 2017, Jan 23.

Central European Training School for attending the “10th Central European Training School on Neutron Techniques” which was held in Budapest, Hungary (Accommodation + 300€ for travelling) 2016, Mar 22.

Awards

Schmidt Science Fellowship nominated as one of the 3 students by Technical University of Denmark and became eligible to apply for the fellowship. 2018, August 31.

Best poster award at the 1st International Symposium on Lipid Oxidation and Antioxidants, Euro Fed Lipid, Porto, Portugal. 2016, June 5-7.

ABBREVIATIONS

<i>AA</i>	antioxidant dimer	<i>LOOH</i>	lipid hydroperoxide
<i>ALG</i>	sodium alginate	<i>M</i>	metal
<i>ALGs</i>	alginates	<i>MAYO</i>	mayonnaise
<i>ANOVA</i>	analysis of variance	<i>MS</i>	mass spectrometry
<i>App.</i>	appendix/appendices	<i>mod</i>	modified
<i>BHA</i>	butylated hydroxyanisole	<i>n-3</i>	omega-3
<i>CAT</i>	conjugated autoxidizable triene	<i>NP</i>	non-adsorbed protein
<i>CAS</i>	sodium caseinate	<i>OH[•]</i>	hydroxyl radical
<i>CI</i>	creaming index	<i>Pap.</i>	paper/papers
<i>CITREM</i>	citric acid esters of monoglycerides	<i>PC</i>	phosphatidylcholine
<i>com</i>	commercial	<i>PCA</i>	principal component analysis
<i>DATEM</i>	diacetyl tartaric acid ester of mono- and diglycerides	<i>PTMIO</i>	4-phenyl-2,2,5,5-tetramethyl-3- imidazoline-1-oxyl nitroxide
<i>DHA</i>	docosahexaenoic acid	<i>PUFAs</i>	polyunsaturated fatty acids
<i>DS</i>	droplet size	<i>PV</i>	peroxide value
<i>TOC</i>	tocopherol	<i>PSL</i>	protein surface load
<i>EPA</i>	eicosapentaenoic acid	<i>RSM</i>	response surface methodology
<i>EPR</i>	electron paramagnetic resonance	<i>RT</i>	room temperature
<i>GC</i>	gas chromatography	<i>SANS</i>	small angle neutron scattering
<i>H</i>	hydrogen	<i>SAXS</i>	small angle X-ray scattering
<i>HLB</i>	hydrophilic – lipophilic balance	<i>SAC0</i>	succinic anhydrate
<i>HMWE</i>	high molecular weight emulsifiers	<i>SAC12</i>	dodecyl succinic anhydrate
<i>H[•]</i>	hydrogen radical	<i>SLN</i>	solid lipid nanoparticle
<i>HO[•]</i>	hydroxyl radical	<i>TEMPO</i>	4-hydroxy-2,2,6,6- tetramethylpiperidine 1-oxyl
<i>HOO[•]</i>	hydroperoxyl radical	<i>V</i>	viscosity
<i>HPLC</i>	high performance liquid chromatography	<i>VC</i>	volatile compounds
<i>I</i>	initiator	<i>ZP</i>	zeta potential
<i>IT</i>	interfacial tension		
<i>LACTEM</i>	lactic acid esters of monoglycerides		
<i>LC</i>	long chain		
<i>LH</i>	unsaturated lipid, reduced lipid		
<i>LMWE</i>	low molecular weight emulsifiers		
<i>L[•]</i>	lipid alkyl radical		
<i>LO[•]</i>	lipid alkoxy radical		
<i>LOO[•]</i>	lipid peroxy radical		
<i>LOH</i>	lipid alcohol		

TABLE OF CONTENT

PREFACE.....	ii
ACKNOWLEDGEMENTS	iii
SUMMARY	v
RESUMÉ.....	vii
LIST OF PUBLICATIONS.....	ix
LIST OF ATTENDED CONFERENCES	x
AWARDS AND GRANTS	xi
ABBREVIATIONS	xii
Chapter 1: Introduction.....	1
1.1. Background	1
1.2. Aim and hypotheses of the thesis.....	3
Chapter 2: Emulsions, emulsifiers, and oil-water interface.....	5
2.1. Emulsions.....	5
2.1.1. Oil-in-water emulsions.....	5
2.2. Emulsifiers	6
2.2.1. High molecular weight emulsifiers (HMWE).....	6
2.2.2. Low molecular weight emulsifiers (LMWE).....	10
2.3. Emulsion formation and stability	14
2.4. Physical stability of emulsions.....	17
2.5. Oil-water interfacial structure of emulsions (SAXS & SANS).....	18
2.6. Distribution of emulsifiers in the emulsion system (EPR).....	21
Chapter 3: Lipid oxidation.....	25
3.1. Autoxidation.....	25
3.2. Volatile secondary oxidation products and their impact on sensory properties.....	27
3.3. Lipid oxidation in oil-in-water emulsions.....	29
3.4. Factors influencing lipid oxidation in complex systems.....	31
Chapter 4: Antioxidants in oil-in-water emulsions.....	35
4.1. Antioxidants	35
4.2. Antioxidant strategies in oil-in-water emulsions	37

4.2.1. Phenolic compounds	37
4.2.2. Phenolipids.....	37
4.2.3. Antioxidant emulsifiers.....	39
4.2.4. Modified emulsifiers with phenolic acids and various lipophilicity	39
Chapter 5: Experimental design and methodology.....	41
5.1. Emulsifiers used in various studies.....	42
5.2. Experimental set up for the optimization studies	43
5.3. Experimental set up for the application of modified emulsifiers	45
5.4. Experimental set up for the application of n-3 delivery system in mayonnaise.....	46
5.5. Evaluation of physical and oxidative parameters.....	47
Chapter 6: Results and discussion	49
6.1. Part I: Effect of emulsion composition and homogenizer type on physical and oxidative stability	49
6.1.1. Overall comparison of the effect of emulsion formulae on physical and oxidative stability	49
6.1.2. Effect of emulsion formulae on physical stability	54
6.1.3. Effect of emulsion formulae on oxidative stability	57
6.1.4. Optimal formulae	61
6.2. Part II: Influence of modified emulsifiers on physical and oxidative stability	61
6.2.1. Effect of modified alginates on physical and oxidative stability (Paper IV)	63
6.2.2. Effect of modified DATEMs and PCs on physical and oxidative stability (Papers V and VI)	64
6.2.3. Principal component analysis of data obtained with modified emulsifiers.....	71
6.3. Part III: Incorporation of delivery emulsions into mayonnaise (Appendix III)	74
6.3.1. Physical stability of mayonnaises during storage	75
6.3.2. Oxidative stability of mayonnaises during storage	77
Chapter 7: Conclusions and perspectives	81
7.1. Conclusions.....	81
7.2. Perspectives.....	84
REFERENCES	87
APPENDICES	i
A.1. Comparison of colloid mill vs. Stephan mixer and effect on physical and oxidative stability	i
A.2. Distribution of labelled DATEMs in high fat emulsions studied by EPR spectroscopy	vi
A.3. Mayonnaises enriched with high fat n-3 delivery oil-in-water emulsions	xi

Chapter 1: Introduction

This chapter gives the background of the Ph.D. study together with aims and hypotheses.

1.1. Background

Marine long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to have health benefits in the last decades. Research studies indicated that consumption of LC n-3 PUFAs has a relationship with reduced risk of cardiovascular diseases and improved mental health, immune system and infant brain development (Song et al., 2016; Wysoczanski et al., 2016; Nichols, et al., 2014). LC n-3 PUFAs are mainly found in fish and fish products and the consumption of these compounds have been inadequate according to daily recommended intake levels in western countries. Therefore, food researchers and food industry have paid increased attention to producing LC n-3 PUFA enriched food products.

Researchers have focused on providing physically and oxidatively stable LC n-3 PUFA delivery systems for various types of food systems such as milk, yogurt, cream cheese, mayonnaise, and salad dressings. However, susceptibility of LC n-3 PUFAs to lipid oxidation brings along some challenges. These challenges consist of undesired sensory properties and loss of nutritional value of the food stuffs enriched with LC n-3 PUFAs. Therefore, new strategies and approaches have been investigated and applied in order to produce physically and oxidatively stable LC n-3 PUFA delivery systems, which can be incorporated into foods.

As a delivery system for LC n-3 PUFAs, fish oil-in-water emulsions have been studied and compared with adding neat fish oil into the food systems. This has mainly been done for low fat content fish oil-in-water emulsions (Let et al., 2007; Berton-Carabin et al., 2014; García-Moreno et al., 2014). However, a wider range of fat loads for a delivery system needs to be developed in order to increase applicability in food enrichment processes. This involves producing high fat delivery systems. One of the advantages of using a high fat emulsion for enrichment of food products is its lower water and higher fat content compared to low fat emulsions, which means that lower amounts of emulsions would be required for enrichment. Moreover, fat content can significantly affect the final physical properties (e.g., viscosity, texture, etc.) of the delivery system, which is a concern for the food industry regarding the enrichment of food systems without changing its original characteristics. Nevertheless, there have only been a few studies focusing on high fat fish oil-in-

water emulsions (Horn et al., 2011). Therefore, the main aim in this Ph.D. study was the development of high fat fish oil-in-water emulsions with adequate physical and oxidative stabilities.

Physical stability of oil-in-water emulsions has critical importance in terms of creaming and phase separation phenomena, due to the fact that the appearance of the delivery system is associated with it. These instabilities may lead to non-homogenous composition when delivery emulsions are incorporated into food systems. Physical stability has an impact on oxidative stability of oil-in-water emulsions. Therefore, maintaining a decent physical stability in high fat oil-in-water emulsions can be a prerequisite for obtaining high oxidative stability. Physical parameters affecting lipid oxidation in high fat emulsions have only been investigated in a few studies, although the physical characteristics of an emulsion system are expected to be different depending on the concentration of the dispersed phase. For instance, high fat emulsions tend to be densely packed with oil droplets and be more viscous compared to low fat oil-in-water emulsions, which affects the behaviour of prooxidants as well as lipid oxidation mechanisms in high fat oil-in-water emulsion systems. Recently, studies have focused on the properties of the interfacial layer surrounding the oil droplets, which is the location where prooxidants come in close proximity to the unsaturated lipids and promote oxidation.

Emulsifiers play an important role in maintaining physical stability, due to their ability to reduce interfacial tension, by being adsorbed at the oil-water interface, which favors oil droplet formation and stabilization. In addition, since emulsifiers are added in excess, a proportion of the emulsifiers will be dispersed in the aqueous phase of the emulsion. Different strategies regarding emulsifier use, such as bioinspired emulsifier combinations, have been suggested in order to stabilize oil-in-water emulsions. Previous studies reported that combined use of emulsifiers enhances the physical and oxidative stability of emulsions compared to a single emulsifier (Guzey and McClements, 2006; Pallandre et al., 2007; Dickinson, 2011; García-Moreno et al., 2014). For example, proteins have been used in combination with surfactants or polysaccharides as emulsifiers in oil-in-water emulsion systems to enhance the properties of the interfacial layer such as thickness, packing density, permeability and antioxidant activity (Fang and Dalgleish, 1996; Wilde et al., 2004; García-Moreno et al., 2014). Therefore, type and concentration of the applied emulsifiers are important factors which have an impact on the physical and oxidative stability of the emulsions.

Recently, new strategies for improving interfacial layer and bringing antioxidant activity to the oil-water interface have been investigated in compartmentalized systems such as oil-in-water

emulsions, membranes, or living cells. One of the novel approaches for achieving this goal is to modify emulsifiers with phenolic acids and various alkyl chain lengths. These modified emulsifiers are expected to have affinity to oil-water interface due to their right hydrophilic-lipophilic balance (HLB); thereby they will locate at the interface and perform also as an antioxidant due to their phenolic acid covalently attached to the emulsifier itself. If present in excess, these emulsifiers are also expected to partition into the aqueous phase to some extent and act as antioxidants.

1.2. Aim and hypotheses of the thesis

The overall aim of this Ph.D. project was to investigate the impact of emulsion composition and combination of multifunctional emulsifiers on oil-water interfacial structure and emulsifier distribution as well as their influence on physical and oxidative stability of high fat fish oil-in-water emulsions. Furthermore, the development of physically and oxidatively stable high fat (50-70%) omega-3 delivery oil-in-water emulsions and their incorporation into food systems (e.g., mayonnaise) was also studied.

In order to achieve these goals, this Ph.D. study was divided into the following parts:

- **Part 1:** Optimization of physically and oxidatively stable high fat (50-70%) fish oil-in-water emulsions using sodium caseinate (CAS) in combination with commercial polysaccharides (sodium alginate, ALG) or surfactants (diacetyl tartaric acid ester of mono- and diglycerides, DATEM; phosphatidylcholine, PC). Investigating the effects of fish oil content, total emulsifier content, ratio between emulsifiers as well as homogenizer type on physical and oxidative stability. Characterizing interfacial structure of 70% fish oil-in-water emulsions stabilized with combined use of CAS and PC using small angle X-ray and neutron scattering techniques. (Paper I, II, III, IV, Appendix I)
- **Part 2:** Studying the effects of modified stabilizer/surfactants with enhanced surface activity by the attachment of short/long hydrophobic chain (for ALG) or covalently attached caffeic acid and different lengths of alkyl chains (for DATEM and PC), on physical and oxidative stability of 70% fish oil-in-water emulsions when used in combination to CAS. Investigating the distribution of labelled surfactants (DATEM) with different chain lengths in the high fat oil-in-water emulsion system using electron paramagnetic resonance (EPR) spectroscopy. (Paper IV, V, VI, and Appendix II)
- **Part 3:** Incorporation of n-3 delivery 70% fish oil-in-water emulsions produced with modified DATEM into high fat content (80%) mayonnaise. The physical and oxidative

stability of the enriched mayonnaise was studied, including the analysis of sensory characteristics of mayonnaise samples. (Appendix III)

The studies mentioned in the 3 parts above were conducted in order to test the following hypotheses.

Part 1:

Hypothesis I – Physical and oxidative stability of high fat (50-70%) fish oil-in-water emulsions are affected by fish oil content, total emulsifier content, and the ratio between combined emulsifiers (CAS+ALG, CAS+DATEM, and CAS+PC) as well as type of homogenizer. (Paper I, II and V, Appendix I)

Hypothesis II – Combined use of CAS and PC affects the structure of the oil-water interface in 70% oil-in-water emulsions compared to single use of CAS. The concentrations of CAS and PC also have an influence on the interfacial structure such as interfacial thickness and packing density. (Paper III)

Part 2:

Hypothesis III – Different alkyl chain lengths of modified surfactants (DATEM and PC) and a modified stabilizer (ALG) have an impact on the distribution of emulsifiers in high fat emulsions as well as adsorption at the oil-water interface when combined with CAS. (Paper IV, V and VI, and Appendix II)

Hypothesis IV – Enhanced surface activity of ALG by the attachment of short/long hydrophobic chain improves oxidative stability of high fat oil-in-water emulsions. (Paper IV).

Hypothesis V – Having caffeic acid attached to the commercial DATEM or PC improves oxidative stability of high fat emulsions compared to mixtures of commercial surfactants and free caffeic acid when combined with CAS. (Paper V and VI)

Part 3:

Hypothesis VI – Omega-3 enriched mayonnaise containing high fat (70%) fish oil-in-water emulsions stabilized with CAS and modified DATEMs present better physical and oxidative stability when compared to enrichment with neat fish oil. (Appendix III)

Chapter 2: Emulsions, emulsifiers, and oil-water interface

This chapter focuses on emulsions, emulsifiers, emulsion production including homogenizers, physical stability of emulsions, and distribution of emulsifiers in the emulsion system as well as oil-water interfacial structure of emulsions.

2.1. Emulsions

Emulsions are colloidal dispersions of two immiscible liquids, which are mixed together in the presence of emulsifiers and form a structure, where one of the liquids is dispersed as spherical droplets in the other liquid, which is called as continuous phase (McClements, 2005). According to the distribution of droplets, there are several types of emulsions such as oil-in-water, water-in-oil as well as double emulsions (e.g., water-in-oil-in-water or oil-in-water-in-oil). Application of these emulsions is in food, agriculture, cosmetics, drug delivery as well as petroleum. In this Ph.D. project, the focus will be only on oil-in-water emulsions and their applications in food system.

2.1.1. Oil-in-water emulsions

An emulsion, which has oil droplets dispersed in an aqueous phase, is classified as an oil-in-water emulsion (McClements, 2005). Examples from food systems are milk, mayonnaise, cream, dressings, cream cheese and soups. Oil-in-water emulsions can be classified according to their fat content. There have been many studies conducted on low fat (<30%) oil-in-water emulsions (Let et al., 2007; Berton-Carabin et al., 2014; García-Moreno et al., 2016); however, there has been only a few studies reported on high fat (>50%) oil-in-water emulsions (Horn et al., 2011). This Ph.D. study specifically focuses on high fat fish oil-in-water emulsions.

High fat oil-in-water emulsions

Oil-in-water emulsions consisting of more than 50% fat can be categorized as high fat oil-in-water emulsions. These emulsions tend to have a higher viscosity than low fat emulsions due to their high fat content. However, even at the same level of fat content, viscosity of high fat emulsions can be different depending on the type of the emulsifier or emulsifiers, using only proteins vs proteins in combination with surfactants or thickeners etc. Thus, these emulsions can be considered as spoonable or pourable based on the viscosity. Examples of high fat emulsions are dressings, cream cheese and mayonnaise.

Low fat emulsions may contain higher levels of water soluble metal ions and free radicals due to their higher water content. These compounds may act as prooxidants and trigger lipid oxidation in emulsion systems (Coupland and McClements, 1996). In case of using emulsions as delivery systems for n-3 PUFAs, it is an advantage to use high fat emulsions due to the fact that relatively small amounts of delivery high fat emulsions would be adequate for the enrichment of the food system with the targeted bioactive compound. Moreover, high fat delivery emulsions provide easiness for enriching highly viscous food systems such as mayonnaise, cream cheese and dressings due to similar consistency and textural properties.

2.2. Emulsifiers

Emulsifiers are surface active compounds, which are added into two immiscible liquids prior to homogenization in order to form a kinetically stable emulsion for a reasonable period of time (McClements, 2005). Emulsifiers adsorb at the oil-water interface during homogenization, when the droplets are freshly formed, and create an interfacial layer, which keeps the oil droplets dispersed in the aqueous phase. Emulsifiers can be classified according to their molecular weight as high molecular weight emulsifiers and low molecular weight emulsifiers.

2.2.1. High molecular weight emulsifiers (HMWE)

HMWEs are biopolymers such as proteins and polysaccharides, which are the most common emulsifiers used in the food industry.

Proteins

Proteins are amphiphilic biopolymers, which adopt a conformation locating the lipophilic parts in the oil phase and hydrophilic parts in the aqueous phase. Due to their ability to reduce interfacial tension between oil and water, they can be employed as emulsifiers. Proteins consist of amino acids, which vary according to their polarity, dimensions, chemically reactive groups, and interactions with other molecules (McClements, 2005). Characteristics of protein molecules are determined by the type, number and sequence of its monomers.

Proteins can have various configurations due to the high number of amino acids in their structure. Some proteins can be classified according to their configuration adopted in aqueous mediums; e.g., globular and random-coil. Examples of these protein types can be whey protein and casein, respectively, which are both milk proteins and widely used in food industry. These configurations may provide flexibility to proteins when adsorbed at oil-water interface in oil-in-water emulsions. It was reported that compact and globular proteins such as β -lactoglobulin form dense and

interconnected interfacial films, whereas flexible and disordered proteins such as caseins form thicker yet less densely packed interfacial films (Dickinson, 1992; Atkinson et al., 1995). Thick interfacial layer provided by proteins (e.g., casein) contribute physical stability of the emulsions providing steric repulsion forces (McClement, 2005).

The surface activity of the proteins arises due to their hydrophilic and lipophilic parts, which gives the amphiphilic character (Dickinson 2003). Even though proteins are water-soluble biopolymers, the hydrophobic effect due to their lipophilic regions provides the driving force in order to adsorb at the oil-water interface in oil-in-water emulsions (Matsumura and Matsumiya, 2012). Casein consists of individual casein types (e.g., α -casein, β -casein), which exist as linear disordered chains of around 200 amino acid residues in length and have a strong tendency to adsorb at hydrophobic surfaces due to the hydrophobic C-terminal part of the molecule (Dalgleish, 1997; Dickinson, 1997). Due to casein's random coil structure, once they are adsorbed at an oil-water interface, their hydrophilic amino acid domains will project into the water phase and the hydrophobic amino acid domains will attach the oil phase. Casein's high emulsifying properties are related to its high hydrophobicity and conformational adsorption ability (Dalgleish, 1997; Dickinson, 1997).

In this Ph.D. study, sodium caseinate (CAS), which is an ingredient widely used in food industry with high emulsifying capacity, was used as the main emulsifier in high fat oil-in-water emulsions (Dickinson 2006, Horn et al., 2011). CAS is a spray-dried pure milk protein manufactured from fresh pasteurized skimmed milk made by acid precipitation of the casein around pH 4.6, which is the isoelectric point of casein. During the acidification process, the calcium and inorganic phosphate, which are associated with the casein micelle in milk, are dissolved and leached from the curd (Southward and Walker, 1980). The protein composition of CAS is ca. 50% alpha-casein, 35% beta-casein and 15% kappa-casein. The molecular structure of alpha-casein is shown in Figure 2.1.

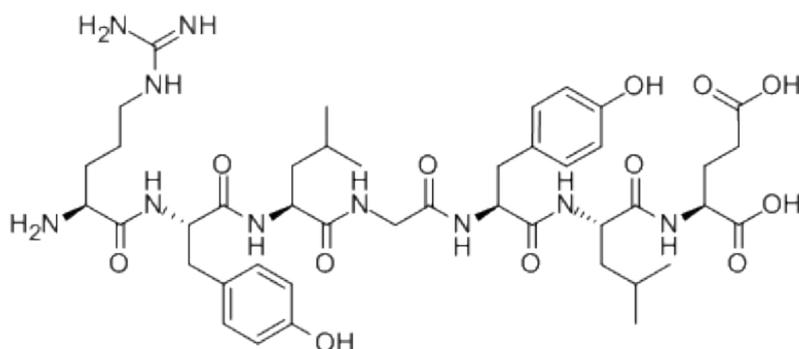


Figure 2.1. Molecular structure of alpha-casein (Alfa chemistry, 2019)

At neutral conditions (pH=7), CAS (above its isoelectric point, 4.6), is negatively charged due to the partial dissociation of the carboxylic groups at pH higher than isoelectric point. When adsorbed at the oil-water interface, the negative charge of the molecules provides electrostatic stability to oil droplets; therefore, flocculation and coalescence of oil droplets is inhibited. In order to have a physically stable protein stabilized emulsions, pH of the aqueous phase should be away from protein's isoelectric point, otherwise electrostatic forces are minimized and may not allow to keep the emulsions physically stable (McClements, 2005; Furtado et al., 2017).

CAS or other proteins (e.g., β -lactoglobulin) also showed antioxidant activity such as free radical scavenging by amino acid residues and metal chelating activities in oil-in-water emulsions (Diaz et al., 2003; Horn et al., 2013). It was reported that sulfhydryl groups scavenge radicals, whereas phosphoserine groups mainly chelate metals (Faraji et al., 2004; Elias et al., 2005). Besides, it was reported that CAS forms a thick oil-water interface providing a good physical barrier, which prevents prooxidants (e.g., metal ions) permeation from aqueous phase to oil phase. Moreover, excess CAS may be distributed in the aqueous phase of the emulsion (Berton-Carabin et al., 2018). These non-adsorbed proteins greatly contribute to the oxidative stability of the emulsions with their metal chelating activities in the aqueous phase of food emulsions, due to the fact that lipid oxidation is catalyzed by metal ions (Elias et al., 2005; Ries et al., 2010).

Polysaccharides

Polysaccharides are mainly used as stabilizers in oil-in-water emulsions, due to their thickening and water-holding properties; however, some of them can act as an emulsifier due to their hydrophobic regions. For example, gum Arabic has surface activity due to its hydrophobic polypeptide chain (Philips and Williams, 1995). Other polysaccharides, e.g., pectin, alginate, modified starch and chitosan, were also used in the formation of oil-in-water emulsions (Dickinson, 2003; Falkeborg et al., 2015). However, the emulsifying properties of these polysaccharides are questioned concerning the molecular origin (e.g., nonpolar groups) of the surface activity. Nonpolar regions of some polysaccharides can be methylated groups or exogenous moieties (e.g., lipids or proteins), which may be bound covalently or non-covalently to the polysaccharide (Berton-Carabin et al., 2018). Nevertheless, the contribution of the polysaccharides to the stabilization/emulsification of the oil droplets is suspected to be due to their thickening properties in the aqueous phase or surface activity at the oil-water interface (McClements, 2005).

Polysaccharides may also interact with other polymers such as other polysaccharides and proteins. These interactions may affect the behaviour and functionality of polysaccharides in emulsions

(Aken, 2006; Sosa-Herrera et al., 2008; Evans et al., 2013). Therefore, the combined use of polysaccharides with proteins may have a potential for improving emulsification of oil droplets due to the fact that polysaccharide-protein complexes may have a higher affinity to the oil-water interface than polysaccharides alone due to the hydrophobic regions of the protein, and thereby they may form a thicker interfacial layer. This is more likely to happen with the electrostatic attraction when the protein and polysaccharide molecules are oppositely charged, but also naturally occurring complexes (e.g., gum Arabic and protein residues) or Maillard conjugates may form protein-polysaccharide complexes (Evans et al., 2013).

Sodium alginate is one of the commonly used polysaccharides as thickener in food industry. It is composed of α -L-guluronate and β -D-mannuronate, arranged as linear homopolymeric and heteropolymeric blocks as shown in Figure 2.2. It has been shown that alginate has antioxidant activity such as radical scavenging activity in oil-in-water emulsions (Falkeborg et al., 2014).

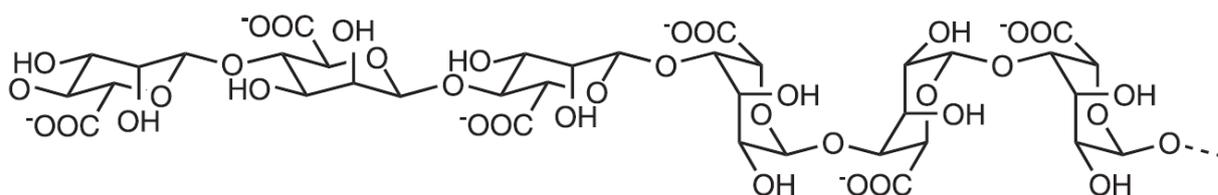


Figure 2.2. Molecular structure of alginate (Falkeborg et al., 2015).

Sodium alginate (ALG) has been used in combination with sodium caseinate for stabilizing water-in-water emulsions due to their ability to form a ternary water-caseinate-alginate system (Capron et al., 2001; Antonov et al., 2004). Dickinson and Euston (1991) reported that surface rheology was used to show the interfacial protein-polysaccharide interactions at pH 3, where CAS is positively and propylene glycol alginate is negatively charged, respectively. The same study also showed the formation of some surface complex at pH 7. It was indicated that charge overcompensation can occur due to the hydrophobic interactions between the surfactant tails and the hydrophobic backbone of the polymer chains (Guzmán et al., 2016; Goddard, 2002). Interaction between poly vinylacetate and SDS with the same charge was also pointed out by Isemura and Imanishi (1958).

Salvia-Trujillo et al. (2016) reported that sodium alginate in combination with Tween 80 improved oxidative stability of 2.5% (w/w) fish oil-in-water emulsion due to the metal chelating ability of its anionic groups. Same study also remarked that droplet flocculation was observed at sodium alginate concentrations exceeding 0.05% (w/w), which was attributed to depletion flocculation due to expulsion of non-adsorbed sodium alginates from the vicinity of oil droplets. After all, the

interaction between polysaccharides and proteins/surfactants are dependent on the type of the molecules, their concentration, charge density of the chains, hydrophobicity and ionic character of the surfactant, the interactions (electrostatic, Van der Waals, hydrogen bond or other types of weak interactions) between the chains and the surfactant molecules or micelles the pH and on the ionic strength of the system (Guzmán et al., 2016).

2.2.2. Low molecular weight emulsifiers (LMWE)

LMWEs are surfactants, which lowers the surface tension between immiscible liquids such as oil and water.

Surfactants

Surfactants are relatively small molecules, which consist of a hydrophilic head group and a lipophilic tail group (McClements, 2005). They are used for improving emulsion formation and stability. Surfactants can be classified as synthetic or natural, and also depending on the nature of the head and tail groups. Examples of synthetic surfactants are mono- and diglycerides, sucrose esters, derivatives of monoglycerides (e.g., CITREM [citric acid esters of monoglycerides], DATEM [diacetyl tartaric acid esters of monoglycerides], LACTEM [lactic acid esters of monoglycerides]), Tweens (e.g., polyoxyethylene sorbitan esters, Tween 20, 60, or 80). Natural and bio-based surfactants are phospholipids (e.g., lecithin, phosphatidylcholine (PC)), saponins, and glycolipids, which might have animal, plant or microbial of origin.

Head groups of surfactants may be nonionic, anionic, cationic, and zwitterionic. Tail groups may consist of one or more alkyl chains with various number of carbon atoms. These alkyl chains may be saturated or unsaturated. All these characteristics of surfactants have an influence on their adsorption behaviour at the oil-water interface as well as molecular organization in various structures (e.g., micelles, reverse micelles, bilayers, or vesicles) in solution such as an aqueous phase of the emulsion.

Surfactants may have an influence on emulsion properties in several ways such as by altering oil-water interfacial structure, interacting with other emulsifiers or biopolymers at the interface and/or in the aqueous phase, or forming micellar structures at the interface and/or in the aqueous phase. It was reported that surfactants (e.g., Tween 20, lecithin) replaced already adsorbed proteins at the oil-water interface in oil-in-water emulsions (Mackie et al. 2000; van Aken, 2003). These interactions or behaviours of the surfactants have great potential for improving physical and oxidative stability of the oil-in-water emulsions (Fang and Dalgleish, 1993; Dalgleish et al., 1995). On the other hand, replacement of proteins by other surfactants (e.g. Tween 20) makes the interface weaker (breaks

protein/protein interaction at the interface by having lateral domains) and reduces physical stability of emulsions (Mackie et al., 2001; Berton et al., 2012). Moreover, it was reported that CITREM might have partly interacted with ascorbyl conjugated linoleic acid and ascorbyl palmitate at the interface or participated in micelles formed with citrem in the aqueous phase and thereby have an impact on the antioxidant efficacy (Sørensen et al., 2011).

In this Ph.D. study, DATEM (Figure 2.3) and PC (Figure 2.4), which are anionic and zwitterionic surfactants at pH 7, respectively, were employed for the emulsification of high fat fish oil-in-water emulsions. Both DATEM and PC have 8 as a HLB¹ value, which is an intermediate HLB value (7-9), indicating that emulsions produced with these surfactants may not be stable against coalescence due to the low decrease of interfacial tension provided. Therefore, these surfactants are combined with CAS, since synergistic effects of PC and CAS have been previously reported (Fang and Dalgleish, 1996; García-Moreno et al., 2014).

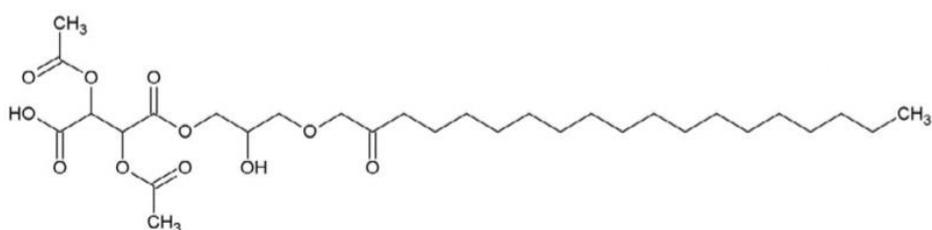


Figure 2.3. Molecular structure of DATEM (Wang et al., 2016)

DATEM is an ionic oil-in-water emulsifier. DATEMs are more hydrophilic compared to the mono- and diglycerides that they are made of due to the hydrophilic head part of the molecule, which consists of diacetyl tartaric acid anhydride and free hydroxyl groups (Gaupp and Adams, 2004). As the hydrophilic region of DATEMs is large, it results in high HLB value (Gaupp and Adams, 2004).

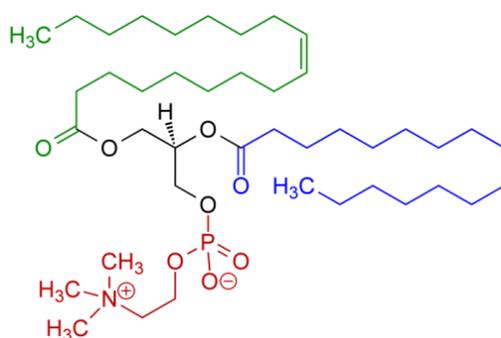


Figure 2.4. Molecular structure of PC (Wikipedia, 2019)

¹ Hydrophilic-lipophilic balance (HLB) is the balance of the size and strength of the hydrophilic and lipophilic moieties of a surfactant molecule.

PC is a type of phospholipid, which is naturally found in cell membranes of living organisms. As shown in Figure 2.4, PC has a ‘head’ group consisting of choline group and two fatty acid chains as ‘tails’. The type of the tails is dependent on the source of the PC; e.g., soy PC contains around 55% of linoleic acid, whereas egg PC mainly consists of oleic acid and palmitic acid (Gornall and Kuksis, 1971; van Nieuwenhuyzen and Tomás, 2008). PC mainly forms a lamellar structure when dispersed in water (Bergensstahl and Fontell, 1983). PC, like other phospholipids, swells in the case of hydration, which also alters the distances between bilayers.

Combined use of emulsifiers

Even though emulsions can be formed using a single emulsifier in some applications, emulsion formation, stability as well as functional properties of an emulsion can be improved by the use of emulsifier combinations (Dickinson, 2011; McClements and Jafari, 2018). Combining emulsifiers should be based on strategies for improving functional and physicochemical attributes of these compounds in emulsion systems. These strategies can be built on the interactions between emulsifiers used in the aqueous phase or at the oil-water interface and their impact on emulsification (Guzmán et al., 2016; McClements and Jafari, 2018).

The structure and composition of mixed interfaces has an effect on packing density, thickness, surface charge and rheology of the oil-water interface (Dickinson, 2011). These parameters impact not only physical stability of the emulsion system, but also oxidative stability. The advantage of having more than one emulsifier enhances the possibility to improving these mentioned characteristics as each emulsifier has its own properties, which can be tuned in by altering the concentration of the emulsifiers and the ratio between them considering their individual characteristics as well as their properties when interacting. Therefore, in this Ph.D. study, various emulsifier combinations were employed instead of using a single emulsifier for the emulsification of high fat oil-in-water emulsions.

Even though proteins are known as good emulsifiers in terms of providing both physical and oxidative stability to oil-in-water emulsions, it was reported that protein-stabilized interfaces are less efficient at protecting emulsified lipids against oxidation than surfactant-stabilized interfaces (Berton et al., 2011). Moreover, a previous study showed that mixtures of CAS and soy lecithin produced smaller droplets for thyme oil-in-water emulsion compared to the use of either emulsifier individually (Xue and Zhong, 2014). In the case of proteins combined with surfactants, and the adsorption of these molecules at the oil-water interface is considered, competition between LMWEs

and protein may occur. This may lead to adsorption of LMWEs (e.g., surfactants) first and then HMWEs (e.g., biopolymers) attach to oil-water interface later. It was also reported that small molecule surfactants displaced protein at the interfacial layer (Mackie, 2004), form a mixed emulsifier layer (Fang and Dalgleish, 1996) or comprises laterally separated domains of emulsifiers (Pugnaroni et al., 2004; Waninge et al., 2005; Berton-Carabin et al., 2018). As better physical stability is obtained by the mixed emulsifier layer compared to laterally heterogeneous layers, mixed emulsifier layer is preferred to be obtained as an interfacial structure for oil-in-water emulsions.

When different types of PC (e.g. DPPC [1,2-dipalmitoyl-sn-glycero-3-phosphocholine], DOPC [1,2-dioleoyl-sn-glycero-3-phosphocholine]) are adsorbed at the interface of an oil-in-water emulsion with CAS as emulsifier, it was observed that DPPC did not show a detectable competition at the oil-water interface, whereas DOPC not only competed with CAS during emulsification for adsorption but also replaced some of the already adsorbed casein from oil-water interface (Fang and Dalgleish, 1996). Another study reported that improved emulsification activity of lecithin was obtained by removing one of the fatty acid tails (lysolecithin), which results in higher HLB number thereby making the molecule more surface active in oil-in-water emulsions (Casado et al., 2012, Choi et al., 2011).

Interaction of surfactants with proteins and polysaccharides, which results in formation of surfactant-biopolymer complexes, may influence the functional properties of the surfactant compared to its individual state. The effects are caused by the changes in the surfactant conformation due to different ways of binding. Binding may either be promoted or inhibited by different physicochemical mechanisms such as electrostatic or hydrophobic interactions, and hydrogen bonding (McClements and Jafari, 2018). Goddard and Hannan (1977) also reported that the polymer/surfactant interaction is most favorable in the following conditions; the longer the hydrocarbon chain of the surfactant or the straighter the chain, and when the head group is terminal to the chain.

This approach regarding use of combined emulsifiers can also benefit retarding lipid oxidation in oil-in-water emulsions. It was shown that the electrostatic deposition of beet pectin on silk fibroin-coated surfaces inhibited lipid oxidation (Chen et al., 2011). It has also been reported that combined use of CAS and lecithin prevented lipid oxidation to a higher extent than when CAS was used alone in 10 % fish oil-in-water emulsions, which is presumably due to a more favorable structure and

thickness of the interfacial layer when CAS and lecithin were used together (García-Moreno et al., 2014).

Functional properties of the emulsifiers can be enhanced with some modifications such as increasing their antioxidant properties by the attachment of a phenolic acid or altering HLB number by changing the lipophilicity of the molecule. These strategies regarding the modification of emulsifiers, stabilizers and antioxidant compounds are introduced in detail in Chapter 4.

2.3. Emulsion formation and stability

To form a simple emulsion, two immiscible liquids (oil and water) and emulsifiers are subjected to emulsification in a homogenizer, which is a mechanical device designed to carry out homogenization. Homogenization of an emulsion can be achieved in one or more steps. Primary homogenization is applied for the creation of a coarse emulsion, whereas secondary homogenization is applied for the reduction of the droplet size of the already existing emulsion (McClements, 2005). Physicochemical and sensory properties of an emulsion depend on the type and concentration of the ingredients and homogenization technique and conditions (McClements, 2005).

There are several types of emulsification equipment, which have been used for producing oil-in-water emulsions, namely high shear systems (e.g., Stephan mixer), high speed mixers (e.g., Ultra-turrax), high pressure systems (e.g., high pressure valve homogenizer), membrane systems, microfluidizers, ultrasonic homogenizers and colloid mills. Most of these homogenizers are more suitable for the production of low fat (<30% oil) emulsions such as microfluidizers, high pressure systems, and membrane systems. This is due to the high viscosity obtained when high fat emulsions are produced, which cannot be handled by all the homogenizers. Therefore, this Ph.D. thesis focused on the use of Stephan mixer and colloid mill, which can handle solutions with high viscosity allowing the production of high fat oil-in-water emulsions.

Stability of the oil-in-water emulsions needs to be maintained in order to prevent the occurrence of physicochemical instabilities, which may also be caused by inefficient homogenization. Instability of oil-in-water emulsions may lead to undesirable changes in appearance (e.g, phase separation), texture, and smell as well as palatability. Therefore, formation of a stable emulsion and its preservation has a significant role. Following section is focused on the homogenizers used in the production of high fat oil-in-water emulsions.

2.3.1. Homogenizers used for the production of high fat oil-in-water emulsion

Homogenizers used for the emulsification of high fat oil-in-water emulsions provide emulsions with different characteristics / important properties; some of them are described in Figure 2.5. Depending on the type of applications, the use of a type of homogenizer can be an advantage or a disadvantage. This is further elaborated upon below.

Stephan mixer is a homogenizer which works by a high shear system principle. Homogenization happens in a mixing bowl, which consists of a blade in the center of the bowl. High as well as low viscosity emulsions can be handled with the blade, which is an advantage for the usage of the equipment in a wider range of applications. Moreover, larger droplet size ($D[4,3]$ of $2\ \mu\text{m}$ or higher) and a formation of a broader droplet size distribution are disadvantages of this homogenizer system. Product stress occurs under the conditions of high pressures, flow rates or possible heat generation during homogenization (Mao et al., 2010), which can trigger lipid oxidation. Thus, systems with high product stress may be a disadvantage depending on the susceptibility of the product to oxidation.

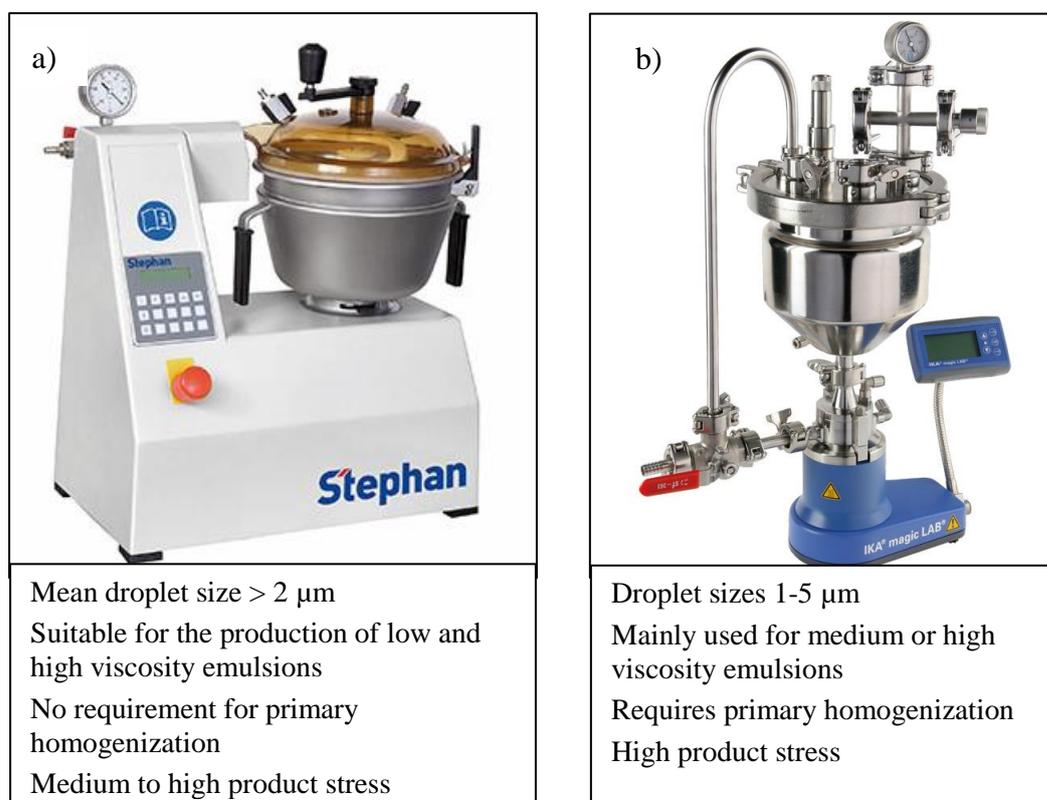


Figure 2.5. Homogenizers employed in the formation of high fat fish oil-in-water emulsions and their important properties; a) Stephan mixer, b) colloid mill.

Colloid mills are the most common type of homogenizer equipment in food industry to homogenize medium and high viscosity emulsions (Walstra, 1993). Primary emulsion should be prepared using a homogenizer such a high speed mixer (e.g., ultra-turrax). Colloid mill breaks oil droplets into smaller pieces when the coarse emulsion flows through a small gap between rotor and stator (Figure 2.6). The intensity of the shear stress can be modified by the adjustment of the gap between rotor and stator. Smaller gaps lead to smaller droplets; however, time spent in the colloid mill and flow rate used as well as number of times the emulsion is passed through the gap may also contribute to the droplet disruption, thereby decreasing droplet size of the emulsions (McClements, 2005).

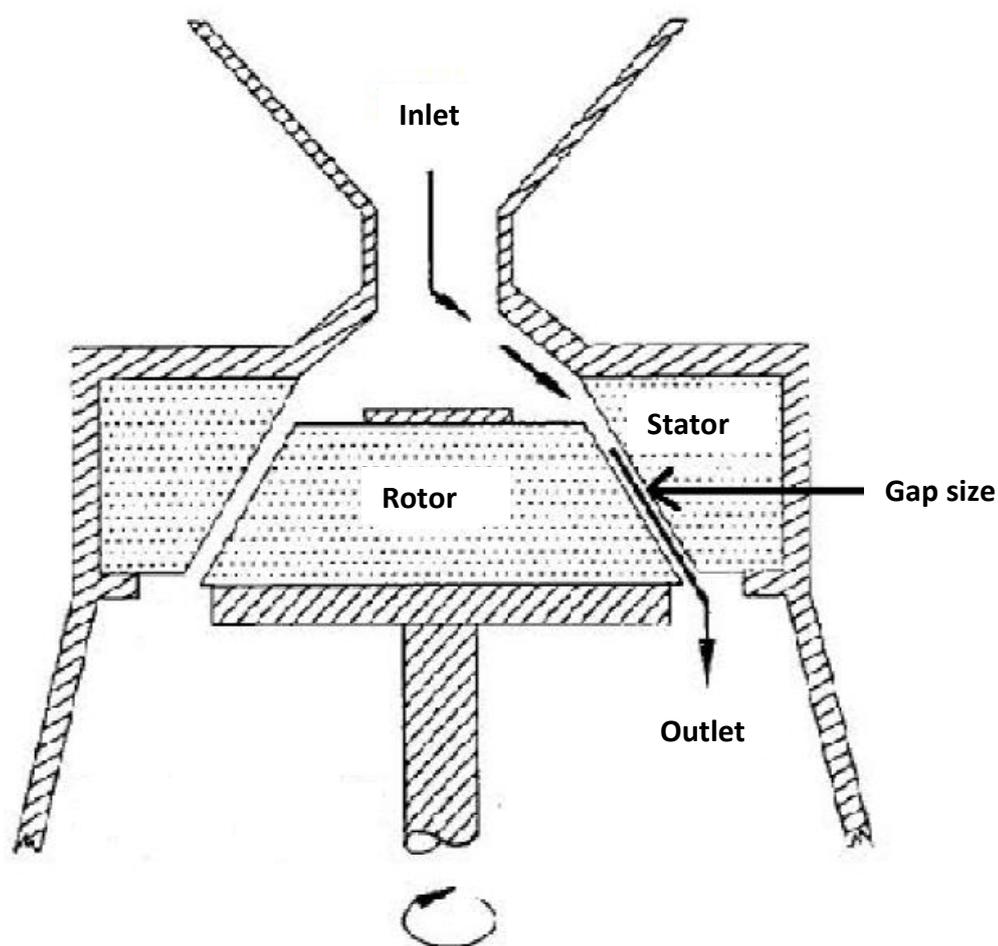


Figure 2.6. Illustration of the working principle of a colloid mill (Adapted from Wikimedia, 2019)

Both Stephan mixer and colloid mill have water jacketed homogenization chambers to keep the temperature low. Temperature control helps to prevent the occurrence of lipid oxidation during the emulsion production. Both homogenizers can provide monomodal droplet size distributions; however, colloid mills provide narrower droplet size distribution compared to Stephan mixers due

to the gap between rotor and stator, which can be controlled in order to adjust droplet size. As both homogenizers have a closed system, there is a possibility to eliminate oxygen during emulsion production in the chambers. Stephan mixer can operate under vacuum, whereas colloid mill can run under nitrogen flow.

2.4. Physical stability of emulsions

Emulsions may demonstrate physical instabilities over time, starting from the moment they are produced. Physical instability leads to alterations in emulsifier organization and their distribution in an emulsion system. For example, this can occur when two or more oil droplets merge and the emulsifiers reorganize their conformations around the newly formed oil droplet in an oil-in-water emulsion. Physical instabilities are creaming, sedimentation, flocculation, coalescence, phase inversion, Ostwald ripening and phase separation, which are illustrated in Figure 2.7 (McClements, 2005).

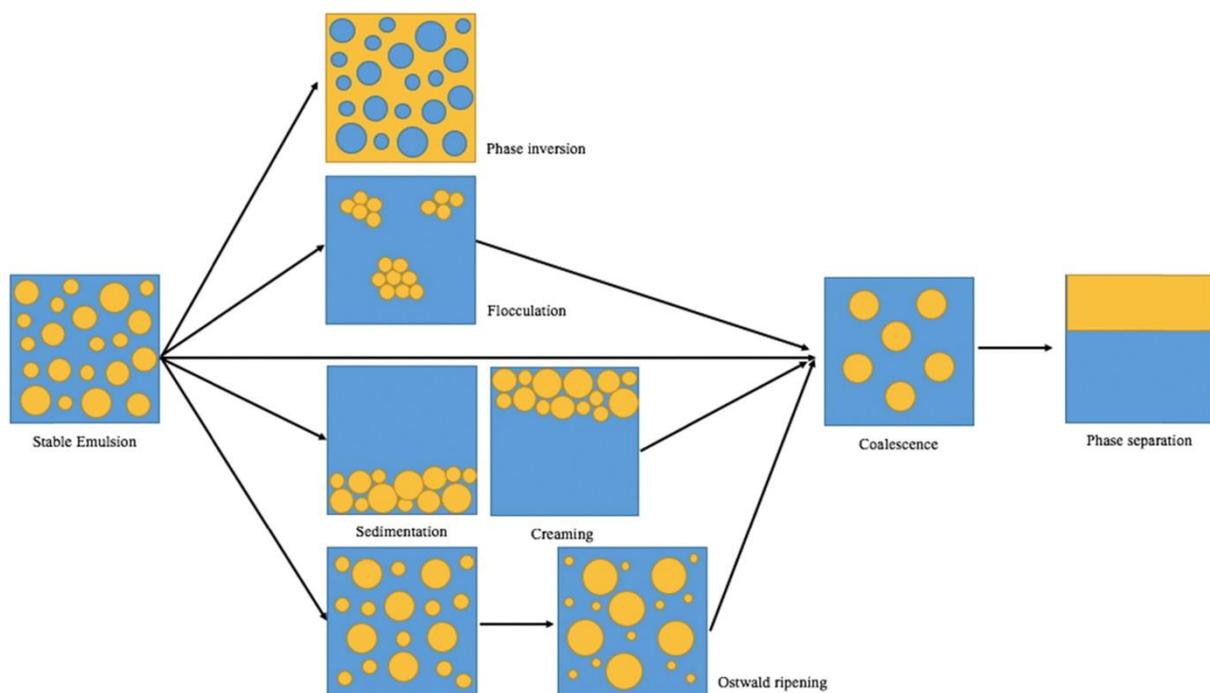


Figure 2.7. Illustration of physical instability mechanisms in oil-in-water emulsions (Hu et al., 2017).

Density difference between the two phases emulsified (e.g., oil and water) causes gravitational phase separation, which are called creaming or sedimentation as shown in Figure 2.7. These instabilities can be prevented by density matching, increasing viscosity of the continuous phase as well as decreasing the droplet size. Therefore, homogenization and addition of stabilizers and

thickening agents are commonly used techniques in order to increase the physical stability of emulsions (Berton-Carabin et al., 2018).

Droplets may aggregate and form flocs, which contribute to a faster creaming due to the increased effective size compared to an individual droplet. The occurrence of flocculation hinges upon the droplets overcoming repulsive forces between droplets such as electrostatic and steric repulsions. Depletion flocculation occurs in the presence of low biopolymer concentrations, whereas bridging flocculation takes place in the presence of excess biopolymer concentrations in the continuous phase (Dickinson, 2009). These flocculated oil droplets may further merge and result in phase separation.

There should be adequate amount of emulsifier available in order to form physically stable emulsions. However, it should be borne in mind that high concentrations of biopolymers (e.g., proteins and polysaccharides) might cause aggregation leading to depletion flocculation, and thereby coalescence of oil droplets and instability of the oil-in-water emulsions (Dickinson and Golding 1997).

Ostwald ripening takes place in polydisperse emulsions, where smaller and larger droplets are present. It occurs due to the larger internal Laplace pressure of the smaller droplets compared to larger ones, which advances the diffusion of small droplets to larger droplets, thereby increasing the size of the large droplets in the emulsion system. It could be critical in some oil-in-water emulsion systems in food industry; therefore, the rate of Ostwald ripening should be controlled (McClements, 2005).

Among these instabilities, phase inversion may also occur, which is a process where oil-in-water emulsion changes to water-in-oil emulsion or *vice versa*. Phase inversion is a common technique used in the production of some food systems such as margarine and butter; however, it is undesirable in other emulsion systems due to its negative effect on texture, appearance, stability and palatability properties (McClements, 2005).

2.5. Oil-water interfacial structure of emulsions (SAXS & SANS)

Interfacial structure of the emulsions is dependent on the type, concentration as well as combination of the emulsifiers used. In general, single emulsifiers result in a monolayer formation. However, it was found that CAS forms multilayers when applied in higher concentrations (Dickinson, 1999). All these diversified behaviours of molecules result in differences in interfacial layer thickness,

packing density and permeability as well as dynamism in the molecular exchange at the oil-water interface such as replacement of proteins by LMWEs; e.g., surfactants. Moreover, some emulsifiers, e.g., proteins, reorganize themselves after the adsorption at the oil-water interface, which allows them to rearrange their conformation and increase the contact points between protein itself and interface (Berton-Carabin et al., 2018). It is also important that time is allowed during emulsification for proteins to adsorb. Hence, too fast mixing or shearing can lead to unstable or lack of formation of emulsions. Therefore, some time is required until the proteins find stability in their conformation and reach equilibrium before doing some analysis on the adsorbed or non-adsorbed protein content in the emulsions (Berton-Carabin et al., 2018).

Physical properties of emulsions are closely related to oil-water interfacial structure, and they also have a considerable importance for the oxidative stability of the emulsion systems (Berton-Carabin et al., 2014). The oil-water interface of the emulsions has been found to be particularly critical as its properties play a significant role in the factors contributing to occurrence of lipid oxidation (Berton-Carabin et al., 2018). Therefore, characterization of oil-water interface is important and in order to achieve that novel technologies using interdisciplinary approach should be applied.

There are some techniques used for the characterisation of the oil-water interface indirectly, such as measuring the protein content in the aqueous phase of an emulsion emulsified with proteins. As proteins can either adsorb at the oil-water interface or locate in the aqueous phase as in monomers or aggregates (non-adsorbed proteins), measuring the protein content in the aqueous phase reveals the information on adsorbed protein content. The separation of phases of emulsions, e.g., oil phase, aqueous phase and oil-water interface, is conventionally done by centrifugation and ultracentrifugation. Separated phases are subjected to analysis of protein content or other compounds.

Using protein content information together with surface area of the oil-water interfacial layer provides information for calculating the protein surface load of the emulsions. However, these indirect measurements might not represent the reality in these complex systems. On the other hand, there are some other techniques such as microscopy, where the information is taken from the sample itself from the interested area, but in this case, the information obtained is location specific. There are other techniques to characterize model interfaces such as ellipsometry or atomic force microscopy. However, for using these techniques, model interfaces need to be built (e.g. by using Langmuir-Blodgett trough); thus, they do not totally represent the real interface. Therefore, even

though in some cases useful information can be obtained to a considerable extent from the above mentioned techniques; alternative techniques are needed in order to elucidate oil-water interfacial structure in oil-in-water emulsions.

SAXS and SANS techniques have been used in various fields from healthcare to energy. Despite its great potential, these techniques have been poorly applied in food science for elucidating the structure of various food systems. However, coupling SAXS and SANS techniques are suggested as powerful tools for understanding the nature, behaviour and structure of complex systems such as food emulsions. Several applications in other food systems are also presented in a previous review (Lopez-Rubio and Gilbert, 2009). The mechanisms behind these techniques are illustrated in Figure 2.8 and 2.9.

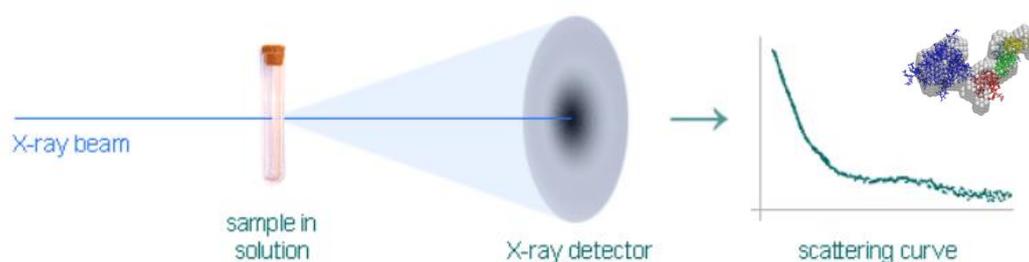


Figure 2.8. Illustration of the principle of small angle X-ray scattering experimental technique (Biosaxs, 2019)

SAXS is an experimental technique, where the sample is illuminated by X-ray photon beams, which are provided by particle accelerators known as synchrotrons. X-rays hit the sample and scattered radiation from the electron-dense groups of the molecules in the sample is collected by a detector (Figure 2.8). Scattering curve (intensity versus scattering angle) was used to model a protein molecule that is shown on the upper right part of the plot in Figure 2.8. This technique helps to determine the size of the molecule, molecular shape in 3 dimensional matrix as well as to model the separate domains of the molecule of interest, which provides information about internal structure. Moreover, SAXS is used in combination with SANS in order to investigate the molecular dynamics of the molecules (e.g., proteins) in solutions, emulsions or in natural food system (e.g., milk) (Larson-Smith et al., 2012; de Kruif et al., 2012; Ingham et al., 2016).

SANS technique requires a neutron source from a nuclear reactor, which provides the neutron beam to the instrument. As shown in Figure 2.9, incident beam comes from neutron source and targets the sample. After neutrons hit the molecules in the sample, they interact with the nucleus and scatter with a small exit angle less than 5 degrees. The small angle between scattered beam vector and

incident beam vector is shown as 2θ . The scattering vector (momentum transfer, q) is the difference between the incident and scattered beam vectors, which is formulized as $q = k_f - k_0$. The intensity of the scattered vectors is collected by the detector and the Intensity, $I(q)$, is presented as a function of the momentum transfer, q .

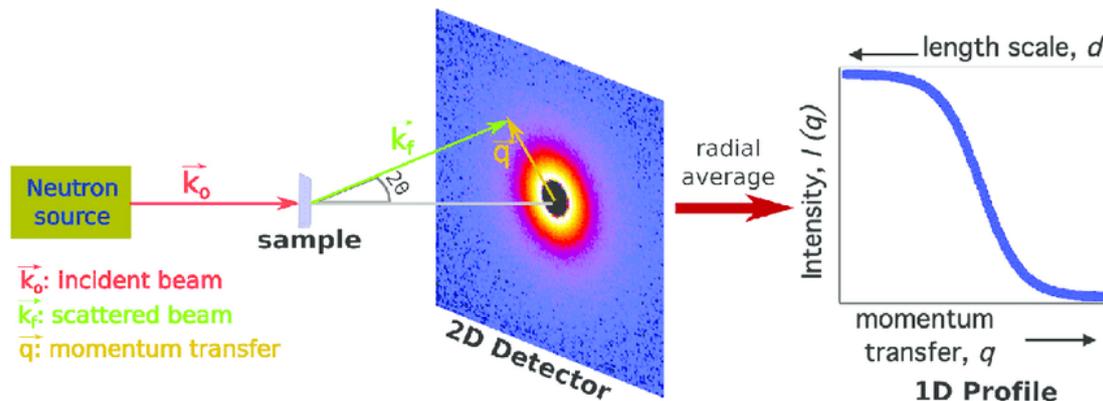


Figure 2.7. Illustration of the principle of small angle neutron scattering experimental technique (Castellanos et al., 2016).

An important tool in SANS is the possibility to use D_2O , which is a form of water called heavy water and contains deuterium (isotope of hydrogen) instead of hydrogen, for adjusting the contrast between emulsifiers, oil, and aqueous phases of the emulsion. Contrast adjustments permit to selectively extinguish the signal of one of the component to probe specific objects in multicomponent samples, e.g. the shell or the core of the core-shell particles (Banc et al., 2016). Contrast may be matched either for oil and water phases, which reveals the information about emulsifiers, or varied for aqueous phase in a systematic way, which gives information about the molecules in the sample based on the scattering intensity $I(q)$ compared to contrast variation employed. Mathematical models are fitted to the curves obtained from each sample, which are further used for the interpretation of the results.

2.6. Distribution of emulsifiers in the emulsion system (EPR)

Emulsions consist of higher amounts of emulsifiers than is actually needed for the emulsification of the oil droplets, which means that the excess emulsifier content will be distributed between the water or oil phases. Distribution of emulsifiers in the emulsion system mainly depends on the hydrophobicity of the emulsifiers (Berton-Carabin et al., 2014). For instance, biopolymers such as proteins and polysaccharides are generally water-soluble; therefore non-adsorbed molecules are present in the form of monomers, oligomers or aggregates in the aqueous phase. On the other hand, depending on the HLB, surfactants can be either water-dispersible or oil-dispersible and thereby excess surfactants are dispersed in the water or oil phases in forms of single molecules, micelles,

reverse micelles or vesicles if the concentration is higher than their critical micelle concentration (CMC). Lifetime of these structures is very short, e.g., around 10^{-3} s for a micelle (Berton-Carabin et al., 2014), meaning that the molecular exchanges in these micellar structures are very fast and dynamic. Therefore, special techniques are required which are time sensitive and has a principle fast enough to analyse these short-lived structures.

Distribution of the emulsifiers can be measured using conventional methods such as separating the different phases of the emulsion and applying further analysis for the targeted compound in each phase, as described in section 2.5. However, these techniques are usually destructive and they do not provide information about the dynamics in the system. Therefore, results might not represent the real picture of the distribution of emulsifiers in the emulsion system. EPR spectroscopy is one of the non-destructive techniques, which has been applied in emulsion systems in order to understand the distribution mechanism of the emulsifiers/surfactants *in situ*. However, this technique requires the use of an EPR-active molecule such as spin probes. Examples of commercial spin probes are hydrophilic 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL, Figure 2.3) and 4-phenyl-2,2,5,5-tetramethyl-3-imidazoline-1-oxyl nitroxide (PTMIO). This could also be a drawback of this technique, due to the fact that the emulsifier of interest needs to be modified with an EPR-active molecule such as nitroxide. This might affect the characteristics and behaviour of the compound, which could be unrepresentative of the real emulsifier.

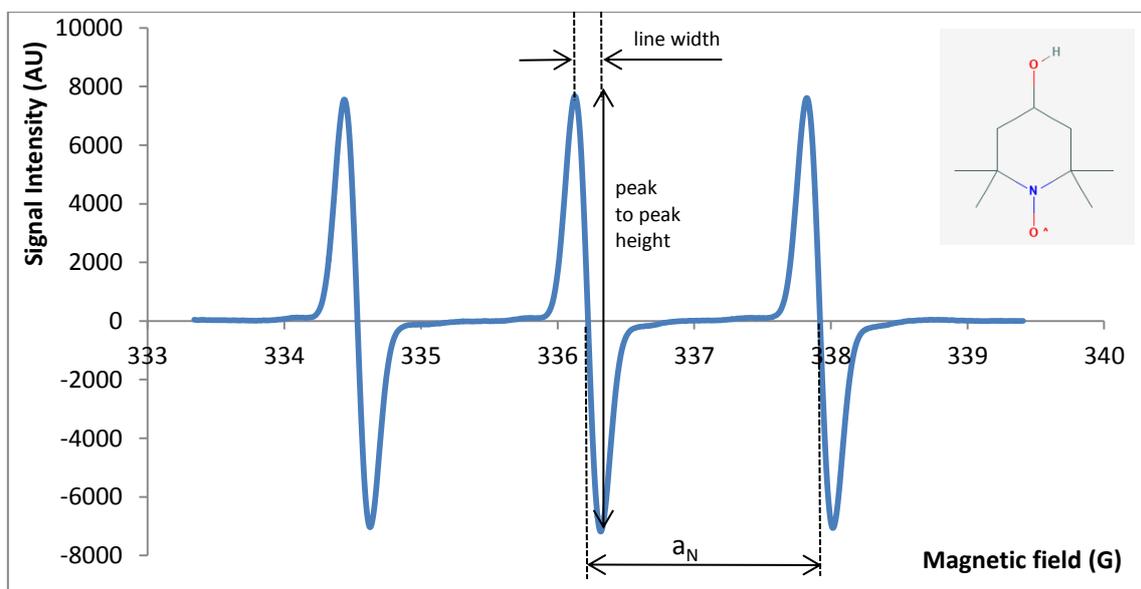


Figure 2.3. EPR spectrum of 50 μM TEMPOL in water. Molecular structure of TEMPOL is shown on the upper right corner of the spectrum.

EPR spectrum obtained from TEMPOL is shown in Figure 2.3 with the parameters used in order to analyse and interpret the data collected. Intensity of a spectrum is calculated by the double integration of the simulated spectra, which gives information about the amount of spin-probe in different environments such as oil or water phase of the emulsion. Peak to peak height is calculated from the difference between minima and maxima of the center peak. This information can be used for chemical stability of the spin-probe used in the study. Hyperfine splitting constant (a_N) is calculated from the distance between the centers of center peak and high-field peak. This indicates the polarity of the environment, where the spin-probe dissolves/disperses in and gives the signal. For instance, a_N value of the compound in the lipid phase should be lower than the a_N value of the compound in the aqueous phase. Line width is calculated from the distance between the centers of upper and lower points of the center peak, which indicates the mobility of the spin-probes, e.g., smaller values imply higher mobility. Line width is also affected by the interaction of the probe/spin labelled emulsifier with other paramagnetic substances such as oxygen or even the same probe/spin labelled emulsifiers. These parameters can be used for understanding the distribution of emulsifiers in the oil-in-water emulsion systems.

Previous study reported that model ingredients (TEMPOL, 16-DS [16-doxy]-stearic acid], and PTMIO) showed different distribution patterns between oil and water phases of 10% tetradecane-in-water emulsions depending on their lipophilicity (Berton-Carabin et al., 2013a). PTMIO was distributed between the lipid and aqueous phases; 16-DS was distributed between the lipid phase and the interface, whereas TEMPOL was present in the aqueous phase. Berton-Carabin et al. (2013b) showed in another study that PTMIO partitioned between the aqueous phase, the lipid droplet core, and the surfactant micellar pseudo-phase. In addition, the reduction rate of the nitroxide groups of PTMIO by ascorbate anions was faster in DTAB-stabilized emulsions than in SDS-stabilized emulsions, which showed the impact of droplet surface charge on the reactivity of PTMIO (Berton-Carabin et al., 2013b). Pegi et al. (2003) investigated the influence of lipophilicity and structure of different model molecules on their distribution in solid lipid nanoparticle (SLN) dispersions. The authors reported that TEMPOL was distributed in the solid lipid core, the phospholipid layers (deeper in SLN layer or in liposomes and closer to the surface of SLN) and water in the ratios of 0:0:100, whereas labelled PC was distributed in these phases in the ratios of 10:89(26:3:60):1.

Chapter 3: Lipid oxidation

This chapter will focus on summarizing lipid oxidation mechanisms in oil-in-water emulsions. Lipid oxidation has been researched for more than a century and the knowledge gained has been used not only in food research but also in health related studies such as understanding aging or curing cancer and other diseases (Schaich, 2013).

Lipid oxidation can occur through three different mechanisms; autoxidation, photooxidation, and enzyme catalysed oxidation. However, focus was directed only to autoxidation in this Ph.D. study due to the fact that experiments were performed in darkness and there were no active catalysing enzymes, which can affect oxidation.

3.1. Autoxidation

Autoxidation is a free radical chain mechanism, which is the reaction of oxygen with organic compounds (Frankel, 2012). Autoxidation consists of three stages, which are initiation, propagation, and termination. Main initiation and propagation stages as well as decomposition of lipid hydroperoxides to secondary volatile compounds are shown in Figure 3.1.

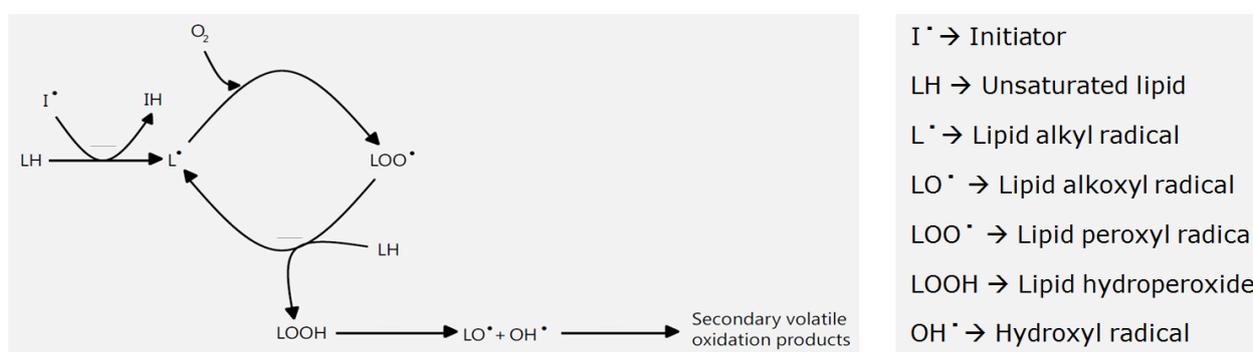


Figure 3.1. Illustration of the lipid autoxidation mechanism including initiation, propagation and termination stages.

Initiation.

In this first step of autoxidation, an unsaturated lipid (LH) lose a hydrogen radical (H•) and form an alkyl free radical (L•) in the presence of initiators such as heat, transition metal ion, or an existing free radical as shown in reaction 1 (Frankel, 2012).



In general, abstraction of hydrogen radical takes place at the bis-allylic positions of PUFAs. Number of bis-allylic bonds can be an indication of oxidative stability. EPA and DHA are thus highly prone to oxidation due to their 4 and 5 bis-allylic carbon positions, respectively.

Hydroperoxides already existing in the emulsion system may be decomposed by the trace metal ions present in the system as illustrated in reactions 2 and 3.



These reactions result in formation of lipid alkoxy radical (LO^\bullet) and lipid peroxy radical (LOO^\bullet). These radicals formed can further initiate lipid oxidation, which is another initiation mechanism for lipid oxidation.

Propagation.

Alkyl radicals are unstable intermediates; therefore, they immediately react with triplet oxygen and generate peroxy radicals as shown in reaction 4.



As formed peroxy radicals are also unstable products, they abstract hydrogen atoms from other unsaturated fatty acids to form hydroperoxides and other alkyl radicals as shown in reaction 5. Lipid hydroperoxides formed at this stage are named as primary oxidation products. Reaction 5 repeats thousand times during the propagation stage until the reaction is interrupted by an antioxidant or hydrogen source is unavailable.

The oxidizability of n-3 PUFAs are linearly related to the number of bis-allylic positions in the fatty esters (Frankel, 2012). The most important LC n-3 PUFAs are EPA and DHA, which have double bonds in *cis*-5,8,11,14,17 and *cis*-4,7,10,13,16,19 positions, respectively. Frankel (2012) identified that EPA produced the eight 5-, 8-, 9-, 11-, 12-, 14-, 15-, and 18-hydroperoxides, whereas DHA produced the ten 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17-, and 20-hydroperoxides.

Termination.

Termination is the final stage of autoxidation, where stable non-radical compounds form as a result of lipid radicals' reaction with each other as shown in reactions 6 to 10.



Formation of the stable non-radical compounds stops the radical chain reaction. Termination stage can be also promoted by antioxidants (see section 4.1).

3.2. Volatile secondary oxidation products and their impact on sensory properties

Lipid hydroperoxides decompose further leading to the formation of secondary volatile oxidation products, which give the food products undesirable flavours and odours. This Ph.D. study focuses on the formation of volatile compounds originating from omega-3 PUFAs in order to understand to which extent the protection of these compounds has succeeded when using emulsions as n-3 PUFA delivery systems.

The formation of secondary volatile compounds is illustrated in Figure 3.1. Unstable hydroperoxides decompose into lipid alkoxy radicals (LO^{\bullet}) and hydroxyl radicals (OH^{\bullet}) through homolytic cleavage, thereafter these alkoxy radicals form secondary oxidation products by β -scission (Frankel, 2012).

Volatile secondary oxidation products can be alcohols, aldehydes, ketones, furans, alkanes, and alkenes, which are formed during lipid oxidation depending on the original lipid hydroperoxide structures. The mechanism for the formation of aldehydes from EPA is shown in Figure 3.2. Propanal, 3-hexenal, 2,4-heptadienal, and 2,4,7,-decatrinal are specific products formed from n-3 PUFAs (e.g., EPA). In the presence of methyl linoleate and linoleate, 2,4-heptadienal is more rapidly oxidized than these fatty esters. Therefore, the saturated aldehydes accumulate and the

unsaturated aldehydes are further oxidized to lower aldehydes and dialdehydes during the more advanced stages of oxidation (Frankel, 2012).

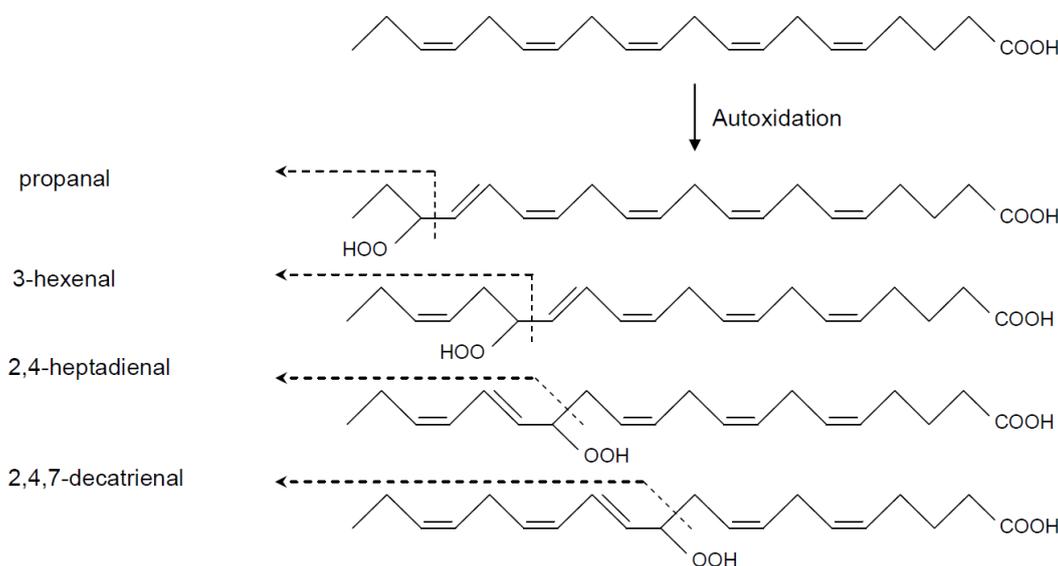


Figure 3.2. Aldehydes formed from EPA hydroperoxides (adapted from Kulås et al., 2003)

Volatile secondary oxidation products have a significant sensory impact on food products due to the fact that they cause flavour deterioration, even at parts per billion (ppb) levels (Frankel 2012). Some of the volatile compounds derived from omega-3 PUFAs are described in Table 3.1.

Table 3.1. Omega-3 PUFA originated volatile secondary oxidation products and their odour descriptions (Genot et al., 2003)

Volatile compound	Odour description
Propanal	sharp-irritating
t-2-Butenal	old cheese
1-Penten-3-ol	sweet
1-Penten-3-one	pungent, rancid green, sharp fishy, glue
t-2-Pentenal	pungent, glue, green, grassy, apple
t-2-Hexenal	sour, green
2,4-Heptadienal	t,c: fishy, burnt, rotten apples; t,t: nasty, green, rancid hazel nuts
t,c-2,6-Nonadienal	cucumber, cucumber-like
2-Ethyl-furan	flower

Sensory evaluation and volatile compound analysis of foods enriched with n-3 PUFA oils (e.g., milk, mayonnaise, granola bars) have been performed in several studies using the senses of taste, smell, sight and mouthfeel (Jacobsen et al., 1999b; 2000a; Hartvigsen et al., 2000; Vankateshwarlu et al., 2004; Sørensen et al., 2010a; Karadağ et al., 2017). In these studies, relationship between volatile compounds and sensory analysis has been investigated and understood that an individual volatile compound did not represent one specific odour by itself, instead, development of fishy and rancid off-flavours due to lipid oxidation described by a sensory panel were represented by a combination of volatile compounds.

Aldehydes, which are formed as secondary oxidation products during lipid oxidation, may chemically or physically interact with proteins adsorbed at oil-water interface. Reactions between aldehydes and amine groups on proteins and peptides can lead to formation of other oxidation products called Strecker aldehydes and pyrroles, which also have off-flavours (Lu et al., 2013). These compounds may also impact the results obtained from some of the analytical methods used for measuring lipid oxidation such as gas chromatography head space analysis, leading to an underestimation of the actual amount of oxidation products formed due to the fact that some of the secondary oxidation products are bound to the proteins and not detected (McClements and Decker, 2018).

3.3. Lipid oxidation in oil-in-water emulsions

In this Ph.D. thesis, lipid oxidation in high fat fish oil-in-water emulsions was studied. Therefore, the focus in this section will be on providing an overview of lipid oxidation in model emulsion systems and real food systems, which are in the form of oil-in-water emulsions. LC n-3 PUFAs such as EPA and DHA are bioactive compounds, which have health-promoting effects and prevent diseases, as mentioned in Chapter 1. Therefore, these compounds needed to be protected from being oxidized when they are incorporated into food systems in order to provide the maximum delivery of these compounds before they are degraded.

Due to the fact that fat is found naturally in dispersed condition in most of the food systems, fat soluble bioactive compounds such as n-3 PUFAs are chosen to be delivered in oil-in-water emulsion systems. However, these systems increase the likelihood of occurrence of lipid oxidation compared to bulk oil systems due to the larger interfacial area between oil and water phases created in oil-in-water emulsions, which exposes lipids to prooxidants present in the aqueous phase.

Nevertheless, there are strategies for providing chemically stable oil-in-water systems by the use of high quality and multifunctional ingredients as well as right homogenization techniques, which will be further discussed in the section 3.4.

As the reaction mechanism and factors that influence oxidation are considerably different for emulsified lipids compared to bulk lipids, lipid oxidation in oil-in-water emulsions has been widely studied and strategies have been developed in order to prevent oxidation (Coupland and McClements, 1996; McClements and Decker, 2000; Berton-Carabin et al., 2013c; Berton-Carabin et al., 2014; Jacobsen et al., 2008; 2015; Decker et al., 2017). In the previous studies, oil phase content varied between 1-70% oil-in-water emulsions. Moreover, food systems in the form of oil-in-water emulsions (e.g., milk, mayonnaise, yogurt, cream cheese, etc.) have also been studied (Nielsen et al., 2007; Sørensen et al., 2010a; Horn et al., 2012b; Alemán et al., 2015).

Jacobsen et al. (2000) reported that use of extra emulsifier (DATEM) in combination to egg yolk improved the viscosity of the mayonnaise; however, this increase in the viscosity did not decrease the formation of fishy and rancid off-flavours. Moreover, same study also showed that addition of tocopherols (Toco 70) did not have an impact on the formation of volatiles and sensory perception. Fish oil enriched cream cheese was investigated to study if fish oil delivery emulsion produced with sodium caseinate, whey protein isolate, or a combination of milk proteins and phospholipids provided better oxidative stability compared to the addition of neat fish oil (Horn et al., 2012b). Results indicated that the oxidative stability of fish oil enriched cream cheeses was highest when fish oil was added as neat oil or in a delivery emulsion prepared with a combination of milk proteins and phospholipids compared to delivery emulsions prepared with either sodium caseinate or whey protein isolate.

Sørensen et al. (2010b) substituted egg yolk as an emulsifier in light-mayonnaise with milk-based emulsifiers due to the fact that iron content in the egg yolk increase lipid oxidation especially in the presence of oils rich in LC n-3 PUFAs (e.g., fish oil). Results indicated that the initial quality of the emulsifiers plays a more important role than the iron content in the emulsifier. In another study, oxidative stability of fish oil enriched drinking yoghurt was found to be higher even when compared to yoghurt with added rapeseed oil or a mixture of rapeseed oil and fish oil stored for up to 29 days (Nielsen et al., 2007).

3.4. Factors influencing lipid oxidation in complex systems

Factors such as ingredients, type of homogenizer, surface charge, pH, interfacial layer, droplet size, viscosity have an effect on lipid oxidation in complex systems such as oil-in-water emulsions.

Ingredients

Model oil-in-water emulsions consist of oil, water and emulsifier or combination of emulsifiers. In some cases, there are antioxidants or other ingredients such as stabilizers (e.g., polysaccharides) or surfactants (e.g., lecithin). The quality of the oil used for the production of the emulsion is important in terms of the amount of oxidation products due to the fact that the lipid oxidation can be initiated by lipid radicals already existing in the oil. Moreover, degree of unsaturated fatty acids also influences the oxidative stability of the oil-in-water emulsions. Fish oil is a good example of how a high degree of unsaturated fatty acid composition can lead to increased lipid oxidation (Frankel, 2012). Metal ions dissolved in the water used in the production of oil-in-water emulsions may also act as prooxidants (Jacobsen, 1999a).

Emulsifiers

Emulsifier types, concentrations and combinations used in an oil-in-water emulsion system may vary largely. Milk proteins such as sodium caseinate and whey protein are commonly applied emulsifiers in the food industry due to their ability to form and stabilize oil droplets in emulsions (Singh, 2011). It has been suggested that CAS provides better physical stability, which forms thicker interfacial layer compared to whey or soy protein, which form thinner layers due to the steric hindrance effects (Hu et al., 2003). Even though first role of emulsifiers is to form an emulsion, which is related to their surface activity, they also show antioxidant activities such as radical scavenging and metal chelating and provide oxidative stability as well (Elias et al., 2008). Similarly, lecithin also inhibits oxidation in oil-in-water emulsions (García-Moreno et al., 2014).

Emulsions can be produced using a single emulsifier or combined use of emulsifiers. They may show synergistic effects when they are used in combination due to enhanced interfacial properties such as packing density, thickness, permeability. The structure of interfacial layer may not only provide a protective membrane for the oil droplets, but also improves oxidative stability of the emulsions by forming a physical barrier against the diffusion of prooxidants present in the aqueous phase (Berton-Carabin et al., 2014).

Distribution of emulsifiers with antioxidant activity in an emulsion is also important due to the fact that non-adsorbed emulsifiers may chelate metals and scavenge radicals in the aqueous phase. Contribution of these non-adsorbed emulsifiers to physical and oxidative stability may be as important as the composition of the oil-water interface depending on the emulsifier type and concentration. On the contrary, formation of micellar structures by surfactants may interact with antioxidants and enhance or inhibit their radical scavenging and metal chelating activities in the aqueous phase of an emulsion system (Richards et al., 2002; Chat et al., 2011; Berton-Carabin et al., 2013b).

Type and initial quality of the emulsifier used in a food system may influence oxidative stability. In mayonnaise, egg yolk at low pH (around 4) releases iron, which is bound to egg yolk proteins such as phosvitin, lipovitelin and low-density lipoprotein (Jacobsen et al., 2001). Released iron (Fe^{3+}) may also react with ascorbic acid, which is present in lemon juice as a common ingredient in mayonnaise, and reduced to more active form of iron as an oxidation catalyst (Fe^{2+}). Combination of these factors such as emulsifier type, pH and interaction with other ingredients results in lipid oxidation in mayonnaise (Gorji et al., 2016).

Droplet size

Mean droplet size ($D[3,2]$) of an emulsion system indicates the total area of the interfacial layer. As the oxidation is initiated at the oil-water interface, surface area of the oil droplets is associated with the rate of the oxidation. This is due to the fact that interfacial layer is the contact area where the prooxidants in the continuous phase comes to the vicinity of the lipid phase in emulsions (Berton-Carabin et al., 2018). However, there is no certain correlation between droplet size and lipid oxidation in emulsion systems. It is dependent on various other conditions of the emulsion system, such as emulsifier type and concentration, surface charge, viscosity, and homogenization conditions (Horn et al., 2012a; 2012c).

Surface charge and pH

In oil-in-water emulsions, oil droplet surface is where the lipid oxidation is initiated and propagated (Berton-Carabin et al., 2014). Therefore, surface charge of the oil droplets plays an important role in oxidative stability. Emulsions produced with anionic surfactants attract cationic metal ions and thereby have higher susceptibility to oxidation compared to cationic surfactants (Mei et al., 1998).

Oxidative stability of CAS stabilized emulsions at lower pH was found to be lower compared to emulsion at neutral pH, irrespective of the iron addition and fat content (Horn et al., 2011; Horn et al., 2012a). Moreover, formation of peroxides and volatile compounds was faster at pH 3.0 compared to pH 7.0 irrespective of the emulsifier type (Tween 80, Citrem, CAS, or lecithin) and iron addition (Jacobsen, 2008). Sørensen et al. (2008) also reported that decreasing pH decreased oxidative stability in Tween 80 and Citrem stabilized emulsions. These results could be attributed to the fact that iron is more active in acidic conditions to react with hydroperoxides (Berton-Carabin et al., 2014).

Viscosity

High fat emulsions yields high viscosity compared to low fat emulsions due to the fact that oil has a higher viscosity itself compared to water. Increase in viscosity in an emulsion system affects the mobility of the molecules in the emulsion system. For instance, prooxidants such as metal ions and free radicals in the emulsion slow down and thereby rate of the oxidation is expected to be decreased in viscous emulsion systems. Shimada et al. (1996) reported that Fe^{2+} induced oxygen consumption was affected by the viscosity of the aqueous phase in the emulsions, which were stabilized with polysaccharides such as xanthan.

Homogenization type and conditions

Type and condition of the homogenization may have an influence on the physical and oxidative stability of the oil-in-water emulsions. Horn et al. (2012c) reported that lipid oxidation in emulsions produced with whey protein were affected by the type of the homogenizer used. In contrast, same study showed that emulsions prepared with CAS was not influenced by the type of homogenizer used. These results are attributed to the effect of homogenizer on the distribution of the emulsifiers in the emulsion. Dalgleish et al. (1996) reported that the milk proteins were located differently between oil-water interface and aqueous phase according to the homogenizer type (microfluidizer vs. valve homogenizer) leading to differences in structure of the interfacial layer.

Antioxidants

Antioxidants are definitely one of the most important factors affecting lipid oxidation in complex systems. Therefore, detailed information about antioxidants and antioxidants used in oil-in-water emulsions are held in Chapter 4.

Chapter 4: Antioxidants in oil-in-water emulsions

This chapter will focus on antioxidants, their mechanisms and antioxidant strategies in oil-in-water emulsions.

4.1. Antioxidants

Antioxidants are the compounds that inhibit or slow down oxidation. They can be classified as natural and synthetic antioxidants. Most natural antioxidants are common food components, such as ascorbic acid and tocopherols, which have been used in the diet for thousands of years (Pokorný, 2001). Synthetic antioxidants such as propyl gallate and butylated hydroxyanisole (BHA) are used in some food products in order to prevent oxidation.

Antioxidants are also classified according to their mechanism of action as primary and secondary antioxidants. Some antioxidants are capable of behaving as both primary and secondary antioxidants, and are referred to as multiple-function antioxidants (Reische et al., 2002). Primary antioxidants, which are also named as chain-breaking antioxidants, disrupt the initiation or propagation step of oxidation reactions by donating hydrogen atoms to lipid peroxy radicals, lipid alkoxy radicals, and lipid radicals (Frankel, 2012). Secondary antioxidants are also called preventive antioxidants, which inactivate prooxidants by chelating metals, scavenging oxygen, and destroying hydroperoxides (Elias and Decker, 2010).

Reactions regarding antioxidant activity of primary antioxidants are presented as follows:



Reactions 11, 12 and 13 happen during initiation and propagation stages of autoxidation, whereas reactions 14, 15, 16 and 17 terminates the free radical chain reactions. Antioxidants (AH) may react with lipid peroxy radicals (LOO^\bullet), lipid alkoxy radicals (LO^\bullet) and lipid alkyl radicals (L^\bullet) and form reduced lipids (LH), lipid alcohol (LOH) and lipid hydroperoxides (LOOH). As part of termination reactions, antioxidant radicals (A^\bullet) may react with lipid radicals (L^\bullet , LO^\bullet , and LOO^\bullet) or antioxidant radical and form lipid conjugates (LA, LOA, and LOOA) or antioxidant dimer (AA).

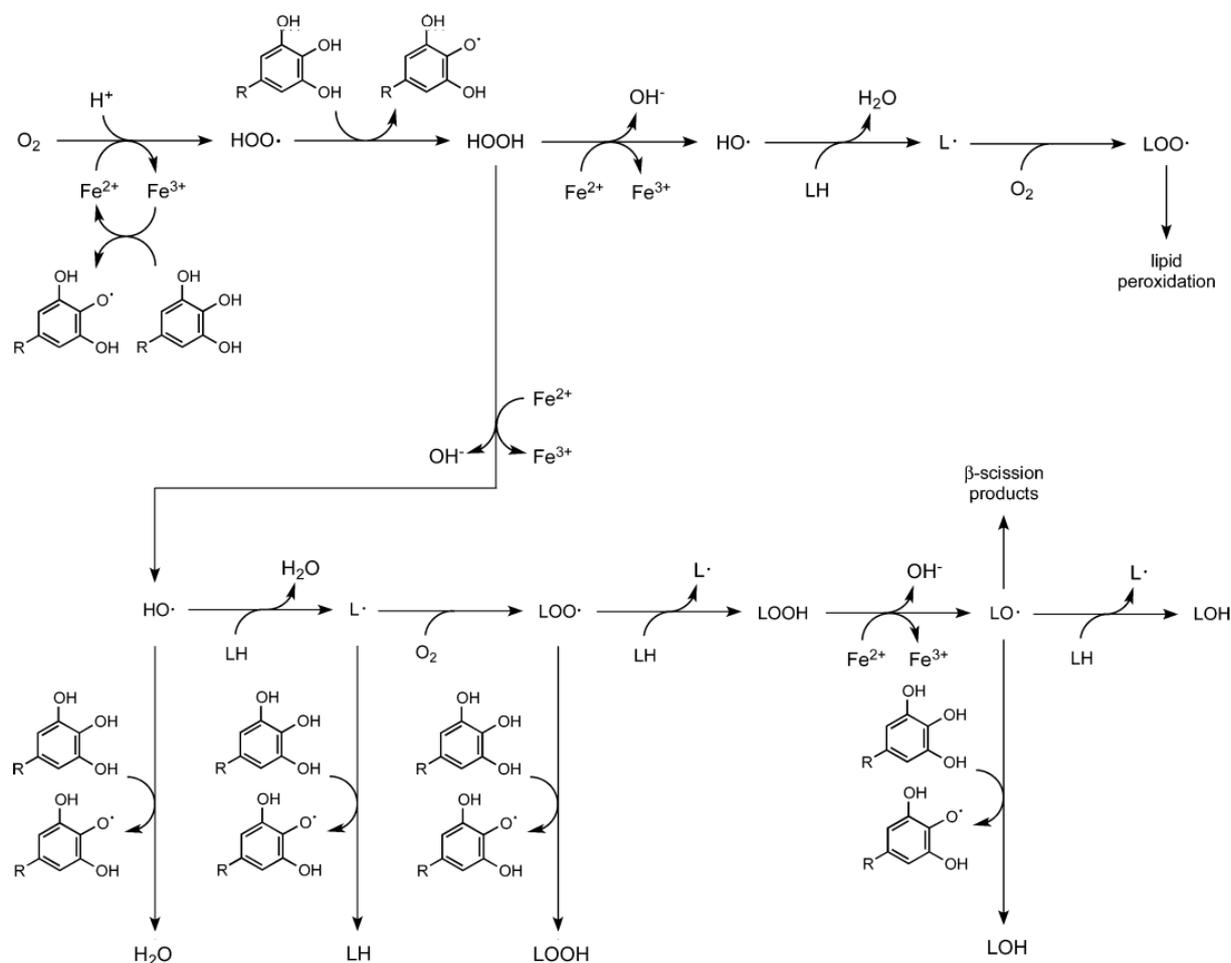


Figure 4.1. Oxidation of a simple gallate group is depicted for illustration of proposed mixed mechanism of polyphenol-mediated lipid oxidation and polyphenol radical scavenging antioxidant activity (Zhou and Elias, 2012). Abbreviations: hydroperoxyl radical (HOO^\bullet), hydroxyl radical (HO^\bullet), reduced lipid (LH), lipid alkyl radical (L^\bullet), lipid hydroperoxyl radical (LOO^\bullet), lipid hydroperoxide (LOOH), lipid alkoxy radical (LO^\bullet).

Polyphenols are reported to have both prooxidative and antioxidative effects in oil-in-water emulsions. Oxidation of a gallate group is shown in Figure 4.1 in order to illustrate the polyphenol-mediated lipid oxidation as a prooxidant and polyphenol radical scavenging antioxidant activity as an antioxidant. Nonenzymatic, metal-catalysed oxidation of polyphenols results in hydroperoxide

formation, which then further undergoes metal-catalysed reduction to generate highly reactive hydroxyl radicals (HO^\bullet) as shown in the upper half of the Figure 4.1. On the other hand, these gallate groups also inhibited lipid oxidation by donating a hydrogen atom to hydroxyl radical, lipid alkyl radical, lipid hydroperoxyl radical, and lipid alkoxy radical, which is shown in the lower half of the Figure 4.1.

4.2. Antioxidant strategies in oil-in-water emulsions

Lipid oxidation can be controlled by the use of phenolic compounds (e.g., phenolic acids, flavonoids, phenolipids), antioxidant emulsifiers and modified emulsifiers with antioxidant activities in oil-in-water emulsions.

4.2.1. Phenolic compounds

Phenolic compounds (e.g., phenolic acids, flavonoids) are secondary plant metabolites commonly found in fruits and vegetables. Molecular structure of a phenolic compound consists of at least one phenol group, which is a benzene ring with at least one hydroxyl group (Zhou and Elias, 2013). Phenolic compounds are added into food stuffs in order to inhibit lipid oxidation. Examples of phenolic compounds used in oil-in-water emulsions in order to prevent lipid oxidation are caffeic acid, ferulic acid, rutin, coumaric acid, rosmarinic acid, and chlorogenic acid (Sørensen et al., 2008; Narita et al., 2012; Kittipongpittaya et al., 2016).

In this Ph.D. study, the main focus is on caffeic acid due to its antioxidant activity by scavenging free radicals and chelating metals especially iron (Gülcin, 2006). Caffeic acid efficacy in oil-in-water emulsions was shown to be dependent on pH, iron addition, and emulsifier type (Sørensen et al., 2008).

4.2.2. Phenolipids

The number of phenolipids extracted from natural sources and performing satisfactory in emulsions is only a few (Shahidi and Zhong, 2011). Therefore, synthesis of phenolipids with varied lipophilicity has become one of the new antioxidant strategies. These compounds are inspired by naturally occurring caffeates and ferulates, which are long-chain alkyl esters of caffeic acid and ferulic acid, respectively. Their enhanced lipophilicity assists them to locate at the oil-water interface, where the oxidation initially occurs.

Earlier studies based on antioxidant activity in oil-in-water emulsions indicated that non-polar antioxidants are generally more efficient than polar antioxidants, which in turn are more effective in bulk oil than non-polar antioxidants. This phenomenon is called the ‘polar paradox’. This was attributed to the fact that the higher the lipophilicity of the compounds, the higher the affinity will be to the oil-water interface, where lipid oxidation is initiated (Porter, 1980; Schwarz et al., 2000).

However, this explanation was not confirmed by some other studies indicating that maximum antioxidant activity was often obtained at an intermediate hydrophobicity of the antioxidant molecule, which allowed it to be located at the oil-water interface (Laguerre et al., 2010; Laguerre et al., 2017). This phenomenon was named as ‘cut-off effect’. Laguerre et al. (2009) reported that the highest oxidative stability obtained with lipophilized chlorogenic acids, which had alkyl chain lengths from 1 to 20 carbons, was obtained for C12 alkyl chain length in a model emulsion with Brij as emulsifier using conjugated autoxidizable triene (CAT) assay.

Another study conducted with alkyl caffeates (C0-20) in a model emulsion with Brij as emulsifier reported that the highest antioxidant activity was found for alkyl caffeates with the chain lengths C8 and C12 using CAT assay (Sørensen et al., 2014). In the same study, antioxidant properties and efficacy of alkyl caffeates, ferulates, and coumarates have been investigated, and only caffeic acid and caffeates were able to form a complex with iron via their catechol group in the phenolic ring. Moreover, it was also reported that caffeic acid and alkyl caffeates showed the highest radical scavenging activity and reducing power, compared to ferulic acid and alkyl ferulates, as well as coumaric acid and alkyl coumarates, which showed the lowest efficiency. Another study with alkyl caffeates also reported that caffeates with short to medium chain length (C4, C8, and C12) were found to be more effective antioxidants in fish oil enriched mayonnaise (Alemán et al., 2015). In the same study, the most effective caffeates, which were used in fish oil enriched milk emulsions, were the ones with shorter alkyl chains (methyl and butyl) rather than the ones with medium and long chains (octyl, dodecyl, hexadecyl and eicosyl). These results indicated that the optimum alkyl chain length is phenolipid type and emulsion system dependent.

Phenolipids are not able to form emulsions when used individually; therefore, they need to be combined with an emulsifier such as proteins, phospholipids, polysaccharides or surfactants. The head group of the phenolipids are not sufficiently large to have a steric or electrostatic repulsion for preventing droplet coalescence (McClements and Decker, 2018). When these phenolipids are

combined with proteins, which may also have antioxidant activity, synergistic effects on oxidative stability may also be seen (Huang et al., 2017).

4.2.3. Antioxidant emulsifiers

Some emulsifiers have antioxidant properties naturally. CAS has radical scavenging activity as well as metal chelating ability, which allows them to strongly bind transition metals due to their numerous phosphate groups (Diaz et al., 2003; Kim et al., 2007). Faraji et al. (2004) reported that whey protein isolate, soy protein isolate and CAS in the concentration of 10 mg/L were able to bind 185, 405 and 980 μ moles of iron, respectively, which showed the high iron chelating capacity of CAS compared to other proteins.

Phospholipids such as PC, phosphatidylethanolamine, and phosphatidylinositol show antioxidant activities such as degradation of hydroperoxides, metal chelating as well as regenerating tocopheroxyl to form tocopherol by hydrogen transfer (Bandarra et al., 1999; García-Moreno et al., 2014; Samdani et al., 2018).

4.2.4. Modified emulsifiers with phenolic acids and various lipophilicity

Emulsifiers are surface active compounds, therefore they adsorb at the oil-water interface of emulsions. The strategy with modifying emulsifiers with antioxidants aims to bring the antioxidant activity in closer vicinity of the oil-water interface, where the lipid oxidation is claimed to be initiated. This approach was adopted in a previous study where egg white protein was conjugated with catechin (Gu et al., 2017). It was found that the egg white-catechin conjugates inhibited lipid oxidation significantly more than physical mixture of egg white and catechin.

In another perspective, modified emulsifiers can also be built starting from an antioxidant compound such as alginates. Sodium alginate, which is used as a thickener in oil-in-water emulsions, has shown antioxidant activity (Falkeborg et al., 2014). Due to the fact that lipid oxidation is initiated at the oil-water interfacial layer, modification strategies are built on bringing antioxidant activity to the oil-water interfacial layer. Therefore, sodium alginate was modified with dodecenyl succinic anhydride. This made the whole molecule more lipophilic compared to a regular sodium alginate, and thereby resulted in making it more surface active than a common stabilizer (Falkeborg et al., 2015).

Chapter 5: Experimental design and methodology

The experimental approach of this Ph.D. study is illustrated in Figure 5.1. The first part of the experiments focused on optimization of the high fat fish oil-in-water emulsions' formulae considering physical and oxidative stability for each combination of emulsifiers employed. In this part, oxidative stability of the emulsions was investigated in relation to their physical characteristics including the structure of the oil-water interface (as indicated by the big red arrow in Figure 5.1) for emulsions with combinations of CAS and PC as emulsifiers. The second part of the experiments was designed to investigate the effects of tailor-made emulsifiers, which have improved surface activity as well as antioxidant properties, on physical and oxidative stability of high fat fish oil-in-water emulsions. In this part, distribution of labelled DATEMs in different environments of the emulsion was also studied (as indicated by the big orange arrow in Figure 5.1). Lastly, in the third part, high fat n-3 delivery emulsions were incorporated into mayonnaise in order to reveal the influence of the high fat delivery emulsion system on physical and oxidative stability of the enriched product compared to mayonnaise enriched with neat fish oil including sensory assessment.

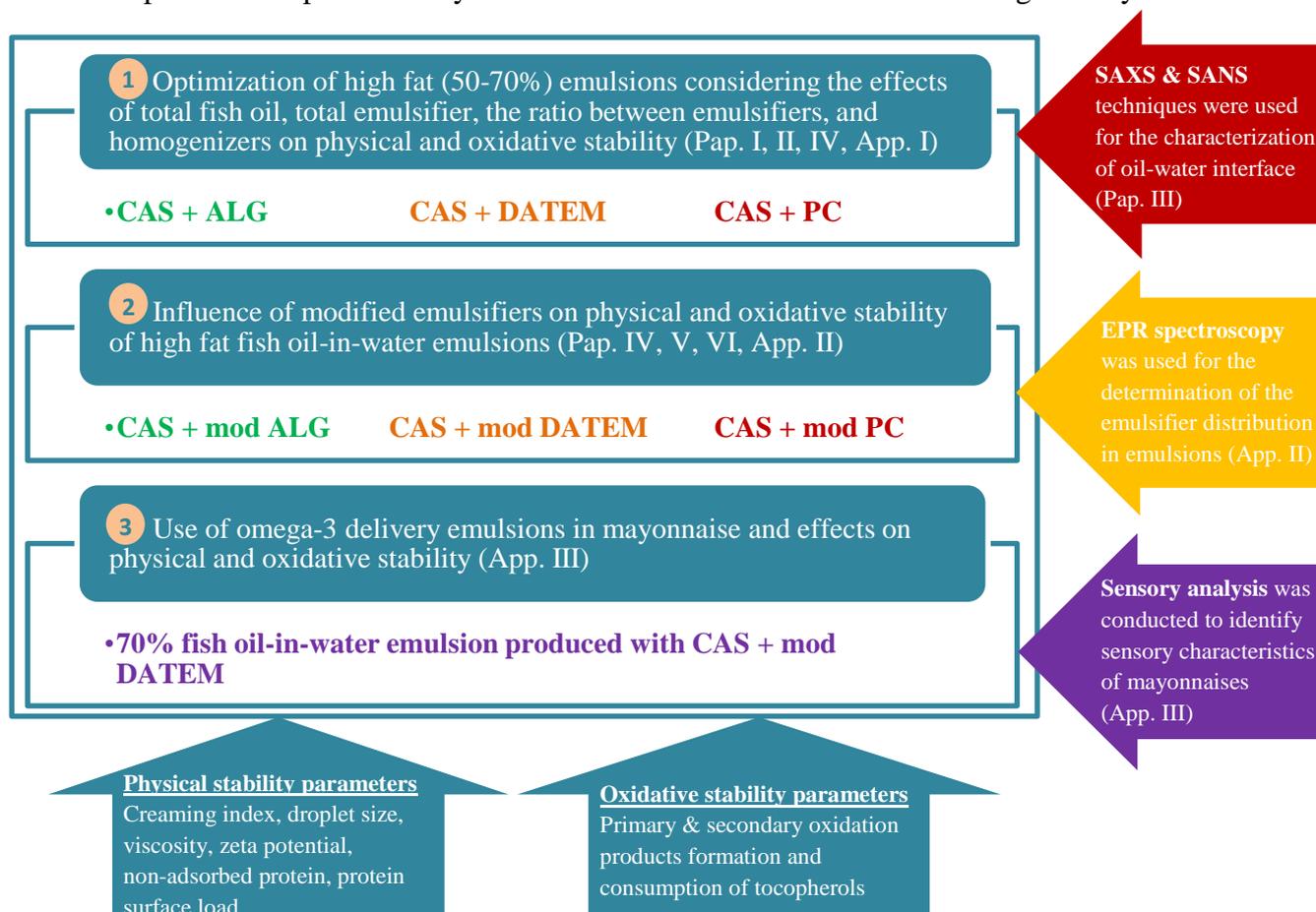


Figure 5.1. Overall experimental approach of the Ph.D. study.

5.1. Emulsifiers used in various studies

This Ph.D. study focuses on producing physically and oxidatively stable high fat emulsions using various emulsifier combinations. First set of experiments defined under Part I employed molecules shown in Figures 2.1, 2.2, 2.3, and 2.4, namely sodium caseinate (CAS), sodium alginate (ALG), diacetyl tartaric acid ester of mono- and diglycerides (DATEM), and phosphatidylcholine (PC), respectively, in the optimization studies in order to find the optimal formulae for each emulsifier combination.

In Part II, optimal formulae were applied in high fat oil-in-water emulsions using modified emulsifiers, which potentially had enhanced antioxidant and/or surface activities. Alginates were modified with succinic anhydride (SAC0) and dodecanyl succinic anhydride (SAC12) to study the effect of hydrophobic chain on surface activity of alginate (Falkeborg et al., 2015). Molecular structures are shown in the Figure 5.2. Short chain modified alginate and long chain modified alginate are abbreviated as SCMA and LCMA, respectively. Although SCMA and LCMA were previously tested in low-fat oil-in-water emulsions (Falkeborg et al., 2015), they were for the first time ever evaluated in high fat fish oil-in-water emulsions in this PhD thesis.

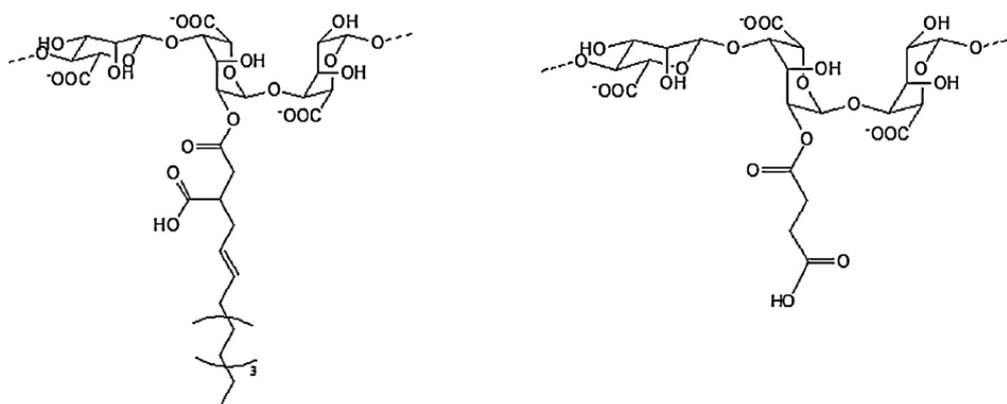


Figure 5.2. Molecular structure of modified alginates; on the left, long chain modified alginate (LCMA) and on the right, short chain modified alginate (SCMA) (Falkeborg et al., 2015)

DATEM and PC were modified with caffeic acid covalently attached to the glycerol backbone of the surfactants in order to enhance their antioxidant activity. In addition, same molecules were also modified with alkyl chains of different carbon chain lengths in order to study the effect of lipophilicity on surface activity and adsorption behaviour at oil-water interface: Carbon chain lengths of 12 and 14 for DATEM (Anankanbil et al., 2017), and 14 and 16 for PC (Anankanbil et al., 2018b) surfactants were evaluated. The reason for selecting modified DATEMs with C12 and C14

chain lengths was based on the provided high surface activity, narrow droplet size distribution and superior antioxidant activity of these compounds in 20% fish oil-in-water emulsions (Anankanbil et al., 2017; 2018a). Similarly, modified PCs with C14 and C16 chain lengths were selected due to their physical stability based on the droplet size distribution and creaming as well as better iron chelating activity compared to modified PCs with C12 and C18 in 20% fish oil-in-water emulsions (Anankanbil et al., 2018b). The molecular structures of these compounds are shown in Figures 5.3 (mod DATEMs) and 5.4 (mod PCs). DATEMs with caffeic acid and C12 or C14 are abbreviated as DATEM_C12 and DATEM_C14. PCs with caffeic acid with C14 and C16 are abbreviated as PC_C14 and PC_C16.

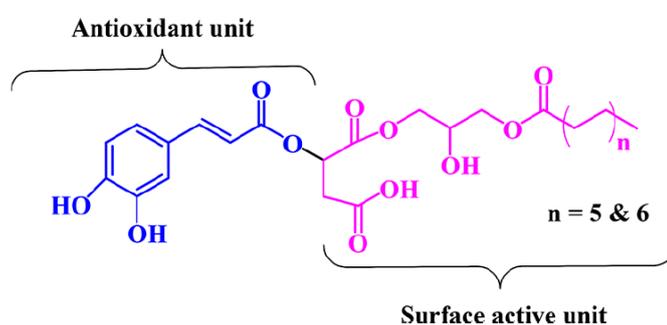


Figure 5.3. Molecular structures of modified DATEMs (DATEM modified with caffeic acid and C12 or C14 alkyl chains) (Anankanbil et al., 2017)

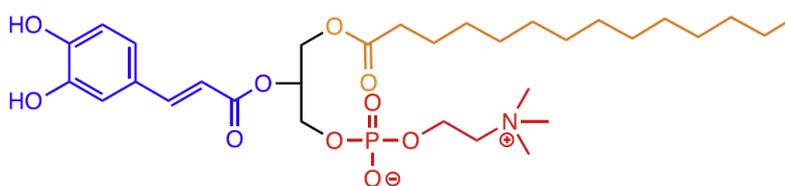


Figure 5.4. Molecular structures of modified PCs (PC modified with caffeic acid and C14 or C16 alkyl chains) (Anankanbil et al., 2018b)

5.2. Experimental set up for the optimization studies

The optimization studies were conducted using Box-Behnken's design, which is a factorial design commonly employed in response surface methodology (RSM) studies (Khajeh, 2010). Box-Behnken's design together with RSM provide the experimental set up for finding the optimal recipe for the production of physically and oxidatively stable high fat oil-in-water emulsions. The experimental data obtained from physical and oxidative parameter analyses were subjected to RSM, where the data were fitted to quadratic models. Influence of the input variables studied (oil content,

total emulsifier concentration, and the ratio between emulsifiers) on the physical and oxidative parameters measured were evaluated by analysis of variance (ANOVA). The quadratic models obtained were used to build the contour plots and optimal values were determined. Fish oil content in three different levels (50, 60, and 70%, w/w), total emulsifier content (1.4, 2.1, and 2.8%, w/w), and ratio between emulsifiers (0.4, 1.2, and 2.0) were the factors studied. The same experimental set up was applied to three different emulsifier/stabilizer combinations: CAS+ALG, CAS+DATEM, and CAS+PC. The numbers used in the emulsion codes in Part I are presented in Table 5.1.

Table 5.1. Emulsion numbers based on Box-Behnken's design (Part I)

Emulsion No	Fish oil %, w/w	Total emulsifier %, w/w	Ratio of CAS to ALG or DATEM or PC
1	50	1.4	1.2
2	70	1.4	1.2
3	50	2.8	1.2
4	70	2.8	1.2
5	50	2.1	0.4
6	70	2.1	0.4
7	50	2.1	2.0
8	70	2.1	2.0
9	60	1.4	0.4
10	60	2.8	0.4
11	60	1.4	2.0
12	60	2.8	2.0
13	60	2.1	1.2
14	60	2.1	1.2
15	60	2.1	1.2

In chapter 6, emulsion codes are stated as ALG-1, ALG-2, ..., ALG-15 for the emulsions produced with CAS+ALG combinations. Emulsion codes are named as DATEM-1, DATEM-2, ..., DATEM-15 for the emulsions produced with CAS+DATEM. Lastly, emulsion codes are described as PC-1, PC-2, ..., PC-15 for the emulsions produced with CAS+PC. Formulae regarding code numbers from 1 to 15 are detailed in Table 5.1.

The influence of homogenizer type on physical and oxidative stability of the emulsions was also assessed in the first part of this Ph.D. study. Sixty% fish oil-in-water emulsions were produced using combinations of CAS+DATEM or PC as emulsifiers and were homogenized either employing Stephan mixer or colloid mill. Physical and oxidative stability results together with the experimental plan are presented in Appendix I.

5.3. Experimental set up for the application of modified emulsifiers

Optimal recipes selected for each type of emulsifier combination CAS+ALG or DATEM or PC was employed in emulsions produced with modified versions of ALG, DATEM and PC. Emulsions produced with mod ALGs had 3 different levels of fish oil content (Paper IV); however, in the comparison made in figures and tables in section 6.2, only 70% fish oil-in-water emulsions were used. This was for the sake of the comparison between mod ALG emulsions with mod DATEM and mod PC emulsions, which all contained 70% fish oil.

Table 5.2. Experimental design and emulsion codes used in section 6.2 (Part II)

Study code	Emulsion code	Total emulsifier (% , w/w)	CAS:ALGs or CAS:total DATEM or CAS:total PC (ratio)	DATEM:mod DATEM or PC:mod PC (ratio)	Caffeic acid (ppm)
mod ALG* (Pap. IV)	CAS	1.4	-	-	-
	CAS+ALG	1.4	1.2	-	-
	CAS+SCMA	1.4	1.2	-	-
	CAS+LCMA	1.4	1.2	-	-
mod DATEM** (Pap. V)	DATEM_com	2.8	2.0	-	-
	DATEM_C12_10%	2.8	2.0	9.3	305
	DATEM_C12_30%	2.8	2.0	2.3	914
	DATEM_C12_60%	2.8	2.0	0.7	1828
	DATEM_C14_10%	2.8	2.0	9.3	290
	DATEM_C14_60%	2.8	2.0	0.7	1740
	DATEM_com_caf_low	2.8	2.0	-	290 [§]
DATEM_com_caf_high	2.8	2.0	-	1740 [§]	
mod PC*** (Pap. VI)	1 CAS	2.8	-	-	-
	2 PC_com	2.8	1.2	-	-
	3 PC_C14_360	2.8	1.2	9.6	360
	4 PC_C14_1080	2.8	1.2	2.4	1080
	5 PC_C14_2160	2.8	1.2	0.7	2160
	6 PC_C16_360	2.8	1.2	8.8	360
	7 PC_C16_1080	2.8	1.2	2.3	1080
	8 PC_C16_2160	2.8	1.2	0.6	2160
	9 PC_com_caf_360	2.8	1.2	-	360 [§]
	10 PC_com_caf_2160	2.8	1.2	-	2160 [§]

All the emulsions produced are 70% fish oil-in-water emulsions. [§] Added free caffeic acid.

*In mod ALG study, dose-response effect was not tested; either com ALG or mod ALGs were used.

In mod DATEM study, dose-response effect of mod DATEMs was investigated. The ratio between com DATEM and mod DATEMs was fixed for different mod DATEMs, therefore the final caffeic acid concentration varied slightly for different mod DATEMs due to their different molecular weights. *In mod PC study, dose-response effect of mod PCs was investigated. The ratio between PC and mod PCs was adjusted to obtain an equivalent caffeic acid concentration (e.g., 360, 1080, and 2160 ppm) in the final emulsion.

In these three studies, emulsions were stored at room temperature in darkness for 12 days and oxidation was promoted using iron addition (100 μ M of Fe²⁺ in the emulsion).

5.4. Experimental set up for the application of n-3 delivery system in mayonnaise

Mayonnaise samples were prepared with added delivery emulsions (Appendix III). Details of delivery emulsions are presented in Table 5.3, which were selected based on the results from Paper V. All delivery emulsions consisted of 70 wt% fish oil and 2.8 wt% total emulsifier with a ratio of CAS to total DATEM of 2. The ratio between commercial DATEM to modified DATEMs was 0.7, which means that ~60% of the commercial DATEM was replaced by the modified DATEMs in the final emulsion. This results in a concentration of 2160 ppm caffeic acid in the final delivery emulsion. Therefore, a control delivery emulsion was included, which had commercial DATEM and equivalent amount of free caffeic acid added. Emulsifiers (CAS, DATEM, and modified DATEMs) were dissolved in distilled water and stirred overnight at 4°C. Aqueous phases were adjusted to pH 7 using 2M NaOH. Emulsions were produced in a Stephan Universal mixer (Stephan, UMC5, 1995, Hameln, Germany) in 500 g batches as described by Horn et al. (2011).

Table 5.3. Emulsifiers and their amounts used in the production of delivery emulsions

Emulsion codes	Description	CAS (% w/w)	DATEM (% w/w)	Mod DATEM (% w/w)	Caffeic acid (% w/w)
CAS	CAS	2.8	-	-	-
DATEM	CAS + DATEM	1.87	0.93	-	-
DATEM_C12	CAS + DATEM + DATEM C12	1.87	0.37	0.56	0.18*
DATEM_C14	CAS + DATEM + DATEM C14	1.87	0.37	0.56	0.17*
DATEM_CA	CAS + DATEM + caffeic acid	1.87	0.93	-	0.17

All the delivery emulsions consist of 70% w/w fish oil.

*The caffeic acid amount (colored in light grey) is already present in the modified DATEM surfactants; therefore it was not added in the delivery emulsions.

The amounts mixed for the preparation of mayonnaise samples are described in Table 5.4. A control mayonnaise sample was produced using neat fish oil added directly in the mayonnaise sample during its production. Ingredients of mayonnaise samples are described in Appendix III.

Table 5.4. Experimental design including the sample codes and descriptions for mayonnaise samples. (MAYO: Mayonnaise; DE: Delivery emulsion)

Mayonnaise codes	Descriptions	MAYO (g)	DE (g)
1_MAYO_FO	Mayonnaise produced with 16% fish oil	600	-
2_MAYO_CAS	Mayonnaise + delivery emulsion CAS	467	133
3_MAYO_DATEM	Mayonnaise + delivery emulsion DATEM	467	133
4_MAYO_DATEM_C12	Mayonnaise + delivery emulsion DATEM_C12	467	133
5_MAYO_DATEM_C14	Mayonnaise + delivery emulsion DATEM_C14	467	133
6_MAYO_DATEM_CA	Mayonnaise + delivery emulsion DATEM_CA	467	133

All the mayonnaises consist of 80% w/w oil (16% fish oil + 64% rapeseed oil). Refer to Table 5.3 for the details of the DEs.

5.5. Evaluation of physical and oxidative parameters

Assessment of the physical and oxidative parameters was carried out by using the methods described in Table 5.5. Further details are present in the referred papers and appendices.

Table 5.5. Methods used in this Ph.D. study

Method	Short description	Papers/Appendices
Droplet size	Measurement of particle sizes using laser diffraction	Pap. I, II, III, IV, V, VI, App. I, and III
Apparent viscosity	Measured using a stress-controlled rheometer at 20 s ⁻¹ shear rate for emulsions and 1.5 s ⁻¹ shear rate for mayonnaise samples	Pap. I, II, IV, V, VI, App. I, and III
Non-adsorbed protein	Emulsions were separated using centrifugation + ultracentrifugation and the protein content in the water phase was analysed using BSA or DUMAS method	Pap. IV, V, VI, and App. III
Protein surface load	Protein surface load was calculated using non-adsorbed protein content (C _{SER}), initial protein content (C _{INI}), droplet surface area (d ₃₂), and oil fraction (Φ), according to the formula as follows: $\Gamma = \frac{(1-\Phi)d_{32}}{6\Phi} (C_{INI} - C_{SER})$	Pap. VI and App. III
Interfacial tension	Interfacial tension of the emulsifiers at the oil-water interface was determined using an automated drop tensiometer OCA20 (DataPhysics Instruments GmbH, Filderstadt, Germany) at 25°C	Pap. VI
Zeta potential	Surface charge of the oil droplets was measured using Zetasizer Nano 2S	Pap. I, II, V, VI, App. I, and III
Creaming index	Creaming was determined by the formula CI (%) = b/a*100; where (a) is the height of total emulsion and (b) is the height of aqueous phase	Pap. I, II, IV, V, VI, App. I, and III
Peroxide value	Lipids were extracted according to Bligh & Dyer method and peroxide value was determined on the lipid extracts by colorimetric determination of iron-thiocyanate complex using a spectrophotometer	Pap. I, II, IV, V, VI, App. I, and III
Volatile compounds	Dynamic head space (DHS) was used to collect volatile compounds, which were then trapped on Tenax GR tubes and separated, identified and quantified using in Gas chromatography – Mass spectroscopy (GC-MS)	Pap. I, II, IV, V, VI, App. I, and III
Tocopherols	Lipid extracts were analyzed according to AOCS method(1998) and analyzed by HPLC	Pap. I, II, IV, V, VI, App. I, and III
Sensory	Eight assessors were employed for sensory profiling of mayonnaises and physical appearance, texture and smell attributes were tested. Samples were not tasted by the assessors.	App. III

Table 5.5. Methods used in this Ph.D. study (continues)

Method	Short description	Papers/Appendices
SAXS & SANS	SAXS was done using CREDO apparatus at the Research Centre for Natural Sciences, Hungarian Academy of Sciences (Hungary) SANS measurements were performed on the Yellow Submarine instrument at the Budapest Neutron Center (Hungary) and the Larmor Instrument, Rutherford Appleton Laboratory (England)	Pap III
EPR	Miniscope MS 300 was used for the measurements, TEMPOL and labelled DATEMs with various alkyl chains were employed at Penn State University	App. II
Statistics	ANOVA and RSM were carried out using Statgraphics XVII (Statpoint Technologies, Inc., Virginia, USA) Multivariate data analysis (e.g., PCA) was carried out using Latentix 2.12 (LatentiX, Copenhagen, Denmark)	Pap. I, II, IV, V, VI, App. I, and III

Finally, the different studies conducted in this Ph.D. thesis are presented in Table 5.6 including the emulsifiers and methods used and storage conditions applied.

Table 5.6. Emulsifiers, methods and storage conditions applied in various studies.

Papers/Appendices	Emulsifiers	Methods used*	Storage conditions
Paper I	CAS, ALG	CI, DS, V, ZP, PV, TOC, and VC.	28 days, at RT, in darkness
Paper II	CAS, PC	CI, DS, V, ZP, PV, TOC, and VC.	12 days, 100µM Fe ²⁺ , at RT, in darkness
Paper III	CAS, PC	DS, NP, SAXS, and SANS.	Measurements were done within 1-3 days after sample preparation
Paper IV	CAS, ALG, SCMA, LCMA	CI, DS, V, ZP, NP,	12 days, 100µM Fe ²⁺ , at RT, in darkness
Paper V	CAS, DATEM, DATEM_C12, DATEM_C14	CI, DS, V, ZP, NP, PV, TOC, and VC.	12 days, 100µM Fe ²⁺ , at RT, in darkness
Paper VI	CAS, PC, PC_C14, PC_C16	CI, DS, V, ZP, NP, PSL, ST, PV, TOC, and VC	12 days, 100µM Fe ²⁺ , at RT, in darkness
Appendix I	CAS, DATEM, PC	CI, DS, V, PV, TOC, and VC	15 days, at RT, in darkness
Appendix II	CAS, labelled DATEM	EPR	6 days, at RT, in darkness
Appendix III	CAS, DATEM, DATEM_C12, DATEM_C14	CI, DS, V, ZP, NP, PSL, PV, TOC, and VC.	28 days, at RT, in darkness

*Abbreviations for the methods: CI – creaming index, DS – droplet size, V – viscosity, ZP – zeta potential, NP – non-adsorbed protein, PSL – protein surface load, IT – interfacial tension, SAXS & SANS – small angle X-ray and neutron scattering, EPR – electron paramagnetic resonance spectroscopy, PV – peroxide value, TOC – tocopherols, VC – volatile compounds, RT – room temperature (20 °C).

Chapter 6: Results and discussion

This chapter includes the summary of the findings of the Ph.D. study together with an overall discussion of the results in comparison to related literature. Refer to manuscripts and appendices for detailed information regarding individual studies. The structure of this chapter is presented below:

Part I: Optimization of high fat (50-70%) emulsions considering the effects of total fish oil, total emulsifier, and ratio between emulsifiers as well as type of homogenizer on physical and oxidative stability. The combinations of emulsifiers studied were as follows:

- Sodium caseinate and sodium alginate
- Sodium caseinate and DATEM or PC (including results from SAXS & SANS techniques)

Part II: Influence of modified emulsifiers on physical and oxidative stability of high fat fish oil-in-water emulsions. The different combinations of emulsifiers evaluated were as follows:

- Sodium caseinate and modified alginates
- Sodium caseinate and modified DATEMs (including results from EPR spectroscopy) or modified PCs

Part III: Use of omega-3 delivery emulsions containing modified DATEM in mayonnaise and effects on physical and oxidative stability (including results from sensory analysis).

6.1. Part I: Effect of emulsion composition and homogenizer type on physical and oxidative stability

6.1.1. Overall comparison of the effect of emulsion formulae on physical and oxidative stability

Fish oil content, total emulsifier content and ratio between emulsifiers were selected as important factors affecting physical and oxidative stability parameters of high fat (50-70%) emulsions. The effects of these factors as well as type of homogenizer on the physical and oxidative stability of high fat fish oil-in-water emulsions stabilized with different type of emulsifiers combined with CAS are first summarized in Table 6.1 in order to illustrate the overall results.

Table 6.1. Influence of factors selected in the optimization of high fat n-3 delivery oil-in-water emulsions

Factors	Parameters	CAS+ALG (Pap. I)	CAS+DATEM (Pap. V)	CAS+PC (Pap. II)
Increase in fish oil content (50 to 70%)	Creaming	Decreased*	Decreased*	Decreased*
	Droplet size	Increased*	Decreased*	Decreased*
	Zeta potential	Increased	Increased*	Decreased*
	Viscosity	Increased*	Increased*	Increased*
	Peroxide value	Increased	Decreased	Decreased
	2,4-heptadienal	Increased	Decreased	Increased*
Increase in total emulsifier content (1.4 to 2.8%)	Creaming	Decreased	Decreased*	Decreased*
	Droplet size	Increased	Decreased*	Decreased*
	Zeta potential	Increased*	Increased*	Decreased*
	Viscosity	Increased*	Increased	Increased
	Peroxide value	Increased	Decreased	Decreased
	2,4-heptadienal	Increased	Decreased	Decreased
Increase in the ratio of CAS to ALG/ DATEM/ PC (0.4 to 2)	Creaming	Increased	Decreased	Decreased*
	Droplet size	Decreased*	Decreased*	Decreased*
	Zeta potential	Decreased	Increased	Decreased*
	Viscosity	Decreased	Increased	Increased
	Peroxide value	Decreased	Decreased	Increased
	2,4-heptadienal	Increased	Decreased*	Increased
Type of homogenizer (Colloid mill compared to Stephan mixer) (App. D)	Creaming	N/A	Decreased	Decreased
	Droplet size	N/A	Decreased*	Decreased*
	Zeta potential	N/A	Decreased*	Increased*
	Viscosity	N/A	Increased*	Increased*
	Peroxide value	N/A	Increased*	Increased*
	2,4-heptadienal	N/A	Increased	Increased

Increased;
 Significantly increased;
 Decreased;
 Significantly decreased;
 N/A; not available; *Change is significantly different when $p < 0.05$.

It was observed that, in general, increasing fish oil and total emulsifier content increased the viscosity of the emulsions; thereby improving physical stability as also observed from the finding that creaming decreased (Paper I, II, IV, and V). The increase in viscosity was, however, dependent on the type of emulsifier, which was combined with CAS. Thus, sodium alginate (ALG) behaved as a thickener, whereas surfactants (DATEM and PC) were more surface active compared to ALG and replaced CAS at the oil-water interface, but did not increase viscosity to the same extent as ALG.

Increasing fish oil and total emulsifier content as well as ratio between CAS to surfactants significantly decreased the droplet size for CAS and DATEM or PC emulsions, whereas similar trend was not observed for CAS+ALG emulsions. Zeta potential and oxidative stability parameters did not reveal a general trend for different types of emulsifiers combined with CAS.

PCA analysis revealed that emulsions with ALG were located closer to viscosity and droplet size (D[4,3]), whereas they were further away from oxidation parameters compared to emulsions with DATEM or PC (Figure 6.1). This indicates that emulsions stabilized with combinations of CAS and ALG presented higher viscosity and larger droplet size but higher oxidative stability compared to emulsions stabilized with combinations of CAS and DATEM or PC. Due to the fact that ALG, as a water soluble polysaccharide, primarily acts as a thickening and water-holding agent, its presence increased the viscosity of the aqueous phase. Thickening effect of ALG in the aqueous phase of the emulsion further increased the viscosity of the whole emulsion. This was attributed to the higher viscosity of the water phase, which enabled better disruption of the oil, thereby favouring the formation of smaller droplets (Antonov et al., 2004; Aken, 2006). As smaller droplets provided larger oil-water interfacial area, viscosity increased because of the friction between droplets, which restrained the mobility. Increased viscosity improved physical stability of the emulsions by enhancing creaming stability with the mechanism of slowing down the mobility of the oil droplets (Hiemenz, 1986, Dickinson, 1992). On the contrary, surfactants (DATEM and PC) did not have thickening properties in the aqueous phase of the emulsions, but instead they were surface active and adsorbed at the oil-water interface.

In general, emulsions (e.g., numbers 1, 3, 5, 7, and 9) with either the lowest fat content (50%) or total emulsifier content (1.4%) were located in the lower part of the PC2 axis compared to others. These emulsions had larger droplet sizes and lower viscosity, confirming that the high fish oil content and total emulsifier content enhanced physical stability by forming smaller droplets and increasing viscosity regardless of the emulsifier type used in combination with CAS. This is also confirmed by the finding that the Fish Oil category variable was located opposite to the D[4,3] variable.

Importance of the alginate concentration in the aqueous phase (ratio between alginate and the aqueous phase) for forming a stable emulsion is due to the fact that depletion flocculation and bridging flocculation might occur in the presence of high ALG concentration as explained in chapter 2. Besides, high concentration of HMWEs (e.g., CAS) and stabilizers (e.g., ALG) in the

water phase could lead to a slower movement of the emulsifiers (e.g., CAS) during emulsification, thereby resulting in coalescence of oil droplets (Paper I). On the contrary, LMWEs (e.g. surfactants) are also surface active as CAS, and may therefore not yield high concentrations of the molecules in the aqueous phase as is the case for thickeners. Thereby, bridging or depletion flocculation is less likely to occur for the surfactants compared to high molecular weight thickeners. Further discussion on physical stability of emulsions is shown below in section 6.1.2.

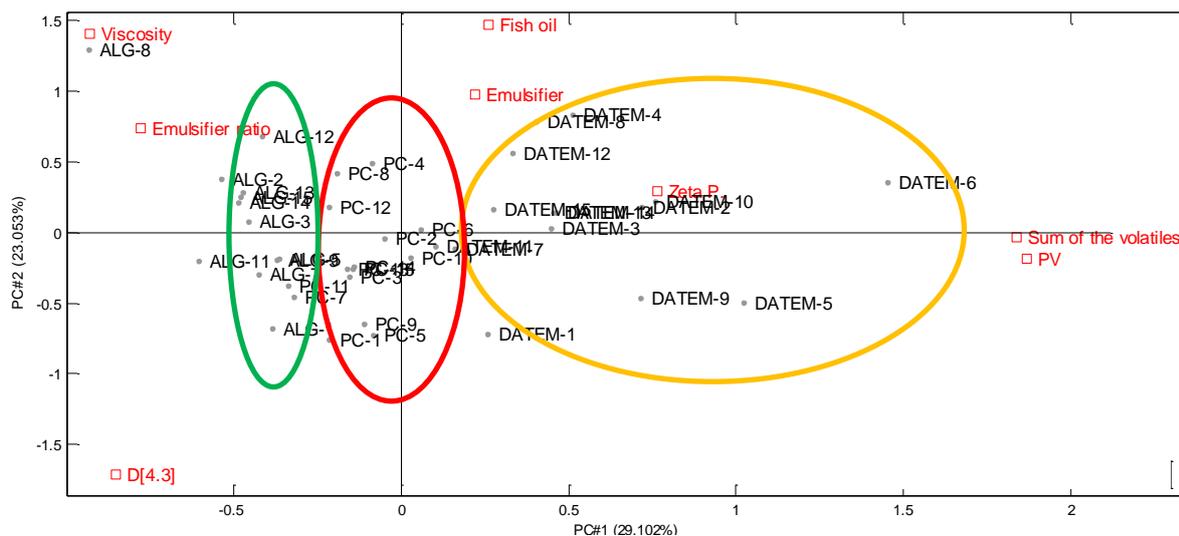


Figure 6.1. PCA biplot of emulsions produced with CAS and ALG or DATEM or PC. For the emulsion codes, refer to Table 5.1 and the description below the table. ‘Emulsifier ratio’ refers to the ratio of CAS to ALG or DATEM or PC; ‘Emulsifier’ refers to total emulsifier content; ‘Fish oil’ refers to total fish oil content.

As physical stability of the high fat oil-in-water emulsions has an effect on oxidative stability, contribution of emulsifiers combined with CAS to lipid oxidation was also investigated in this Ph.D. study. It should be borne in mind that emulsions produced with CAS and ALG were exposed to 28 days of storage without added iron (Fe^{2+}). Therefore, the storage conditions were not identical to emulsions produced with CAS and DATEM or PC, which were stored for 12 days with addition of iron ($100\mu\text{M}$) to promote lipid oxidation. The reason for adding iron in these 2 studies was due to the finding that low amounts of primary ($\text{PV} < 1$ meq. peroxides/ kg oil) and secondary (volatile compounds $< 60\text{ng/g}$ sample) oxidation products were obtained during 28 days of storage for emulsions produced with CAS+ALG. Therefore, it would be misleading to compare CAS+ALG emulsions with emulsions produced with CAS+DATEM/PC due to different storage conditions. However, since the PC emulsions were located further away from the PV and volatiles, the PCA

model suggests that PC emulsions had better oxidative stability than DATEM emulsions. Further discussion on oxidative stability of emulsions is shown below in section 6.1.3.

Homogenizer type also had an influence on physical stability of the emulsions produced with CAS+DATEM or PC (Table 6.1). As described in Appendix I, 60% fish-oil-in-water emulsions were produced using either Stephan mixer or a combination of ultra-turrax and colloid mill. Droplet size of the emulsions were smaller when colloid mill was used compared to Stephan mixer. This was due to the possibility to adjust a gap for the emulsion to pass through, which led to a smaller droplet size formation. Viscosity was higher for the emulsions produced with colloid mill compared to Stephan mixer, which was presumably due to the smaller droplet sizes formed in the colloid mill. Creaming stability was also higher for the emulsions produced with colloid mill compared to Stephan mixer, which was due to having a higher overall viscosity for the emulsions produced with colloid mill.

Changes in the physical stability affected the oxidative stability of the emulsions produced using Stephan mixer and colloid mill homogenizers. Emulsions produced with Stephan mixer had significantly higher primary oxidation product formation and increased (not significant) 2,4-heptadienal formation regardless of the emulsifier types used (Table 6.1.), which could be due to the less stress applied during the production using Stephan mixer, as opposed to colloid mill, which also had a pre-homogenization step employed using ultra-turrax. Nevertheless, these effects were not visible at day 0 results of peroxide value and volatile compounds (Appendix I). Therefore, the difference in oxidative stability could be also attributed to the physical stability of the emulsions provided by different homogenizers.

As explained above, creaming instability in the emulsions produced with Stephan mixer might have increased the oxidative stability by keeping prooxidants away from oil droplets. Besides, smaller droplets formed by the colloid mill led to a larger surface area, which increased the contact area between prooxidants in the water phase and lipids, thereby favoured lipid oxidation. In summary, colloid mill provided higher physical stability, whereas inferior oxidative stability compared to Stephan mixer for CAS+DATEM and CAS+PC emulsions. The emulsification equipment can significantly affect the distribution of emulsifiers between the different phases; however, this was not investigated in this study, which could be a subject for further studies.

6.1.2. Effect of emulsion formulae on physical stability

Creaming stability of the emulsions varied depending on the formula as well as type of emulsifier, which was combined with CAS. Among emulsions produced with ALG, only two of them showed creaming, whereas most of the emulsions produced with DATEM or PC showed creaming (Figure 6.2a). As stated before, thickening properties of ALG improved the creaming stability of the emulsions by increasing the viscosity of the aqueous phase, which hindered the mobility of the oil droplets. Therefore, it was obtained that the increase in the ratio of CAS to ALG resulted in increase in creaming (Table 6.1).

On the other hand, surfactants (DATEM and PC) do not act as stabilizers. Therefore, increasing the ratio of CAS to DATEM or PC decreased the creaming (Table 6.1, Figure 6.2). DATEM and PC are surface active compounds, therefore they compete with CAS to adsorb at the oil-water interface and replaced the protein at the oil-water interface and/or interacted with the protein forming thicker interfacial layer (Fang and Dalgleish, 1996, Paper III). Nevertheless, these phenomena might not necessarily improve creaming stability of the oil-in-water emulsions. Combined use of DATEM or PC with CAS resulted in decrease in physical stability for the most of the formulae applied; such as decrease in viscosity, increase in creaming and droplet size (Table 1, Figure 6.2a, c, and d). This loss in physical stability could be explained by the replacement of protein, which is an efficient emulsifier forming viscoelastic films, at the oil-water interface by DATEM or PC. Combinations of proteins and surfactants have been reported to form lateral domains at the interface which also affected physical stability (Berton et al., 2012). Therefore, mixtures of CAS and DATEM or PC might have also resulted in the formation of lateral domains, which provided less rigid interfaces compared to emulsions produced with only CAS, thereby leading to physical instability (Berton-Carabin et al., 2014). This was also indicated by the SAXS and SANS studies conducted on the emulsions produced with CAS and PC (Figure 6.4, Paper III). Besides, increased CAS concentration (from 1.4 to 2.8%, w/w) in an emulsion produced with only CAS provided better physical stability yielding higher viscosity, creaming stability and smaller droplet size.

Zeta potential indicates the surface charge of the oil droplets in an emulsion system and is an important tool to characterize both physical and oxidative stability of the emulsions (McClements, 2005). All emulsifier combinations provided sufficiently negative surface charge for providing a sufficient repulsion between oil droplets in order to prevent coalescence (> -30 mV) (Figure 6.2b). Thus, surface charge of the emulsions contributed to the conservation of the physical stability after

the emulsion formation. It was expected that the emulsions with DATEM would have a more negative charge compared to PC due to the nature of the molecule's head group, which was zwitterionic for PC and anionic for DATEM (McClements, 2005). The PCA model indicates that this was the case since zeta potential was located closest to the DATEM emulsions (Figure 6.1). However, inspection of Figure 6.2b revealed that there was no clear correlation between zeta potential values and the use of DATEM vs PC.

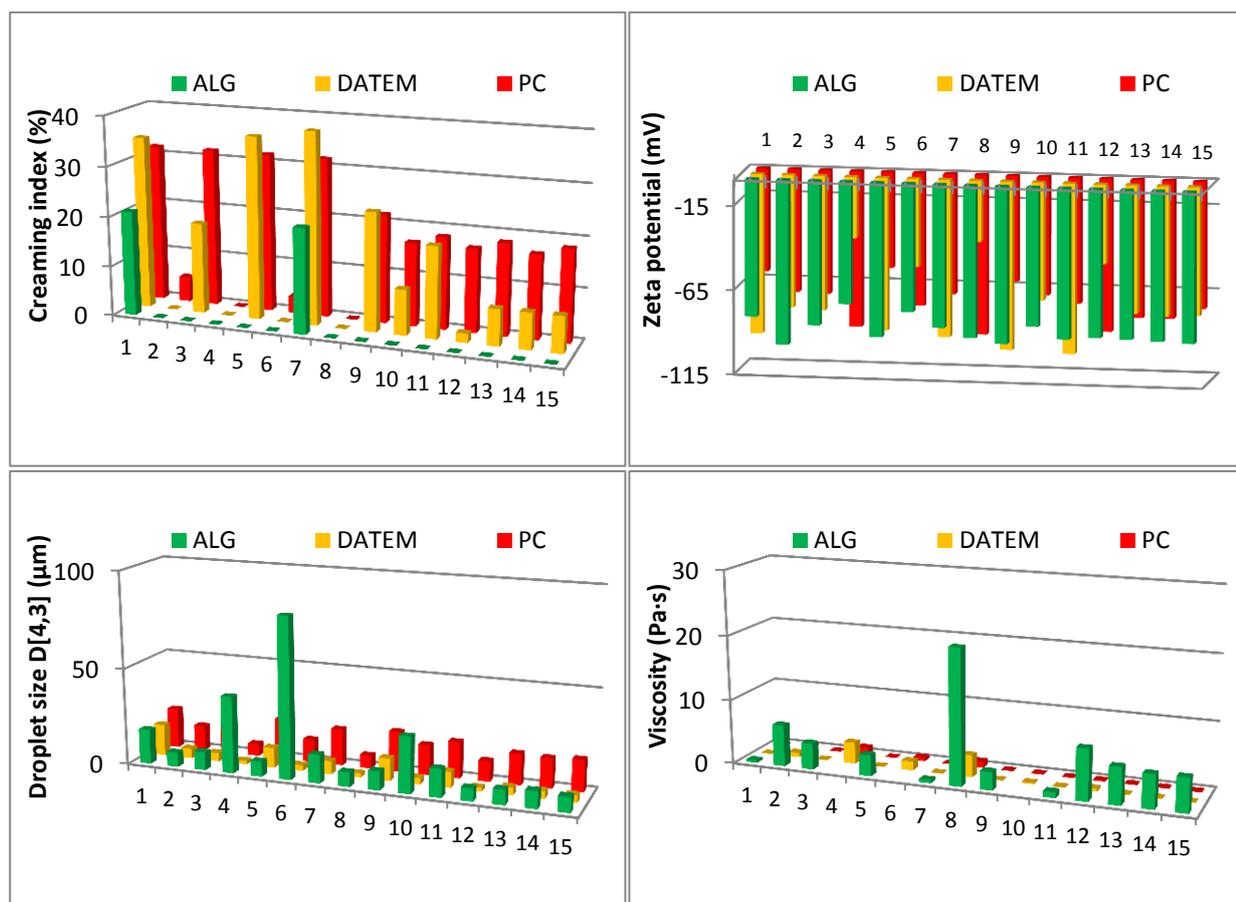


Figure 6.2. Physical stability parameters a) creaming index at the end of storage (day 28 for ALG and day 12 for DATEM and PC), b) zeta potential (day 4 for ALG and day 2 for DATEM and PC), c) droplet size (day1), and d) apparent viscosity at 20 s^{-1} (day 28 for ALG and day 1 for DATEM and PC) of emulsions produced with CAS and ALG or DATEM or PC. ALG refers to CAS+ALG emulsions; DATEM refers to CAS+DATEM emulsions; and PC refers to CAS+PC emulsions. Refer to Table 5.1 and the description below Table 5.1 for the emulsion codes. (Papers I, II, and V).

Droplet size of the emulsions varied for each emulsifier used in combination with CAS, with the following ranking at day 1: CAS+PC > CAS+ALG > CAS+DATEM (Figure 6.2c). Due to the fact that these stabilizers/emulsifiers have a different mechanism during emulsification, it was expected to obtain differences between droplet sizes. As ALG mostly stayed in the water phase and thickened

the water phase, during emulsification oil-water interface was almost exclusively formed by adsorbed CAS. Even though ALG is not a surface active compound, some of the ALG might also have interacted with CAS and adsorbed at the interface due to weak attractive interactions. This may occur between anionic polysaccharides and food proteins carrying a net negative charge. Despite of both polymers carry the same net charge, electrostatic interaction between the polysaccharide and positively charged patches on the protein may occur (Dickinson and Euston, 1991). However, this contribution is not expected to be sufficient to yield small droplet formation. Therefore, decreased droplet size was attributed to the thickening effects of ALG in the water phase, which then led to better disruption of the oil droplets during homogenization.

On the other hand, DATEM or PC, as surface active compounds, competes with CAS in order to adsorb to the oil-water interface during homogenization. As surfactants are LMWE, they can move faster compared to bigger molecules such as CAS. Thereby, they may adsorb at the oil-water interface before CAS, or even displace already adsorbed proteins at the oil-water interface. Emulsions produced with CAS+DATEM formed smaller droplets compared to emulsions with CAS+PC, which could be due to the higher surface activity of DATEM compared to PC during homogenization. It has been shown that lysolecithins are more surface active compared to lecithins, which indicates that the removal of one tail contributed to the surface activity (Casado et al., 2012, Choi et al., 2011). Even though DATEM consists of a mixture of mono- and di-glycerides, mono-glycerides were reported to be dominating (Personal communication from Guo Zheng, Århus University). Therefore, DATEM might have performed better as an emulsifier compared to PC, which had two fatty acid chains in all molecules.

Emulsions stabilized with CAS+ALG had the highest apparent viscosity (shear rate 20 s^{-1} : $0.38 - 20.88 \text{ Pa}\cdot\text{s}$) compared to emulsions stabilized with CAS+DATEM or PC. This could be attributed to the thickening effect of ALG in high fat oil-in-water emulsions (Figure 6.2d). Apparent viscosity (at 20 s^{-1}) of emulsions with CAS+DATEM ($0.02 - 3.38 \text{ Pa}\cdot\text{s}$) was higher compared to emulsions stabilized with CAS+PC ($0.02 - 1.13 \text{ Pa}\cdot\text{s}$) for the same formula. As mentioned in the previous paragraph, smaller droplet formation of emulsions with DATEM compared to PC promoted higher viscosity due to increased surface area as well as friction between oil droplets in a highly packed high fat emulsion system.

6.1.3. Effect of emulsion formulae on oxidative stability

Emulsions produced with CAS and ALG, where oxidation was not accelerated, showed high oxidative stability, which could be partially attributed to the antioxidant properties of both CAS and ALG (Figure 6.3). Another study also reported that combined use of ALG and Tween 80 enhanced oxidative stability (<1.6 mM hydroperoxides) in 2.5% (w/w) fish oil-in-water emulsions during 16 days of storage due to metal chelating ability of anionic groups of ALG (Salvia-Trujillo et al., 2016). Falkeborg et al. (2014) also reported that ALG had antioxidant activity such as scavenging free radicals. Interaction of ALG with CAS at the oil-water interface also contributed to the steric and electrostatic repulsion between oil droplets by being a large and negatively charged molecule (Figure 6.2). Presence of ALG as a large molecule at the oil-water interface resulted in thicker interfacial layer, which provides a physical barrier for prooxidants in the aqueous phase of the emulsion (Paper IV, Dickinson and Euston, 1991). Moreover, high viscosity of the aqueous phase also slows down the movement of the prooxidants and free radicals, which might inhibit the propagation of oxidation in the emulsion system (Sims et al., 1979). These factors presumably contributed to the oxidative stability of the emulsions produced with ALG. Although these emulsions were highly stable in terms of oxidation, their high viscosity may limit their application into food systems.

It was observed that the ratio between emulsifiers (noted as ‘emulsifier ratio’ in Figure 6.1) and oxidative instability parameters (PV and volatiles) were located at the opposite ends of PC1-axis of the PCA biplot. This indicated that higher emulsifier ratio of CAS to ALG or DATEM or PC led to a better oxidative stability due to the superior radical scavenging and metal chelating activities of CAS. Increasing ratio of CAS to ALG resulted in decreased peroxide value and increased 2,4-heptadienal formation, even though these changes were not significant (Table 6.1). This could be attributed to the effect of CAS in preventing hydroperoxide formation due to its metal chelating activities, whereas ALG contributed more on inhibiting secondary volatile product formation due to its radical scavenging properties.

Overall primary and secondary oxidation product formation in the emulsions is shown in Figure 6.3. As discussed before emulsions produced with ALG provided very stable emulsions towards lipid oxidation; both PV and volatile compounds were very low during 28 days of storage at room temperature in darkness. Formation of primary and secondary oxidation products were more pronounced in emulsions produced with DATEM or PC, which were both stored at room

temperature in darkness for 12 days with added iron. Higher oxidative stability of emulsions with PC was due to the metal chelating and proton donating properties of PC, whereas no antioxidant activity reported for DATEM (Anankanbil et al., 2018a). It should be also considered that the emulsions produced with CAS+PC had higher creaming compared to CAS+DATEM, which can explain the higher oxidative stability of these emulsions. As the creaming separates droplet phase from the aqueous phase, amount of the aqueous phase becomes much lower in a certain volume of the droplet phase. This also means that the amount of prooxidants in that certain volume is lower compared to a non-creamed emulsion. Therefore, creaming might contribute the higher oxidative stability in emulsions produced with CAS+PC.

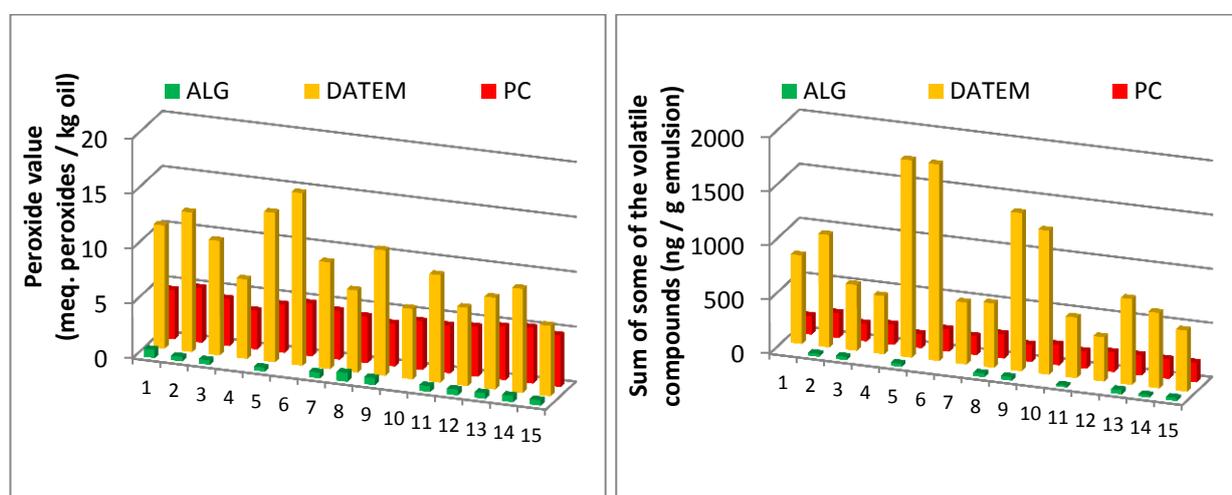


Figure 6.3. Oxidative stability of emulsions produced with CAS and ALG or DATEM or PC assessed with a) PV and b) volatile compounds at the end of the storage day (day 28 for CAS+ALG and day 12 for CAS+DATEM or PC). ALG refers to CAS+ALG emulsions; DATEM refers to CAS+DATEM emulsions; and PC refers to CAS+PC emulsions. For the emulsion codes, refer to Table 5.1 and the description below Table 5.1. (Papers I, II, and V).

On the other hand, emulsions produced with the combination of CAS+DATEM or CAS+PC, which were both prepared under the same conditions also regarding the amount of added iron, showed that emulsions with PC provided better oxidative stability compared to emulsions with DATEM (Figure 6.3). This was attributed to the antioxidant properties of PC, which donates protons to stabilize radicals and chelates metal ions (García-Moreno et al., 2014; Berton-Carabin et al., 2014), whereas there was no antioxidant activity detected for commercial DATEM (Anankanbil et al., 2018a). Therefore, formation of primary and secondary oxidation products was decreased when the ratio of CAS to DATEM was increased (Figure 6.3), which was due to radical scavenging and metal chelating activities of CAS (Faraji et al., 2004; Elias et al., 2008). Oxidative stability was affected

by the decrease in the CAS concentration at oil-water interface. Even though several previous studies reported that the non-adsorbed proteins notably influence oxidative stability of the emulsions, interfacial proteins still have great protection towards lipid oxidation as it has been shown that the oxidation is initiated at the oil-water interface (Berton-Carabin et al., 2014). Thus, replacement of CAS at the oil-water interface could have contributed to the decrease in the oxidative stability in these emulsions produced with CAS+DATEM.

On the contrary, oxidative stability was decreased when the ratio of CAS to PC was increased (Figure 6.3), which was attributed to the radical scavenging and metal chelating activities of PC (Bandarra et al., 1999; García-Moreno et al., 2014). Moreover, PC might show synergistic effect when combined with CAS at right concentrations, which may result in interactions between PC and CAS and lead to a formation of thicker interfacial layer (Fang and Dalgleish, 1996; García-Moreno et al., 2014, Paper II and III).

Surfactants interact with proteins at the oil-water interface or in the aqueous phase of the oil-in-water emulsions. This interaction promotes some changes in the conformation of the molecules, which might affect their properties. Combined use of SAXS and SANS techniques showed that the PC altered the conformation of CAS at the oil-water interface (Paper III). Figure 6.4 illustrates the possible configurations of CAS and PC at the oil-water interface of a 70% oil-in-water emulsion. It was found that the combined use of CAS and PC provided a thicker interface when CAS and PC interacted at the oil-water interface compared to an emulsion produced with only CAS. However, interface was discontinuously covered by a certain emulsifier configuration at the oil-water interface, leading to several types of domains as shown in Figure 6.4.

Thicker interfacial layer domains such as PC multilayers and CAS+PC interactions may lead to better oxidative stability by creating a physical barrier as well as allowing the CAS and PC molecules adjust their configurations and release their antioxidative domains towards the aqueous phase, which may increase the oxidative stability. Similar effect was observed in a study where Tween 20 was used in combination with whey protein isolate in emulsion system (Donnelly et al., 1998). It was also found that PC formed multilayers (Figure 6.4 D), which are attached or in the close proximity of oil-water interface, when CAS and PC was used in combination. However, this heterogeneity in the interfacial structure may lead to the formation of lateral domains, which further affects physical stability of the high fat emulsions leading to droplet coalescence as well as oxidative instability of the high fat emulsions leading to the diffusion of prooxidants through the

gaps between lateral domains. However, it should also be considered that SANS experiments required either contrast match between oil and aqueous phases or contrast variation between aqueous phase and surfactant using D_2O , which might have had a slight effect on the surfactant behaviour.

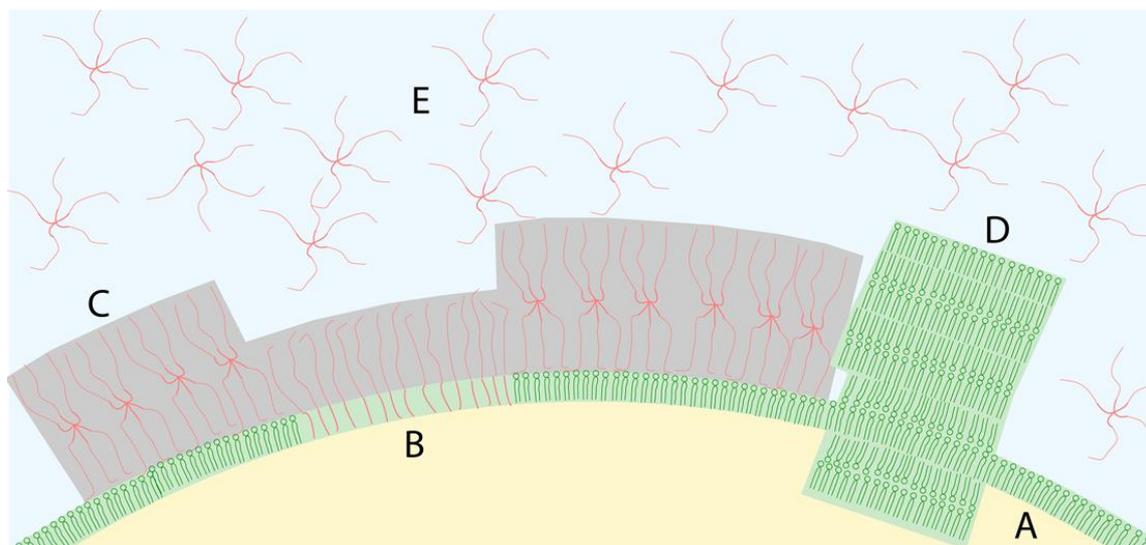


Figure 6.4. Proposed structure for the studied 70% oil-in-water interface. PC monolayer (A) with CAS particles loosely bound on the PC layer without forming a continuous coverage (B-C). Excess PC forms multilayers and some may be bound to the interface without forming continuous coverage (D). The unabsorbed CAS remains in the water phase (E), (Paper III).

It should be also considered that the emulsions produced with CAS+PC had higher creaming compared to CAS+DATEM, which may have contributed to the higher oxidative stability of these emulsions. As the creaming separates droplet phase from the aqueous phase, the amount of the aqueous phase becomes much lower in a certain volume of the droplet phase. This also means that the total amount of prooxidants in that certain volume is lower compared to a non-creamed emulsion. Therefore, creaming might contribute to the higher oxidative stability in emulsions produced with CAS+PC. However, emulsions with no creaming (e.g., PC-4 and PC-8) were still more oxidatively stable compared to DATEM emulsions produced with the same formulae, which suggests that in intact emulsions PC provides better stability due to the other reasons. For example, PC emulsions have larger droplet size compared to DATEM emulsions, which might have provided less contact between prooxidants and lipids due to a smaller surface area. On the other hand, PC emulsions (e.g., PC-4 and PC-8) had more negative surface charge which should make it more prone to oxidation when iron is used to accelerate oxidation; however, this effect apparently did not influence the results. As a consequence, these results suggested that the physical macrostructure as

well as the physical structure of the oil-water interface play very important roles for the oxidative stability differences between DATEM emulsions and PC emulsions, which should be investigated further.

6.1.4. Optimal formulae

Optimal emulsion formulae obtained by the use of Box-Behnken's design and RSM varied for different emulsifiers (ALG, DATEM, or PC) combined with CAS. Emulsions produced with CAS and ALG showed highest physical and oxidative stability at 70% fish oil content, 1.4% total emulsifier content and 1.2 as the ratio between CAS to ALG (Paper I). This optimal formula was applied further in the study where modified ALGs were used (Paper IV). Emulsions with CAS and DATEM provided the best stability at 70% fish oil content, 2.8% total emulsifier content and 2 as the ratio of CAS to DATEM (Paper V). This optimal formula was employed in the following two studies where modified DATEMs were used in high fat fish oil-in-water emulsions (Paper V) and also these delivery emulsions' incorporation into mayonnaise (Appendix III). CAS and PC emulsions were suggested to be produced with 70% fish oil content, 2.8% total emulsifier, and 1.2 CAS to PC ratio (Paper II). This optimal formula was used in the study where modified PCs were employed in high fat fish oil-in-water emulsions (Paper VI).

6.2. Part II: Influence of modified emulsifiers on physical and oxidative stability

Emulsifiers employed in this Ph.D. study had two main approaches: (1) enhancing emulsification ability of a stabilizer (sodium alginate), which already has antioxidant activity naturally (Figure 5.2), and (2) adding/enhancing antioxidant activity of surfactants (DATEM and PC) by covalent attachment of caffeic acid in the glycerol backbone of the molecules, as well as linking an alkyl chain at various lengths (lipophilicity) in order to evaluate the influence on surface and antioxidant activities in high fat oil-in-water emulsions (Figures 5.3 and 5.4). The summary of the results of these three studies are presented in Table 6.2 and then discussed further one by one.

Table 6.2. Summary of the results indicating the influence of the emulsifier modification on physical and oxidative stability of fish oil-in-water emulsions

	Shorter chain	Longer chain
CAS +mod ALG (Pap. IV)	<ul style="list-style-type: none"> ➤ CAS+SCMA had smaller droplet size compared to CAS emulsions, but higher than CAS+ALG. ➤ Viscosity of CAS+SCMA was higher than CAS. ➤ CAS+SCMA replaced more protein compared to CAS+ALG. ➤ CAS+SCMA provided similar oxidative stability as CAS; and higher oxidative stability compared to CAS+LCMA and CAS+ALG. 	<ul style="list-style-type: none"> ➤ CAS+LCMA provided the smallest droplet size compared to CAS+SCMA, CAS+ALG and only CAS. ➤ CAS+LCMA had the highest viscosity compared to the rest. ➤ CAS+LCMA replaced more protein at the oil-water interface compared to CAS+SCMA and CAS+ALG. ➤ CAS+LCMA provided less oxidative stability compared to CAS+SCMA and CAS+ALG.
CAS + mod DATEM (Pap. V)	<ul style="list-style-type: none"> ➤ DATEM_C12_30% and DATEM_C12_60% provided larger droplet size compared to DATEM_com ➤ DATEM_com had lower viscosity compared to DATEM_C12_30%, but higher than DATEM_C12_60%. ➤ Non-adsorbed protein results showed that DATEM_C12 was not as surface active as DATEM_com. ➤ DATEM_C12_10% decreased PV compared to DATEM. DATEM_C12_10% was pro-oxidative compared to DATEM_com_caf_low, whereas DATEM_C12_60% provided lower levels of PV compared to DATEM_com_caf_high. ➤ Formation of volatiles was lower in emulsions containing DATEM_C12 at all concentrations compared to DATEM_com_caf. 	<ul style="list-style-type: none"> ➤ DATEM_C14_10% provided smaller droplet size compared to DATEM_com, whereas DATEM_C14_60% had larger droplets. ➤ DATEM_C14_10% and DATEM_C14_60% provided lower viscosity compared to DATEM_com. ➤ DATEM_C14 was more surface active compared to DATEM_C12 and DATEM_com. ➤ DATEM_C14_10% decreased PV compared to DATEM. DATEM_C14_10% was prooxidative compared to DATEM_com_caf_low, whereas DATEM_C14_60% provided lower levels of PV compared to DATEM_com_caf_high. ➤ DATEM_C14 provided lower PV compared to DATEM_C12. ➤ Formation of volatiles was lower in emulsions containing DATEM_C14 at all concentrations compared to DATEM_com_caf.
CAS + mod PC (Pap. VI)	<ul style="list-style-type: none"> ➤ 3_PC_C14_1080 and 4_PC_C14_2160 provided smaller droplet size compared to 2_PC_com and 1_CAS. ➤ 3_PC_C14_1080 lowered the viscosity compared to 2_PC_com. ➤ PC_C14 did not show significant difference in the content of non-adsorbed protein compared to PC_com(s). ➤ Protein surface load decreased with the increasing concentration of PC_C14. ➤ 3_PC_C14_360 and 5_PC_C14_2160 were more prooxidative regarding PV than 9_PC_com_caf_360 and 10_PC_com_caf_2160, respectively. ➤ PC_C14 had lower PV than PC_C16. ➤ 5_PC_C14_2160 had lower concentrations of volatiles compared to 10_PC_caf_2160. 	<ul style="list-style-type: none"> ➤ 8_PC_C16_2160 provided smaller droplet size compared to 2_PC_com and 1_CAS. ➤ 7_PC_C16_1080 and 8_PC_C16_2160 lowered the viscosity compared to 2_PC_com. ➤ 8_PC_C16_2160 replaced more protein compared to PC_C14(s) and PC_com(s). ➤ Protein surface load decreased with the increasing concentration of PC_C16. PC_C16 provided higher protein surface load compared to PC_C14. ➤ 6_PC_C16_360 and 8_PC_C16_2160 were more prooxidative regarding PV than 9_PC_com_caf_360 and 10_PC_com_caf_2160, respectively. ➤ Emulsions with PC_C16 had lower concentrations of volatiles compared to emulsions with PC_C14 or PC_com.

6.2.1. Effect of modified alginates on physical and oxidative stability (Paper IV)

Emulsions produced with the combinations of CAS and alginates (ALG, SCMA, and LCMA) showed improved stability towards creaming compared to emulsions produced with only CAS (Figure 6.5). This was due to the thickening properties of the alginates in oil-in-water emulsions. Even after modification, the alginates kept their thickening properties which reduced creaming. Increasing fish oil content (from 50% to 70%) also increased viscosity of fish oil-in-water emulsions produced with modified ALGs and CAS (Paper IV).

The alterations in the structure of alginate resulted in differences in their adsorption behaviour at the oil-water interface as well as distribution among aqueous phase and oil-water interface in the oil-in-water emulsions. Protein content in the aqueous phase results showed that the amount of non-adsorbed proteins was the lowest for the 70% oil-in-water emulsion produced with CAS+ALG compared to emulsions produced with CAS+modified ALGs or only CAS (Figure 6.5). This indicated that the modified ALGs had higher surface activity compared to ALG and thereby adsorbed more on the oil-water interface as well as replaced more protein at the interface. Similar findings were reported by Falkeborg et al. (2015) indicating that the commercial ALG behaved as a thickener by forming a viscous solutions and accordingly the viscosity of the final emulsion was increased, whereas LCMA was adsorbed at the oil-water interfacial layer in 30% fish oil-in-water emulsions.

Droplet size of the emulsions was affected both by the alginate type and fish oil content. LCMA provided smaller droplet size compared to ALG and SCMA. Emulsions with only CAS had the largest droplet size compared to emulsions with CAS and LCMA or SCMA or ALG at all levels of fish oil content (Figure 6.5). Higher fish oil content provides a more viscous aqueous phase due to decreased volume of water for the same emulsifier content as well as more viscous total emulsion due to higher viscosity of fish oil compared to water. Thereby, the disruption of oil droplets during homogenization is enhanced.

These results indicated that the LCMA had a better surface activity due to its long alkyl chain attached to the molecule, where it acted more as an emulsifier than a stabilizer. LCMA contributed to the formation of smaller droplets with its advanced emulsifying ability, whereas ALG located in the aqueous phase and acted as a stabilizer. Moreover, LCMA replaced more protein at the oil-water interface compared to SCMA. SCMA also worked as an emulsifier compared to ALG, which dissolved in the aqueous phase as a stabilizer.

Oxidative stability of the emulsions produced with CAS and alginates were highly dependent on the type of the alginate combined with CAS. Replacing some of the CAS with ALG improved the physical stability. However, it negatively affected the formation of hydroperoxides and volatile oxidation products compared to emulsion with only CAS (Paper IV). The decrease in oxidative stability could be due to the decrease in the concentration of CAS, which has a strong metal chelating activity and chelates metal ions (Diaz et al., 2003; Horn et al., 2013).

As mentioned above regarding the non-adsorbed protein content results, modified ALGs not only behaved as stabilizers, but also acted as emulsifiers and competed with CAS for adsorbing at the oil-water interface. As the LCMA was more surface active, it replaced more CAS from the interface compared to SCMA (Figure 6.5) and this may have contributed to the lower oxidative stability in CAS+LCMA emulsions because of the metal chelating and radical scavenging properties of CAS. Even though non-adsorbed CAS might also have shown antioxidant activity in the water phase of CAS+LCMA emulsions, antioxidant effects of CAS at the oil-water interface might have been more effective. In addition to the previous discussions, LCMA had a double bond in its hydrophobic chain, which made it more sensitive towards lipid oxidation. Moreover, differences in droplet size and zeta potential might also have affected the overall oxidative stability differences in these emulsions.

6.2.2. Effect of modified DATEMs and PCs on physical and oxidative stability (Papers V and VI)

Modified DATEMs or PCs had different chain lengths and a caffeic acid covalently attached to the molecules itself (Figure 5.3 and 5.4). In order to investigate the dose-response effect of antioxidant activity of the mod DATEMs or PCs in high fat fish oil-in-water emulsions, concentration of these compounds was increased gradually. Therefore, emulsions were produced with the combination of CAS, com DATEM or PC and mod DATEMs or PCs (Table 5.3). This experimental setup was applied to both of the emulsifiers regardless of the differences in their optimal formulae. Therefore, it is important to bear in mind that the emulsions with mod DATEMs included lower concentration of modified surfactant and thereby lower concentration of caffeic acid compared to emulsions including mod PCs. For example, for the highest concentration of caffeic acid was 1740 ppm in the final emulsion for mod DATEM emulsion, whereas it was 2160 ppm for mod PC emulsion. The difference in caffeic acid concentrations was smaller for the lower concentrations of modified emulsifiers.

Modifications indicated in the previous paragraph affect the HLB of these compounds due to the attachment of hydrophobic (e.g. alkyl chain) or hydrophilic (e.g., phenolic compound) molecules, which could potentially alter their adsorption behaviour in a complex system such as oil-in-water emulsion. When the lipophilicity of the compound is increased, it may result in higher affinity to the oil phase of the emulsion. However, the lipophilicity of the emulsifier should be in balance with its hydrophilicity to be able to locate at the oil-water interface and form a stable emulsion. Moreover, bringing antioxidant activity to an emulsifier compound may result in oil-in-water emulsions with better oxidative stability. This is due to the existence of a phenolic ring attached (e.g. caffeic acid, which is used as an antioxidant), which donates an hydrogen atom to prooxidants such as free radicals and chelate metal ions due to the presence of a catechol group on the ring structure (Zhou and Elias, 2013).

Emulsions in mod DATEM experiment had smaller droplets compared to emulsions in mod PC experiment (Figure 6.5). Emulsions produced with the highest concentration of the mod DATEMs (when 60% of commercial DATEM was replaced) yielded significantly larger droplets and lower viscosity compared to emulsions with com DATEM, which indicated that the physical stability of the emulsions were not improved with the addition of the mod DATEMs. As mod DATEMs had shorter carbon chain lengths (C12 and C14) compared to commercial DATEM (mixture of C16 and C18), better surface activity of commercial DATEM could be due to its longer alkyl chains. Moreover, DATEM_C14_60% had significantly lower droplet size compared to DATEM_C12_60%, which was also supported by the findings in another study where the same compounds were tested in 20% fish oil-in-water emulsions and found that the mod DATEM with C14 chain length had the smaller droplet size and narrower droplet size distribution compared to mod DATEM with C12 chain length (Anankanbil et al., 2017).

Apparent viscosity of the emulsions with mod PCs was considerably lower (0.8-1 Pa·s) compared to emulsions with mod DATEMs (5-8 Pa·s), which could be attributed to the smaller droplet size of the DATEM emulsions compared to PC emulsions leading to larger surface area and more friction between droplets resulting in higher viscosity. Even though the viscosity of the emulsions was low, effect of mod PCs on producing smaller droplets indicated their enhanced surface activity compared to commercial PC (soy PC) (Figure 6.5). Same effect was also observed with the mod PCs from soy and egg sources, which had one of the alkyl chains replaced with a covalently attached caffeic acid (data not shown). In that study, the droplet size also decreased with increasing concentration of mod

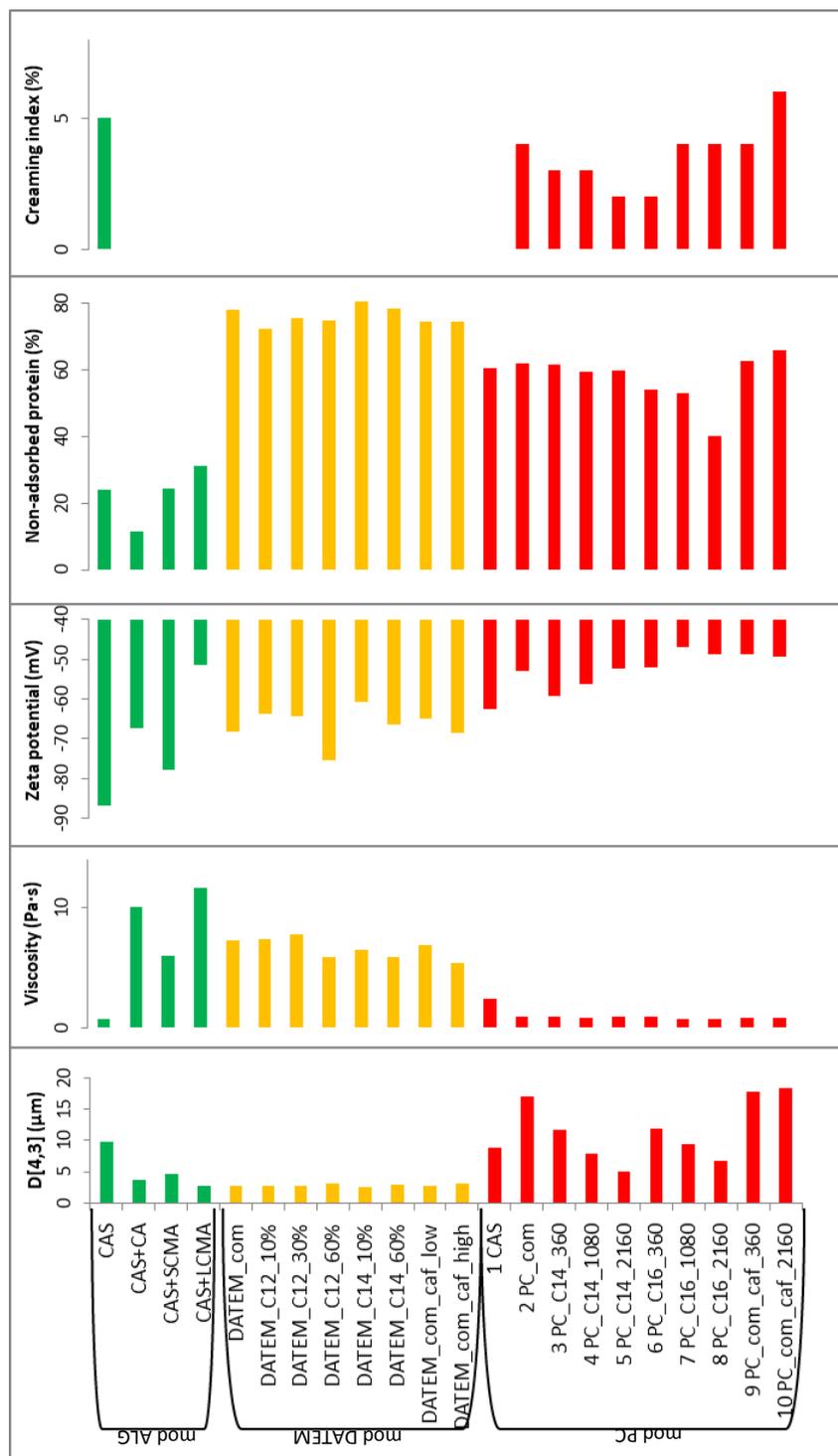


Figure 6.5. Differences in physical parameters (droplet size, viscosity, zeta potential, and non-adsorbed protein) among 70% fish oil-in-water emulsions produced with modified emulsifiers studied in various experiments² (Papers IV, V, VI).

² Relative standard deviation of results are given as follows; mod ALG (DS, $\leq 2\%$; V, $\leq 12\%$; ZP, $\leq 4\%$; NP, $\leq 12\%$), mod DATEM (DS, $\leq 1\%$; V, $\leq 7\%$; ZP, $\leq 3\%$; NP, $\leq 2\%$), and mod PC (DS, $\leq 5\%$; V, $\leq 11\%$; ZP, $\leq 9\%$; NP, $\leq 20\%$). Refer to page xii for the abbreviations of the physical parameters.

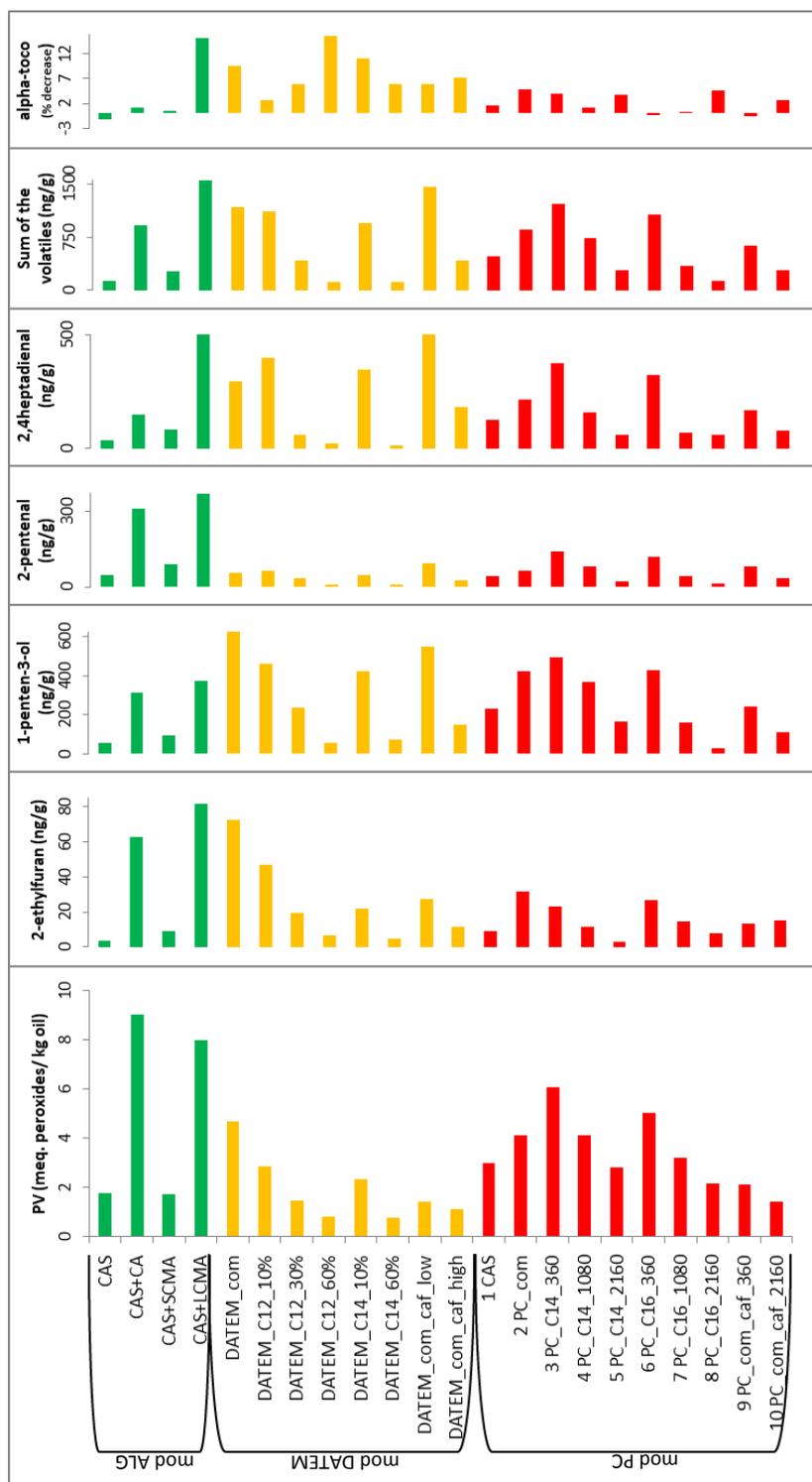


Figure 6.6. Differences in oxidative parameters (peroxide value, relative increase in volatile compounds, and relative decrease in alpha-tocopherol) among 70% fish oil-in-water emulsions produced with modified emulsifiers studied in various experiments³ (Papers IV, V, VI).

³ Relative standard deviation of results are given as follows; mod ALG (PV, $\leq 15\%$; Volatiles, $\leq 14\%$), mod DATEM (PV, $\leq 6\%$; Volatiles, $\leq 39\%$ - except a few values, it is $\leq 20\%$), and mod PC (PV, $\leq 5\%$; Volatiles, $\leq 39\%$ - except a few values, it is $\leq 20\%$).

PCs. This could be attributed to the effect of having only one alkyl chain instead of 2 alkyl chains, which was also reported for lysolecithin being more surface active compared to conventional lecithin (Choi et al., 2011; Casado et al., 2012).

Emulsions produced with the mod PCs decreased in droplet size gradually with the increasing concentration of mod PCs, which confirms the higher surface activity of mod PCs compared to com PC. It was also observed that the mod PC with C16 chain length resulted in smaller droplets compared to mod PC with C14 chain length when used as an emulsifier in 70% fish oil-in-water emulsions. This was also reported by Anankanbil et al. (2018b) indicating that mod PC with C16 alkyl chain provided smaller droplet size compared to mod PC with C14 alkyl chain.

Surface charges of the emulsions with mod DATEMs were more negative compared to emulsions with mod PCs, which could be due to higher concentration of CAS (negative surface charge at pH 7) based on the optimal formulae (CAS content in mod DATEM emulsions $\leq 1.87\%$ and in mod PC emulsions $\leq 1.53\%$). On the other hand, non-adsorbed protein results also indicated that there was more protein in the aqueous phase of the mod DATEM emulsions compared to mod PC emulsions (Figure 6.5). This is explained by the higher surface activity and adsorption ability of mod DATEMs at oil-water interface when compared to mod PCs. In addition, DATEM is an anionic, whereas PC is a zwitterionic surfactant at pH 7, which explains the higher negative surface charge of the DATEM emulsions compared to PC emulsions.

EPR results, which was conducted in order to investigate the distribution of spin labelled DATEMs in 70% tetradecane-in-water emulsions, showed that labelled DATEMs with C10, C12, and C18 alkyl chains largely distributed in the oil phase of the emulsions (Appendix II). Therefore, some of the modified DATEMs could presumably be dissolved in the oil phase as well, which indicated that they were not contributing to the emulsification of the oil droplets. However, it should be considered that emulsifiers (e.g., proteins, LMWE) adsorb differently onto pure hydrocarbon (n-tetradecane) and triglyceride oils (Stevenson et al., 1997), confirming that care must be taken with the interpretation of the results of model emulsion systems produced with hydrocarbons.

Stability of the model DATEM surfactants, which are EPR active spin labelled-DATEMs with C10, C12 and C18 chain lengths, was also determined during 6 days of storage at room temperature in darkness as shown in Figure A.2.4. Spin labelled DATEMs in water and emulsion system gave stable signal during 6 days of storage; whereas the signal was lost when these spin labelled

DATEMs were in CAS, CAS+DATEM or DATEM solutions. This was attributed to the aggregates or micellar structures formed by CAS and/or DATEM, which might have interacted with labelled DATEMs leading to conformational changes of the molecules and quenching their signal. This denotes that mod DATEMs could have interacted with commercial DATEM and/or CAS at the oil-water interface minimizing their presence in the oil phase and favoring emulsification of the oil droplets.

Emulsions produced with CAS and com and/or mod PC emulsions showed creaming, even though the rate of the creaming was very low $\leq 6\%$. Therefore, these emulsions could still be considered as stable emulsion as the creaming rate was lower than 1 mm/day (McClements, 2005). Nevertheless, the reason of the creaming could be due to the formation of laterally separated domains at oil-water interface of the emulsions produced with CAS, com PC and mod PC (Mackie et al., 2000; Fang and Dalgleish, 1996). Combined use of proteins and LMWEs may form laterally heterogeneous interfaces. As shown in Figure 6.4, CAS+PC emulsions resulted in a laterally heterogeneous oil-water interface, which might be the reason for physical instability (Paper III). Lateral domains imply fragmentation of protein film at the interface, which may lead to uncovered surface and thereby result in physical instabilities to some extent (Berton-Carabin et al., 2018). On the other hand, emulsions with com and mod DATEMs did not have any creaming, which could be due to better interactions between CAS and DATEM molecules at the oil-water interface, which yielded smaller droplets and higher viscosity leading to no creaming (Figure 6.5).

Comparison of PV for mod DATEM and mod PC emulsions showed that emulsions produced with highest concentration of mod DATEMs (DATEM_C12_60% and DATEM_C14_60%) had the lowest PV (Figure 6.6). For mod DATEM emulsions, addition of mod DATEMs or DATEM with free caffeic acid improved oxidative stability compared to DATEM_com. It was also found that increasing concentration of mod DATEMs led to higher oxidative stability. This trend was also observed for the formation of volatile compounds. DATEM_C14 provided slightly better oxidative stability compared to DATEM C12, which could be attributed to the difference in alkyl chain length. Indeed, DATEM_C14 was better at replacing proteins at the oil-water interface, which might have contributed to providing better oxidative stability by having more caffeic acid at the interface. Similar results were also found when mod DATEMs were used in the emulsification of 20% fish oil-in-water emulsion that the mod DATEM with C14 had the most powerful effect on

inhibiting lipid oxidation compared to mod DATEMs with C12 and C16 alkyl chains, which was attributed to its superior surface activity (Anankanbil et al., 2018a).

Free caffeic acid addition in the low concentration was prooxidant for most of the volatile compound formation (Figure 6.6). In another study, it was shown that the mod DATEMs with covalent attachment of caffeic acid showed higher DPPH free radical scavenging activity compared to caffeic acid (Anankanbil et al., 2017). Same study also reported that emulsions produced with mod DATEM with C14 alkyl chain showed higher antioxidant activity compared to com DATEM and equivalent amount of added caffeic acid.

Results of mod PC emulsions demonstrated that the replacing some of the CAS with com PC did not improve the oxidative stability in terms of PV and volatile compound formation (Figure 6.6). Besides, addition of mod PCs at their higher concentrations led to a decrease in PV. Moreover, volatile compound formation was decreased by the addition of mod PCs at their middle and higher concentrations. On the other hand, lower concentration of mod PCs showed prooxidant effect compared to 2_PC_com. Overall, it was observed that the increasing concentration of mod PCs increased oxidative stability (Figure 6.6). At the highest concentration of mod PCs, oxidative stability was higher compared to com PC and free caffeic acid at the equivalent concentrations. This finding confirmed that covalent attachment of caffeic acid to the PC molecules was advantageous compared to adding free caffeic acid in the emulsion system.

When two different alkyl chain lengths were compared, it was found that the PC_C16 resulted in better oxidative stability compared to PC_C14, which could be supported by the higher protein surface load in emulsions produced with PC_C16 as a consequence of lower content of non-adsorbed protein and/or larger droplet size (Paper VI). Higher protein surface load indicated a thicker interface by the interaction occurred between CAS, com PC and PC_C16. This was also supported by the results obtained from SAXS and SANS studies (Figure 6.4), which showed that the thicker interfacial layer was observed for the emulsions produced with CAS and PC compared to only CAS emulsions (Paper III).

Regarding antioxidant activities of mod PCs with different chain lengths, Anankanbil et al. (2018b) also reported that the iron chelating ability was found in the following order; C14 > C16 > C12 > C18, which indicated a cut-off effect in metal chelating activity with C14 being the optimal chain length among other mod PCs with various chain lengths. This was not in line with our findings, as

C16 performed better compared to C14, which could be due to the fact that the different total fish oil content, total emulsifier content and types. In our study 70% fish oil and combined use of mod PCs, com PC, and CAS were employed, whereas in the study by Anankanbil et al. (2018b) 20% fish oil-in-water was produced with only mod PCs. In our study, mod PC with C16 alkyl chain yielded higher surface load due to its interaction with CAS at the oil-water interface, which increased the oxidative stability as indicated in the previous paragraph.

In general, consumption of alpha-tocopherols was higher in DATEM emulsions, meaning that the oxidation was also controlled by antioxidant activities of alpha-tocopherol present in the fish oil. It was also decreased in PC emulsions; however, in lower amounts, compared to emulsions with DATEMs. Less consumption of alpha-tocopherol can be explained by the fact that phospholipids have a role in regenerating alpha-tocopherol (Samdani et al., 2018). Bandararra et al. (1999) also reported that PC showed synergistic effect with alpha-tocopherol. Likewise, synergistic effects of tocopherols and soy lecithin were reported on the oxidative stability of the vegetable oil (Judde et al., 2003).

6.2.3. Principal component analysis of data obtained with modified emulsifiers

A PCA biplot is shown in figure 6.7 to present an overview of all variables and emulsion samples from the three studies. First principle component (PC1) explained 37% of the variance and the second principle component (PC2) explained 27%. Emulsions containing mod DATEMs or mod PCs were located in groups, whereas mod ALG emulsions were located more distant to each other, indicating that they behaved differently with respect to the physical and oxidative parameters measured. Viscosity and droplet size variables were located on the opposite ends of PC1 axis, which indicated that larger droplet size correlated well with the lower viscosity. It is possible to see this relationship when looking at the ranking in the following order: mod PC emulsions, only CAS, CAS+SCMA, mod DATEM emulsions, CAS+CA, and CAS+LCMA for increasing viscosity and decreasing droplet size.

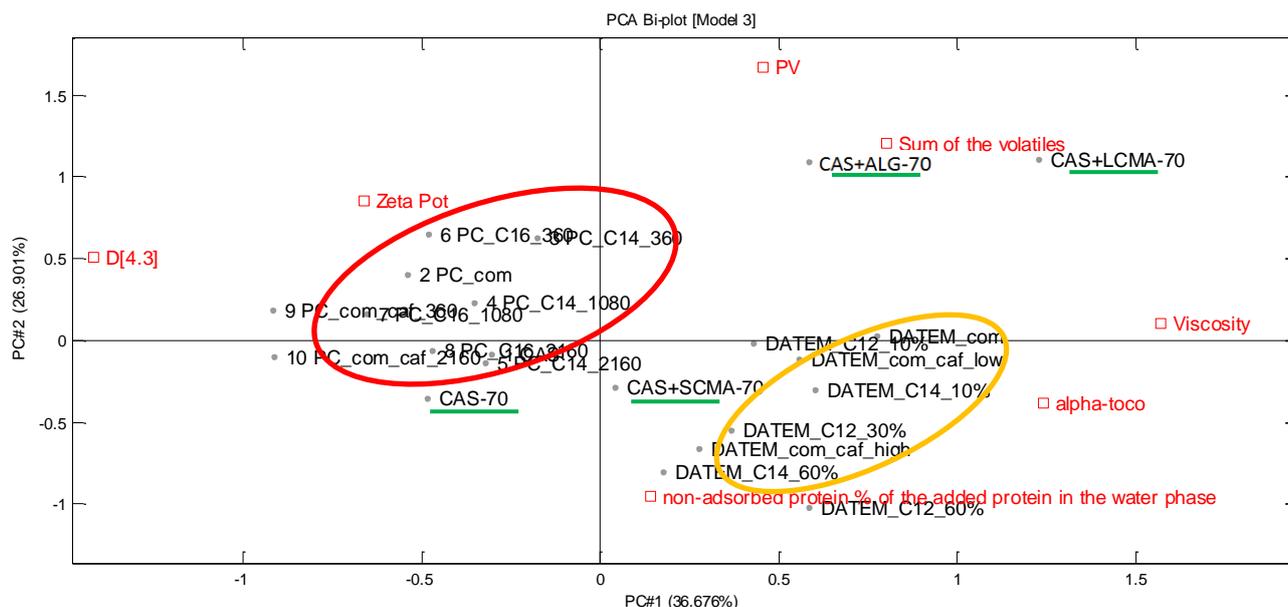


Figure 6.7. PCA biplot for emulsion samples produced with modified emulsifiers as objects and including physical and oxidative parameters as variables. All the emulsions presented in PCA biplot consist of 70% fish oil; total emulsifier and ratio between emulsifiers vary depending on the optimal recipe selected based on the optimization studies discussed in section 6.1. Refer to Table 5.2 for the emulsion codes. Red and yellow circle indicates where the mod PC and mod DATEM emulsions are located; mod ALG emulsions are underlined with green.

Emulsions with mod PCs or only CAS had less negative surface charge (zeta potential) and larger droplets compared to emulsions with ALGs or modified DATEMs, which might also explain the creaming instability (<6%) occurred due to less electrostatic repulsion forces between oil droplets leading to larger droplet size formation. Relative non-adsorbed protein content in the water phase was the highest for the emulsions produced with modified DATEMs or SCMA compared to emulsions with modified PCs, ALG, or LCMA.

Mod PC, mod DATEM, SCMA or only CAS emulsions showed better oxidative stability compared to emulsions with LCMA or ALG. Formation of primary and secondary oxidation products (PV, Figure 6.7) was the highest for emulsions produced with ALG or LCMA in combination to CAS, which was attributed to small droplets leading to larger surface area and thereby increasing contact between prooxidants and lipids. Moreover, double bond in the long chain of LCMA may increase oxidative instability. Relative decrease (%) in alpha-tocopherol content was the highest for the emulsion samples including some of the mod DATEMs, which was presumably due to the lack of antioxidant activity of com DATEM (Anankanbil et al., 2018a). Emulsions produced with higher amounts of mod DATEMs and PCs located in the area with lower PC2, which was furthest away

from PV and volatiles, but closer to non-adsorbed proteins in the aqueous phase. This indicated that the non-adsorbed CAS in the aqueous phase correlated well with less formation of lipid oxidation products and higher concentration of caffeic acid improved oxidative stability of 70% fish-oil-in-water emulsions.

Even though it is difficult to determine the effects of different chain lengths and covalently attached caffeic acid compared to commercial surfactants and added free caffeic acid on oxidative stability in the PCA biplot, it was observed that covalent attachment of caffeic acid to the surfactant molecules inhibited lipid oxidation at their high concentrations compared to a physical mixture of commercial surfactant and equivalent concentrations of added free caffeic acid (Figure 6.6). It was also observed that longer chain lengths provided higher oxidative stability compared to shorter chains in mod DATEM and mod PC emulsions, confirming that the higher lipophilicity enhanced the surface activity and either replaced more protein at the oil-water interface or resulted in interaction of the modified surfactants with proteins to a larger extent and thereby potentially the formation of a thicker interfacial layer.

In previous studies, where phenolipids or lipid-phenolic conjugates were included in oil-in-water emulsion systems, it was required to include an emulsifier due to the fact that phenolipids and lipid-phenolic conjugates are not able to provide strong steric and electrostatic repulsion between droplets because of their small molecular size and low charge in their head groups (McClements and Decker, 2018). In the case of caffeic acid, it was reported that short to medium chain lengths (C4–C12) of lipophilized caffeic acid performed the best in fish oil enriched mayonnaise by adsorbing more at the oil-water interface and inhibiting lipid oxidation (Alemán et al., 2015).

Considering the larger head part of the DATEM molecules compared to a phenolipid, it was expected that longer chain would be needed to balance the hydrophilicity of a larger head group of the DATEM molecule in order to reach a desired HLB. Therefore, we aimed for chain lengths of C12 and C14 for modified DATEMs, a bit longer than the best performed-chain lengths previously reported for caffeic acid, in order to have an adequate surface activity. Moreover, it was also reported that the C12 and C14 chain lengths of mod DATEMs showed superior physical and oxidative stability compared to C10, C16, and C18 chain lengths in a previous study where these modified antioxidant emulsifiers were used in 20% fish oil-in-water emulsions (Anankanbil et al., 2017; 2018a). As mentioned in section 5.1, C14 and C16 chain lengths were selected for mod PC

due to their better physical stability as well as superior metal chelating ability compared to C12 and C18 (Anankanbil et al., 2018b).

Moreover, previous studies indicated that the size of the head group as well as length of the alkyl chain of surfactants have an influence on oxidative stability. Silvestre et al. (2000) showed that the increasing length of the hydrophilic head group on nonionic surfactants decreased the rate of lipid oxidation, which was attributed to steric hindrance against the transition metal ions. Moreover, it was reported that lipid oxidation has also been shown to decrease when the hydrocarbon tail length of surfactants is increased, which again may be due to the ability of the surfactant layer to sterically hinder interactions between hydrophilic pro-oxidants and lipids inside the droplets (Chaiyasit et al., 2000). Nevertheless, the length of the head had an influence to a higher extent compared to tail groups. Therefore, covalent attachment of caffeic acids to a surfactant, which normally has a proper head group, is expected to provide a larger head group, thereby high protection against lipid oxidation compared to a phenolipid. Similarly, head group of mod DATEMs consisted of diacetyl tartaric acid groups and a caffeic acid attached to the glycerol backbone and mod PCs consisted of phosphate group and choline attached to a glycerol backbone, which provided large head groups to these modified surfactants.

In summary, modified stabilizers and surfactants increased physical and oxidative stability of the fish oil-in-water emulsions depending on the chain length, phenolic acid attachment as well as the composition of the delivery system, which was constructed to deliver bioactive compounds. Behaviour of the multifunctional emulsifier was also based on the origin of the stabilizer (e.g., surfactant or thickener) as their working mechanism differs. Modified ALG has a potential to be used in an n-3 delivery system which can be used in the enrichment of a highly viscous food product, whereas modified surfactants (e.g., DATEM or PC) showed potential to contribute lowering the viscosity while providing a good physical stability in terms of creaming as well as better oxidative stability.

6.3. Part III: Incorporation of delivery emulsions into mayonnaise (Appendix III)

Mayonnaise samples produced with neat fish oil or enriched with high fat fish oil-in-water emulsions showed differences in their physical and oxidative stability. Type of emulsifiers (only CAS or combined use of CAS and DATEM) used in the delivery emulsions also resulted in differences on physical and oxidative stability when the delivery emulsions were incorporated into

mayonnaise. Moreover, modification of the DATEM by covalent attachment of caffeic acid as well as different alkyl chain lengths also had an effect on the physical and oxidative stability of the final mayonnaise. These differences between mayonnaises will be further discussed below.

6.3.1. Physical stability of mayonnaises during storage

Physical stability of the fish oil enriched mayonnaise samples was lowered after mixing delivery emulsions (pH 7) and mayonnaises, which had a recipe resulted in a pH of 4. The decrease in the physical stability of these mayonnaises could be explained by the pH adjustment of the delivery emulsions from pH 7 towards ~4, which presumably caused changes in the charges of CAS, DATEM, and modified DATEM molecules. Mayonnaise enriched with n-3 delivery emulsion produced with only CAS (2_MAYO_CAS, Table 5.4) had higher droplet size compared to mayonnaise enriched with neat fish oil (1_MAYO_FO, Table 5.4), which could be due to the lower pH (~4) of the mayonnaise leading to precipitation of CAS adsorbed at oil-water interface around its isoelectric point (pI=4.6). CAS has no net charge around its isoelectric point, meaning that it has both negatively and positively charged groups (McClements, 2005). This presumably resulted in physical instability for oil droplets when delivery emulsion produced with CAS was mixed with mayonnaise, which triggered droplet coalescence and thereby increase in droplet size.

Mayonnaises enriched with n-3 delivery emulsions produced with CAS and DATEM (3_MAYO_DATEM and 6_MAYO_DATEM_CA, Table 5.4) had significantly larger droplets (5.5 and 5.9 μm , Table A.3.3) compared to 1_MAYO_FO (4.8 μm) and lower than 2_MAYO_CAS (9.3 μm). This indicated that involvement of DATEM for stabilizing delivery emulsions also enhanced the physical stability when the delivery emulsion was incorporated into mayonnaise. The presence of DATEM in these emulsions and the interactions of DATEM and CAS at the oil-water interface might have inhibited or reduced the precipitation of CAS. It was reported that emulsifiers/surfactants with free anionic groups, such as DATEM, have the potential to interact with biopolymers (e.g. protein and polysaccharides) in the emulsions via electrostatic forces, hydrophobic or hydrogen bonding (Köhler, 2001).

On the contrary, when 60% of com DATEM was replaced by mod DATEMs (DATEM_C12 or DATEM_C14), droplet size of the final mayonnaise was increased to 13.5 μm for mayonnaise enriched with n-3 delivery emulsion containing DATEM_C14 (5_MAYO_DATEM_C14, Table 5.4), thereby leading to less viscous mayonnaise (Table A.3.3). This is in line with the results

presented in section 6.2.2, where emulsions stabilized with mod DATEMs had larger droplet size compared to com DATEM. Mayonnaise enriched with n-3 delivery emulsion containing DATEM_C12 (4_MAYO_DATEM_C12, Table 5.4) was broken after the addition of delivery emulsion into mayonnaise. Droplet size of the delivery emulsions produced with DATEM_C12 and DATEM_C14 were 4.15 ± 0.01 and 4.05 ± 0.08 μm , respectively (data not shown). The increase in droplet size obtained when modified DATEMs were involved could be attributed to the laterally heterogeneous interfacial structure formation and thereby resulting in instability of the oil droplets (Mackie et al., 2001; Waninge et al., 2005; Berton et al., 2012).

However, even though both of the mod DATEMs yielded less viscous mayonnaises when the n-3 delivery emulsions were mixed, 4_MAYO_DATEM_C12 was much more affected compared to 5_MAYO_DATEM_C14. Therefore, the difference could be attributed to the non-adsorbed protein results (Table 6.5), which indicated that the DATEM_C14 replaced more CAS at the interface leading to a less amount of CAS at the oil-water interface. This could explain that the precipitation of CAS at the oil-water interface was less for mayonnaises containing DATEM_C14 compared to mayonnaises containing DATEM_C12.

Changes in the molecular charge also affected surface charge of the emulsions. Mayonnaises with neat fish oil or enriched with n-3 delivery emulsions produced with only CAS had less negative surface charge (-30.3 and -31.0 mV) than the other mayonnaises (Table A.3.3). Mayonnaises enriched with n-3 delivery emulsions produced with the addition of modified DATEMs had a zeta potential between -44.0 and -44.8 mV, which was more negative compared to both 1_MAYO_FO and 2_MAYO_CAS. The most negative surface charge was observed for the mayonnaise samples enriched with n-3 delivery emulsions produced with com DATEM without and with free caffeic acid added at -51.0 and -55.4 mV, respectively. Zeta potential values give an insight of electrostatic stability in these mayonnaises; basically, < -30 mV zeta potential is considered as a stable emulsion. However, surprisingly, zeta potential value obtained for 4_MAYO_DATEM_C12 did not elucidate the instability of the mayonnaise, which could be due to the fact that the mayonnaise sample still contained stable droplets providing a sufficient surface charge; therefore, mayonnaise was not totally separated.

Alterations in droplet size and surface charge also caused changes in viscosity; larger droplets induced less viscous mayonnaises due to less surface area and thereby less friction between surfaces of oil droplets (Antonov et al., 2004; Aken, 2006). Therefore, viscosity of the mayonnaise samples

was negatively correlated with droplet size (Table A.3.3). 1_MAYO_FO had the highest apparent viscosity compared to other mayonnaises mixed with n-3 delivery emulsions, which indicated that the addition of the delivery emulsions yielded a decrease in viscosity in mayonnaises compared to the control (1_MAYO_FO). 5_MAYO_DATEM_C14 had the lowest apparent viscosity among all others.

The provision of less viscous characteristic of the delivery emulsions could be an advantage for the production of less viscous but still physically stable food products such as salad dressings and soups. Interestingly, lower droplet size of 3_MAYO_DATEM and 6_MAYO_DATEM_CA compared to 2_MAYO_CAS did not lead to higher apparent viscosities (50.16 and 38.99 Pa·s, respectively) than 2_MAYO_CAS (57.81 Pa·s). The reason was presumably the lower amount of CAS in these two emulsions compared to 2_MAYO_CAS, which increased the viscosity of the aqueous phase, thereby resulted in higher viscosity in the final mayonnaise. It should be borne in mind that low viscosity could also be a disadvantage in terms of allowing prooxidants to move in the aqueous phase, interact with lipids and trigger lipid oxidation (Sims et al., 1979).

2_MAYO_CAS had the highest protein surface load at oil-water interface compared to other mayonnaises (Table A.3.3), whereas 1_MAYO_FO had the lowest protein surface load, indicating that the added delivery emulsion emulsified with CAS had thicker interfacial layer compared to egg yolk covered oil-water interfacial layer. Therefore, it might be expected that the 2_MAYO_CAS had a better physical barrier for the diffusion of prooxidants and thereby provides a better oxidative stability compared to 1_MAYO_FO. Similarly, 5_MAYO_DATEM_C14 showed significantly higher protein surface load compared to 1_MAYO_FO, which might inhibit or limit the contact between prooxidants in the aqueous phase and lipids.

6.3.2. Oxidative stability of mayonnaises during storage

Formation of hydroperoxides in mayonnaise samples indicated that 1_MAYO_FO and 2_MAYO_CAS had the highest level of PV compared to the rest of the mayonnaise samples (Figure A.3.1). 2_MAYO_CAS had a shorter lag phase for the hydroperoxide formation compared to 1_MAYO_FO. At the last day of the storage (day 28), 6_MAYO_DATEM_CA had the lowest level of PV compared to the rest of the mayonnaises. Mayonnaises enriched with n-3 delivery emulsions containing com DATEM without any antioxidant or mod DATEMs had similar rate of

oxidation, except for 3_MAYO_DATEM, which had a slightly longer lag phase for hydroperoxide formation compared to the other mayonnaises.

As opposed to the expected regarding the physical stability results (e.g., high protein surface load and thereby thicker interfacial layer), 2_MAYO_CAS was the fastest in producing primary oxidation products. Nevertheless, at the last day of the storage (day 28), 1_MAYO_FO had the same amount of hydroperoxide formation as in 2_MAYO_CAS. Confirming these results and indicating the antioxidant activity of alpha-tocopherol (Figure A.3.2), consumption of alpha-tocopherol content was the most in these two mayonnaises (1_MAYO_FO and 2_MAYO_CAS), which were also oxidized the most. 2_MAYO_CAS had the highest concentration of the following volatile compounds: 2-ethylfuran, 1-penten-3-ol, 2-pentenal, 2-hexenal, 2,4-heptadienal, and 2,6-nonadienal (Figure A.3.3). 1_MAYO_FO had also one of the highest concentration of pentanal, 1-penten-3-ol, and 2,4-heptadienal. As reported by Jacobsen et al. (2000b), larger droplets developed fishy and rancid off-flavours slower and later compared to mayonnaise with smaller droplets in the initial stage of the storage. This was contradicting with the results of this study as 2_MAYO_CAS oxidized faster even though the larger droplet size (9.3 μm) compared to 1_MAYO_FO (4.8 μm).

Previous studies reported that the iron in the egg yolk and low pH are the responsible factors for lipid oxidation in mayonnaise (Jacobsen et al., 2001). It was proposed that the iron bridges in egg yolk proteins (e.g., phosvitin, low density lipoproteins and lipovitellins) may be broken when the pH is lowered from neutral to around 4 resulting in the release of iron from the egg yolk, which further catalyzes lipid oxidation in mayonnaise. Thus, involvement of iron free emulsifiers, such as milk proteins, was expected to decrease the lipid oxidation in mayonnaise samples. However, it was not the case for 2_MAYO_CAS, which had the highest concentrations for most of the volatile compounds detected. Sørensen et al. (2010b) also reported that emulsifier type (milk protein vs. egg yolk) did not contribute in hindering lipid oxidation as expected. Surprisingly, it was found that the initial quality of the emulsifier had a more pronounced effect on oxidative stability than its iron content (Sørensen et al., 2010b). Combining results from the physical and oxidative stability parameters, the reason for the higher oxidation in 2_MAYO_CAS could be attributed to the CAS precipitation at low pH values (lower than the isoelectric point of CAS; $\text{pI}=4.6$), which affected the protein covered droplet surfaces and resulted in droplet coalescence as well as prooxidant diffusion through the interfacial layer.

Mayonnaises enriched with n-3 delivery emulsions produced with the addition of com DATEM or mod DATEMs resulted in lower levels of PV compared to 1_MAYO_FO and 2_MAYO_CAS, which indicated that the presence of DATEM regardless of the type of the DATEM already improved the oxidative stability of the mayonnaise. 5_MAYO_DATEM_C14 had higher protein surface load compared to 6_MAYO_DATEM_CA; however, formation of primary oxidation products was not lower than it was for 6_MAYO_DATEM_CA. Nevertheless, concentration of some of the volatile compounds (e.g., hexanal, 2-hexenal, 2,4-heptadienal, and 2,6-nonadienal, Figure A.3.3) were lower for 5_MAYO_DATEM_C14 compared to 6_MAYO_DATEM_CA, which could be attributed to better metal chelating property of DATEM_C14 at the oil-water interface compared to added free caffeic acid in the aqueous phase of the mayonnaise samples. Same effect was seen in another study, where egg white protein and catechin polymers were conjugated and the conjugates inhibited lipid oxidation in a higher extent compared to physical mixtures of egg white protein and catechin polymers (Gu et al., 2017). This was attributed to the advantage of bringing the antioxidant closer to the oil-water interface where the oxidation is initiated instead of providing the antioxidant activity in the aqueous phase.

On the other hand, 5_MAYO_DATEM_C14 had larger droplets (13.5 μm) compared to 6_MAYO_DATEM_CA (5.9 μm), which might contribute to the slower formation of some of the volatile compounds due to the smaller surface area exposed to prooxidants. 4_MAYO_DATEM_C12 also showed high stability in terms of volatile compound formation, which supposedly high droplets due to droplet coalescence occurred in an observable extent. These findings were in agreement with the study by Jacobsen et al. (2000b) regarding the effect of mayonnaise droplet size. It was reported that the larger droplets are slower and late in the development of fishy and rancid off-flavours compared to smaller droplets. However, 4_MAYO_DATEM_C12 had physical instability, which needs to be borne in mind.

Sensory evaluation results were in line with primary and secondary volatile compound formation, demonstrating that the highest fishy odour was detected for 3_MAYO_CAS at day 28 (Figure A.3.4). Interestingly, 1_MAYO_FO was the lowest, in which fish oil odour was detected at day 28; this contradicts with the volatile compounds found in higher concentration in this sample compared to other mayonnaises. These volatile compounds are pentanal, 1-penten-3-ol, and 2,4-heptadienal, which are reported to release odours such as pungent, glue, green, grassy, apple, sweet, fishy, burnt, rotten apples, nasty, green, rancid hazel nuts. However, sensory analysis results for 1_MAYO_FO

did not report any of the green, fishy, and sweet odour characteristics, which were some of the odours found in the literature and profiled as the sensory attributes for mayonnaise samples by the panel. It was reported that the MAYO_FO had the lowest level of fish oil odour attribute compared to the rest of the mayonnaises at day 28. On the other hand, fish oil odour was also detected higher in 6_MAYO_DATEM_CA compared to both 4_MAYO_DATEM_C12 and 5_MAYO_DATEM_C14. Moreover, 5_MAYO_DATEM_C14 found to have the lowest level of rancid odour attribute compared to the rest of the mayonnaises.

Results from sensory evaluation also revealed some of the textural characteristics regarding the mayonnaise samples. Most importantly, 4_MAYO_DATEM_C12, which showed physical instability during storage, was given lower scores on the firmness and scaled higher for the colour, shininess, and broken, which were classified under appearance attributes. 4_MAYO_DATEM_C12 did not show a total phase separation, therefore after stirring it was possible to compare it with the rest of the mayonnaise samples for the panelists. Panelists also evaluated 5_MAYO_DATEM_C14 as a broken mayonnaise with a number of 3 in a 14 leveled scale, which was attributed its higher droplet size and low viscosity compared to the rest of the emulsions excluding 4_MAYO_DATEM_C12. Moreover, panelists also observed that the textural attributes of mayonnaise samples lost their firmness and adhesiveness for the samples 1_MAYO_FO and 3_MAYO_DATEM during 28 days of storage. These two mayonnaises were the only samples included in the evaluation with day 0 samples.

As a summary, mayonnaise enriched with n-3 delivery emulsions produced with mod DATEMs demonstrated that physical stability of the final mayonnaise was not improved by the addition of modified DATEMs in the delivery emulsions compared to mayonnaise enriched with neat fish oil. However, oxidative stability of the mayonnaises enriched with n-3 delivery emulsions produced with DATEM_C14, in terms of the formation of primary and secondary oxidation products as well as the consumption of the alpha tocopherol, was improved compared to mayonnaise enriched with neat fish oil. Even though sensory analysis results showed that the mayonnaise enriched with neat fish oil showed the lowest development of fish oil odour, mayonnaise enriched with n-3 delivery emulsion produced with DATEM_C14 provided the lowest rancid odour attribute compared to the rest of the mayonnaises.

Chapter 7: Conclusions and perspectives

This chapter presents the conclusion and the future perspectives of this Ph.D. thesis.

7.1. Conclusions

The highest fish oil content (70% w/w) provided the highest creaming stability, smaller droplets and higher viscosity which enhanced overall physical stability of the emulsions. The highest total emulsifier content (2.8% w/w) amongst the three levels tested provided the best physical stability for high fat emulsions stabilized with CAS and DATEM or PC, whereas emulsions stabilized with CAS and ALG were able to reach good stability at 1.4% total emulsifier. This was due to the stabilizing effect of alginate as a polysaccharide with its thickening property; on the contrary, DATEM or PC as surfactants was not efficient in enhancing the physical stability at lower total emulsifier concentration than 2.8% w/w. These findings clearly points out the differences in stabilizing principles between polysaccharides and surfactants.

Moreover, the ratio between CAS and ALG, DATEM or PC emulsifiers was also important for the physical stability. Thus, the optimal ratios varied for emulsions produced with ALG, DATEM and PC in combination with CAS. This indicated that there should be a balance between the amount of emulsifiers used in combination, where the improved physical and oxidative stability was observed. Due to differences in antioxidant activities of ALG, DATEM, and PC when used in combination with CAS, ratio between emulsifiers altered the oxidative stability of the high fat oil-in-water emulsions. Type of homogenizers also affected both physical and oxidative stability. Combinations of CAS and ALG provided high viscosity and creaming stability for the high fat emulsions, whereas combinations of CAS and DATEM or PC yielded emulsions with lower viscosity and creaming stability compared to ALG. High viscosity provided creaming stability by retarding the movement of the oil droplets in the high fat oil-in-water emulsions. However, high viscosity may hamper the incorporation of the delivery emulsions into food products.

Primary and secondary oxidation product formation in the emulsions produced with CAS and ALG was very low, whereas it was more pronounced for the emulsions produced with CAS and DATEM or PC, which was mainly due to the different storage conditions as well as radical scavenging activity of ALG in the water phase. Higher oxidative stability of emulsions with CAS and PC compared to CAS and DATEM was due to the metal chelating and proton donating properties of PC, whereas no antioxidant activity reported for DATEM. Moreover, it was also attributed to the

emulsions produced with CAS+PC had higher creaming compared to CAS+DATEM, which led to a decrease in the amount of the aqueous phase in a certain volume of the droplet phase. Overall, these results confirmed the *hypothesis I* which claimed that the physical and oxidative stability of high fat (50-70%) fish oil-in-water emulsions are affected by fish oil content, total emulsifier content, and the ratio between emulsifiers as well as type of homogenizer.

Small angle X-ray and neutron scattering results indicated that the interfacial structure of the oil-water interface was altered when CAS and PC were used together when compared to only CAS, which confirmed *hypothesis II*. Interaction between CAS and PC occurred at the interface leading to a formation of heterogeneous lateral interfacial structure; proposed domains were CAS+PC adsorbed together, PC monolayer, CAS monolayer attached PC multilayers, and CAS aggregates. Moreover, concentration of CAS and PC had an influence on interfacial thickness and packing density as the distance between PC bilayers were changing with the changes in CAS concentration, hence confirming *hypothesis II*. It was showed that the interfacial thickness was higher when CAS and PC were used in combination, leading to the formation of a physical barrier for the prooxidants, which increase oxidative stability. However, interfacial structure was not homogeneous due to the formation of lateral domains, which may affect physical stability of the high fat emulsions leading to droplet coalescence. The factors leading to heterogeneous lateral interfacial structure should be further investigated due to the fact that oxidative stability of the high fat emulsions can also be affected by the diffusion of prooxidants through the gaps between lateral domains.

Modification of various properties in emulsifier structure had different effects on physical and oxidative stability of the high fat fish oil-in-water emulsions. Modified structures altered the adsorption behaviour at the oil-water interface as well as antioxidant activity in the emulsions, which both had influence on physical and oxidative stability. As claimed in *hypothesis III*, different alkyl chain lengths of modified DATEMs, PCs and ALGs influenced their location in 70% oil-in-water emulsions. Both modified alginates with short and long chain were more surface active compared to commercial alginate, thereby replacing more CAS from the oil-water interface of the high fat emulsions. Modified alginate with a longer chain was more surface active compared to short chain modified alginate, which resulted in smaller droplet size and higher viscosity compared to short chain modified alginate.

On the contrary, modified DATEM with alkyl chain lengths C12 and C14 did not provide smaller droplets and higher viscosity compared to commercial DATEM. However, modified DATEM with

C14 alkyl chain length replaced more CAS from oil-water interface compared to modified DATEM with C12 alkyl chain, which indicated a different trend in the distribution of modified surfactants in the emulsion. EPR results indicated that labelled DATEM with C18 alkyl chain distributed in the oil phase slightly more compared to labelled DATEM with C12 alkyl chain, which showed the higher affinity of the more hydrophobic compounds to the lipid phase. Modified PCs with C16 alkyl chain length showed more interaction with CAS at the oil-water interface according to protein surface load results, which might have led to a thicker interfacial layer in comparison to PC with C14 alkyl chain. These results also confirmed *hypothesis III*.

As hypothesized in *hypothesis IV*, enhanced surface activity of ALG by the attachment of short hydrophobic chain improved oxidative stability of high fat oil-in-water emulsions as the antioxidant activity of ALGs was more effective at the oil-water interface of the emulsions, where the prooxidants interact with oil and lipid oxidation is initiated. However, this effect was not observed for long hydrophobic chain due to the adverse effect of double bond present in the carbon chain, which might have triggered the oxidation in the high fat oil-in-water emulsions. On the other hand, modifying commercial DATEM or PC by covalent attachment of caffeic acid enhanced oxidative stability of high-fat emulsions compared to mixtures of commercial surfactants and free caffeic acid, which confirmed *hypothesis V*. Moreover, adding modified DATEM with C14 alkyl chain length to high fat n-3 delivery emulsions resulted in lower formation of primary and secondary oxidation products compared to modified DATEM with C12 alkyl chain length, which could be attributed to higher amount of DATEM C14 containing caffeic acid at the interface as indicated by the higher non-adsorbed CAS content. Likewise, the formation of secondary oxidation products was lower for modified PC with C16 alkyl chain compared to modified PC with C14 as a consequence of more interaction of PC16 and CAS leading to thicker interface as indicated by the protein surface load results.

Mayonnaises enriched with n-3 delivery emulsions provided larger droplets, higher zeta potential, lower viscosity and higher protein surface load compared to mayonnaises enriched with neat fish oil. Physically stable n-3 delivery emulsions yielded in lower physical stability when incorporated into mayonnaises due to the low pH (~4) of the mayonnaise. Therefore, n-3 delivery emulsion enriched mayonnaises resulted in lower physical stability compared to neat fish oil enriched mayonnaise. However, oxidative stability of n-3 delivery emulsion enriched mayonnaises were higher compared to neat fish oil enriched mayonnaise, which partly confirmed *hypothesis VI* in

terms of oxidative stability. Mayonnaise enriched with n-3 delivery emulsions containing DATEM_C14 had better oxidative stability compared to mayonnaise enriched with n-3 delivery emulsion containing commercial DATEM and free caffeic acid in terms of secondary volatile oxidation product formation and detection of fishy, sweet, and rancid odour attributes, which confirmed that the covalent attachment of caffeic acid to DATEM performed better compared to free caffeic acid in mayonnaise. Moreover, mayonnaises enriched with n-3 delivery emulsions produced with DATEM C14 resulted in better physical stability compared to DATEM C12, due to better surface activity of DATEM C14 leading to a better adsorption at the oil-water interface as well as interacting with CAS, thereby forming a thicker interfacial layer.

Overall, it was observed that the chain length of multifunctional emulsifiers had an influence on their distribution in the oil-in-water emulsion system as well as adsorption behaviour at the oil-water interface and interaction with other emulsifiers (e.g. CAS) when used in combination. Physical characteristics of the multifunctional emulsifiers together with their antioxidant properties affected the oxidative stability and sensory properties of the n-3 delivery emulsions and food systems, which were enriched with the delivery emulsions consisting multifunctional emulsifiers. Thus, studied multifunctional emulsifiers were found to be very important for the food industry, due to their potential use in physically and oxidatively stable n-3 delivery oil-in-water emulsions to enrich food products, which will obtain desired pourability together with physical and oxidative stability during their shelf life.

7.2. Perspectives

This Ph.D. study focused on the effects of combined use of CAS and stabilizers or surfactants on the physical and oxidative stability of high fat fish oil-in-water emulsions. Moreover, oil-water interfacial structure and distribution of multifunctional emulsifiers were further investigated to understand their impact on physical and oxidative stability of high fat fish oil-in-water emulsions. With a similar approach, new tailor-made emulsifiers with enhanced antioxidant activity and improved surface activity can be synthesized and further applied in model emulsions and various food systems due to increasing evidence for the importance of oil-water interface in controlling lipid oxidation in complex systems. In addition, further research for the application of multifunctional emulsifier in emulsion systems is needed since the optimum chain length is dependent on the food system chosen.

Lipid oxidation was the main focus of this Ph.D. study, where primary oxidation and secondary volatile oxidation products were analyzed. However, due to the presence of sodium caseinate in the high fat fish oil-in-water emulsions, secondary volatile lipid oxidation products formed during lipid oxidation can interact with proteins and vice versa. These reactions result in the formation of Strecker aldehydes such as 2-methyl butanal or pyrroles. Even though in this Ph.D. study these compounds were not analysed, it is recommended to analyse these oxidation products due to their contribution to off-flavours and degradation of nutritional and functional properties in the food system.

As the interfacial tension results obtained from the emulsifier combinations including modified PCs contributed significantly to the interpretation of the overall results and directed to a more sensible conclusion, it would be a great benefit to determine the interfacial tension of other modified stabilizers/surfactants used in this Ph.D. study. Therefore, the interfacial tension measurements, which was only determined for the samples containing modified PCs, are recommended to be conducted for the other multifunctional emulsifiers, which has a potential for improving physical and oxidative stability of the high fat oil-in-water emulsions.

Even though access to SAXS and SANS techniques needs prior planning due to the fact that the neutron and synchrotron facilities are scarce and application for beamtime needs to be submitted in advance at specific times of the year, these two techniques can be used together to gain information about the interfacial structure of emulsion systems. In this Ph.D. study, it was possible to focus on only one of the emulsifier combinations (CAS+PC), whereas SAXS and SANS techniques can reveal information regarding interfacial structure for the other emulsifier combinations, which could aid to explain some of the different characteristics and behaviours of these high fat oil-in-water emulsions. Therefore, application of SAXS and SANS techniques is encouraged for other oil-in-water emulsion systems where emulsifier combinations as well as emulsifier and antioxidant combinations were used. Moreover, surface active compounds adsorbed at the oil-water interfacial layer could be interesting to study as a function of time, which may provide information about the conformational changes of these surface active compounds due to their interactions with each other during time.

Advanced fluorescence microscopy techniques may be combined with EPR spectroscopy in order to investigate the distribution of the emulsifiers in different phases of the emulsion system such as the oil phase, the aqueous phase, and interface of the high fat oil-in-water emulsion system. EPR

spectroscopy can be further used to determine the reactivity of the emulsifiers in the different phases they are located. In this Ph.D. study, it was only possible to work with labelled DATEMs, whereas it would be interesting to study labelled PCs with different chain lengths as their interaction with sodium caseinate at the oil-water interface differed significantly depending on the alkyl chain length such as yielding a thicker interface when modified PC with C16 alkyl chain was used compared to modified PC with C14 alkyl chain.

Furthermore, the development of physically and oxidatively stable high fat (50-70%) omega-3 delivery oil-in-water emulsions and their incorporation into different kinds of food systems should be studied. In this Ph.D. study, only mayonnaise was selected as a food system. However, its low pH value resulted in some physical instability for some of the n-3 delivery systems. Therefore, the effects of these n-3 delivery emulsion systems produced with multifunctional emulsifiers on physical and oxidative stability of other food systems, which have neutral pH values, could be interesting to study.

As the main goal of this Ph.D. study is to deliver n-3 PUFAs through emulsion systems, it is also important to test their bioavailability as well as oxidation of both the individual high fat delivery emulsions and food system enriched with the delivery emulsions in the gastrointestinal tract. The bioavailability of hydrophobic bioactive compounds (e.g., n-3 PUFAs) can be improved using specially designed oil-in-water emulsions consisting of lipid droplets dispersed within an aqueous phase. However, the bioavailability is highly dependent on the type of the emulsifiers as well, which are expected not only to provide a good physical and oxidative stability during the shelf-life of the product but also to release the bioactive compounds during ingestion or digestion after consumed.

REFERENCES

- Aken G. A., in: Stephen, A. M., Phillips, G. O. (Eds.), *Food Polysaccharides and Their Applications*, CRC Press, Boca Raton, Florida 2006.
- Alemán, M., Bou, R., Guardiola, F., Durand, E., Villeneuve, P., Jacobsen, C., & Sørensen, A.-D. M. (2015). Antioxidative effect of lipophilized caffeic acid in fish oil enriched mayonnaise and milk. *Food Chem.*, *167*, 236–244.
- Alfa chemistry, α -casein structure. (2019, January 5). Retrieved from <https://www.alfa-chemistry.com/casein-90-95-cas-83471-50-5-item-241869.htm>.
- Anankanbil, S., Pérez, B., Yang, J., Banerjee, C., & Guo, Z. (2017) A novel array of interface confined molecules: Assembling natural segments for delivery of multi-functionalities. *J. Colloid Interface Sci.*, *508*, 230–236.
- Anankanbil, S., Pérez, B., Fernandes, I., Widzisz, K. M., Wang, Z., Mateus, N., & Guo, Z. (2018a). A new group of synthetic phenolic containing amphiphilic molecules for multipurpose applications: Physico-chemical characterization and cell-toxicity study. *Sci. Rep.*, *8*, 832.
- Anankanbil, S., Pérez, B., Banerjee, C., & Guo, Z. (2018b). New phenophospholipids equipped with multi-functionalities: Regiospecific synthesis and characterization. *Journal of Colloid and Interface Science*, *523*, 169-178.
- Antonov, Y. A., Van Puyvelde, P., & Moldenaers, P. (2004). Interfacial tension of aqueous biopolymer mixtures close to the critical point. *International Journal of Biological Macromolecules*, *34*, 29–35.
- Atkinson, P. J., Dickinson, E., Horne, D. S., & Richardson, R. M. (1995). Neutron reflectivity of adsorbed beta-casein and beta-lactoglobulin at the air/water interface. *J. Chem. Soc., Faraday Trans*, *91*, 2847–54.
- Banc, A., Charbonneau, C., Dahesh, M., Appavou, M.-S., Fu, Z., Morel, M.-H., & Ramos, L. (2016). Small angle neutron scattering contrast variation reveals heterogeneities of interactions in protein gels. *Soft Matter*, *12*, 5340-5352.
- Bandarra, N. M., Campos, R. M., Batista, I., Nunes, M. L., & Empis, J. M. (1999). Antioxidant synergy of α -tocopherol and phospholipids. *J. Am. Oil Chem. Soc.*, *76*, 905–913.

- Bergenståhl, B., & Fontell, K. (1983). Phase-equilibria in the system soybean lecithin water. *Progress in Colloid and Polymer Science*, 68, 48-52.
- Berton, C., Ropers, M. H., Viau, M., & Genot, C. (2011). Contribution of the interfacial layer to the protection of emulsified lipids against oxidation. *J. Agric. Food Chem.*, 59, 5052–61.
- Berton, C., Genot, C., Guibert, D., & Ropers, M.-H. (2012). Effect of lateral heterogeneity in mixed surfactant-stabilized interfaces on the oxidation of unsaturated lipids in oil-in-water emulsions. *Journal of Colloid and Interface Science*, 377, 244–250.
- Berton-Carabin, C. C., Coupland, J. N., & Elias, R. J. (2013a). Effect of the lipophilicity of model ingredients on their location and reactivity in emulsions and solid lipid nanoparticles. *Colloids and Surfaces A: Physicochem. Eng. Aspects*, 431, 9–17.
- Berton-Carabin, C. C., Coupland, J. N., & Elias, R. J. (2013b). Reactivity of a model lipophilic ingredient in surfactant-stabilized emulsions: Effect of droplet surface charge and ingredient location. *Colloids and Surfaces A: Physicochem. Eng. Aspects*, 418, 68-75.
- Berton-Carabin, C. C., Genot, C., Gaillard, C., Guibert, D., & Ropers, M. H. (2013c). Design of interfacial films to control lipid oxidation in oil-in-water emulsions. *Food Hydrocolloids*, 33, 99-105.
- Berton-Carabin, C. C., Ropers, M. H., & Genot, C. (2014). Lipid oxidation in oil-in-water emulsions: involvement of the interfacial layer. *Comprehensive Reviews in Food Science and Food Safety*, 13 (5), 945-977.
- Berton-Carabin, C. C., Sagis, L., & Schroën, K.. (2018). Formation, Structure, and Functionality of Interfacial Layers in Food Emulsions. *Annual Review of Food Science and Technology*, 9, 24.1–24.37.
- Biosaxs, small angle X-ray scattering. (2019, January 5). Retrieved from <http://biosaxs.com/technique.html>.
- Capron, I., Costeux, S., Djabourov, M. (2001). Water in water emulsions: Phase separation and rheology of biopolymer solutions. *Rheol. Acta*, 40, 441–456.
- Casado, V., Martin, D., Torres, C., & Reglero, G. (2012). Phospholipases in food industry: a review. *Lipases Phospholipases Methods Protoc*, 861, 495–523.

- Castellanos, M. M., McAuley, A., & Curtis, J. E. (2017). Investigating structure and dynamics of proteins in amorphous phases using neutron scattering. *Computational and Structural Biotechnology Journal*, 15, 117-130.
- Chaiyasit, W., Silvestre, M. P. C., McClements, D. J. & Decker, E. A. (2000). Ability of surfactant hydrophobic tail group size to alter lipid oxidation in oil-in-water emulsions. *J. Agric. Food Chem.*, 48 (8), 3077–3080.
- Chat, O. A., Najar, M. H., Mir, M. A., Rather, G. M., & Dar, A. A. (2011). Effects of surfactant micelles on solubilization and DPPH radical scavenging activity of Rutin. *Journal of Colloid and Interface Science*, 355, 140–149.
- Cheatham, C. L., Colombo, J. & Carlson, S. E. (2006). n-3 Fatty acids and cognitive and visual acuity development: methodologic and conceptual considerations. *Am. J. Clin. Nutr.*, 83, 1458S–1466S.
- Chen, B. C., Li, H. J., Ding, Y. P., & Rao J. J. (2011). Improvement of physicochemical stabilities of emulsions containing oil droplets coated by non-globular protein-beet pectin complex membranes. *Food Research International*, 44, 1468–75.
- Choi, S. J., Decker, E. A., Henson, L., Popplewell, L. M., Xiao, H., & McClements, D. J. (2011). Formulation and properties of model beverage emulsions stabilized by sucrose monopalmitate: influence of pH and lyso-lecithin addition. *Food Res. Int.*, 44, 3006–12.
- Coupland, J. N., & McClements, D. J. (1996). Lipid oxidation in food emulsions. *Trends in Food Science & Technology*, 7, 83-90.
- Dalgleish, D. G., Srinivasan, M., & Singh, H. (1995). Surface Properties of Oil-in-Water Emulsion Droplets Containing Casein and Tween 60. *J. Agric. Food Chem.*, 43, 2351-2355.
- Dalgleish, D. G., Tosh, S. M., & West, S. (1996). Beyond homogenization: The formation of very small emulsion droplets during the processing of milk by a Microfluidizer. *Netherlands Milk and Dairy Journal*, 50, 135–148.
- Dalgleish, G. D., (1997). Food emulsions stabilized by proteins. *Current Opinion in Colloid & Interface Science*, 2, 573-577.
- de Kruif C. G., Huppertz, T., Urban, V. S., & Petukhov, A. V. (2012). Casein micelles and their internal structure. *Adv. Coll. Interface Sci.*, 171–172, 36–52

- Diaz, M., Dunn, C. M., McClements, D. J., & Decker, E. A. (2003). Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, *51* (8), 2365–2370.
- Dickinson, E., & Euston, S. R. (1991). Stability of emulsions containing both protein and polysaccharide. In *Food polymers, gels and colloids*, E. Dickinson (Ed.), pp. 132-146. Cambridge, UK: Royal Society of Chemistry.
- Dickinson, E., Hunt, J. A., & Horne, D. S. (1992). Calcium induced flocculation of emulsions containing adsorbed β -casein or phosvitin. *Food Hydrocolloids*, *6*, 359.
- Dickinson, E., & Golding, M. (1997). Depletion flocculation of emulsions containing unadsorbed sodium caseinate. *Food Hydrocolloids*, *11*, 13-18.
- Dickinson, E., Pinfield, V.J., Horne, O. S., & Leermakers, F. A. M. (1997). Self-consistent-field modelling of adsorbed casein. Interaction between two protein-coated surfaces. *J Chem Soc Faraday Trans*, *93*, 1785-1790.
- Dickinson, E. (1999). Adsorbed protein layers at fluid interfaces: interactions, structure and surface rheology. *Colloids and Surfaces B: Biointerfaces*, *15*, 161–176.
- Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids*, *17*, 25.
- Dickinson, E. (2006). Structure formation in casein-based gels, foams, and emulsions. *Colloids and Surfaces A: Physicochem. Eng. Aspects*, *288*, 3–11.
- Dickinson, E. (2009). Hydrocolloids as emulsifiers and emulsion stabilizers. *Food Hydrocolloids*, *23*(6), 1473–82.
- Dickinson, E. (2011). Mixed biopolymers at interfaces: Competitive adsorption and multilayer structures. *Food Hydrocolloids*, *25*, 1966-1983.
- Elias, R. J., McClements, D. J., & Decker, E. A. (2005). Antioxidant activity of cysteine, tryptophan, and methionine residues in continuous phase beta-lactoglobulin in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, *53*, 10248-10253.
- Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant Activity of Proteins and Peptides. *Critical Reviews in Food Science and Nutrition*, *48*, 430-441.

- Elias, R. J., & Decker, E. A. (2010). Protein antioxidants for the stabilization of lipid foods: current and potential applications. In *Oxidation in foods and beverages and antioxidant applications: Understanding mechanisms of oxidation and antioxidant activity*, Decker, E. A., Elias, R. J., Evans, M., Ratcliffe, I., & Williams, P. A. (2013). Emulsion stabilization using polysaccharide–protein complexes. *Curr. Opin. Colloid Interf. Sci.*, *18*, 272–282.
- Falkeborg, M., Cheong, L., Gianfico, C., Sztukiel, K. M., Kristensen, K., Glasius, M., Xu, Xuebing, & Guo, Z. (2014). Alginate oligosaccharides: Enzymatic preparation and antioxidant property evaluation. *Food Chemistry*, *164*, 185–194.
- Falkeborg, M., Paitaida, P., Shu, A. N., Pérez, B., & Guo, Z. (2015). Dodecyl succinylated alginate as a novel material for encapsulation and hyperactivation of lipases. *Carbohydrate Polymers*, *133*, 194–202.
- Fang, Y., & Dalgleish, D. G. (1993). Casein adsorption on the surfaces of oil-in-water emulsions modified by lecithin. *Colloids and Surfaces B: Biointerfaces*, *1*, 357-364.
- Fang, Y., & Dalgleish, D. G. (1996). Comparison of the effects of three different phosphatidylcholines on casein-stabilized oil-in-water emulsions. *Journal of the American Oil Chemists' Society*, *73*(4), 437-442.
- Faraji, H., McClements, D. J., & Decker, E. A. (2004). Role of continuous phase protein on the oxidative stability of fish oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, *52*, 4558–4564.
- Frankel, E. N. (2012). *Lipid Oxidation*, Woodhead Publishing Limited, 2012.
- Furtado, G. F., Mantovani, R. A., Consoli, L., Hubinger, M. D., & Cunha, R. L. (2017). Structural and emulsifying properties of sodium caseinate and lactoferrin influenced by ultrasound process. *Food Hydrocolloids*, *63*, 178-188.
- Garcia-Moreno, P. J., Horn, A. F., & Jacobsen, C. (2014). Influence of casein-phospholipid combinations as emulsifier on the physical and oxidative stability of fish oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, *62*(5), 1142-1152.
- Gaupp, R.; Adams, W. Di-acetyltartaric esters of monoglycerides (DATEM) and associated emulsifiers in bread making. In *Emulsifiers in Food Technology*; Whitehurst, R. J., Ed.; Blackwell Publishing Ltd.: Northampton, United Kingdom, 2004; pp 86–109.

- Guzey, D., & McClements, D. J. (2006). Formation, stability and properties of multilayer emulsions for application in the food industry. *Advances in Colloid and Interface Science* 128–130, 227–248.
- Guzmán, E., Llamas, S., Maestro, A., Fernández-Pena, L., Akanno, A., Miller, R., Ortega, F., & Rubio, R. G. (2016). Polymer–surfactant systems in bulk and at fluid interfaces. *Advances in Colloid and Interface Science*, 233, 38–64.
- Goddard, E. D., & Hannan, R. B. (1977). Polymer/surfactant interactions. *Journal of the American Oil Chemists' Society*, 54, 561-566.
- Goddard, E. D. (2002). Polymer/surfactant interaction: interfacial aspects. *J. Colloid Interface Sci.*, 256, 228–35.
- Gorji, S. G., Smyth, H. E., Sharma, M., & Fitzgerald, M. (2016). Lipid oxidation in mayonnaise and the role of natural antioxidants: A review. *Trends in Food Science & Technology*, 56, 88-102.
- Gornall, D. A., & Kuksis, A. (1971). Molecular species of lyceophosphatides and triglycerides of egg yolk lipoproteins. *Can. J. Biochem.*, 49 (1), 51-60.
- Gu, L. P., Peng, N., Chang, C. H., McClements, D. J., Su, Y. J., & Yang, Y. J. (2017). Fabrication of Surface-Active Antioxidant Food Biopolymers: Conjugation of Catechin Polymers to Egg White Proteins. *Food Biophysics*, 12 (2), 198–210.
- Guzey, D., & McClements, D. J. (2006). Formation, stability and properties of multilayer emulsions for application in the food industry. *Adv Colloid Interface Sci.*, 128, 227–48.
- Gülcin, I. Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). *Toxicology 2006*, 217, 213–220.
- Hartvigsen, K., Lund, P., Hansen, L. F., & Holmer, G. (2000). Dynamic headspace gas chromatography/mass spectrometry characterization of volatiles produced in fish oil enriched mayonnaise during storage. *Journal of Agricultural and Food Chemistry*, 48(10), 4858–4867.
- Hiemenz, P. C., *Principles of Colloid and Surface Chemistry*, Dekker, New York, 1986.
- Horn, A. F., Nielsen, N. S., Andersen, U., Sogaard, L. H., Horsewell, A., & Jacobsen, C. (2011). Oxidative stability of 70% fish oil-in-water emulsions: Impact of emulsifiers and pH. *European Journal of Lipid Science and Technology*, 113, 1243-1257.

- Horn, A. F., Nielsen, N. S., & Jacobsen, C. (2012a). Iron-mediated lipid oxidation in 70% fish oil-in-water emulsions: effect of emulsifier type and pH. *International Journal of Food Science and Technology*, *47*, 1097–1110.
- Horn, A. F., Green-Petersen, D., Nielsen, N. S., Andersen, U., Hyldig, G., Jensen, L. H. S., Horsewell, A., & Jacobsen, C. (2012b). Addition of Fish Oil to Cream Cheese Affects Lipid Oxidation, Sensory Stability and Microstructure. *Agriculture*, *2*, 359-375.
- Horn, A. F., Nielsen, N. S., Andersen, U., Jensen, L. H. S., Horsewell, A., & Jacobsen, C. (2012c) The choice of homogenisation equipment affects lipid oxidation in emulsions. *Food Chemistry*, *134*, 803–810.
- Hu, M., McClements, D. J., & Decker, E. A. (2003). Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate. *Journal of Agricultural and Food Chemistry*, *51* (6), 1696–1700.
- Hu, Y.-T., Ting, Y., Hu, J.-Y., & Hsieh, S.-C. (2017). Techniques and methods to study functional characteristics of emulsion systems. *Journal of Food and Drug Analysis*, *25*, 16-26.
- Huang, Y., Li, A. J., Qiu, C. Y., Teng, Y. L., & Wang, Y. (2017). Selfassembled colloidal complexes of polyphenol-gelatin and their stabilizing effects on emulsions. *Food Funct.*, *8* (9), 3145–3154.
- Ingham, B. Smialowska, A., Erlangga, G. D., Matia-Merino, L., Kirby, N. M., Wang, C., Haverkampe, R. G., & Carr, A. J. (2016). Revisiting the interpretation of casein micelle SAXS data. *Soft Matter*, *12*, 6937-6953.
- Isemura, T., & Imanishi, A. (1958). The dissolution of water-insoluble polymers in the surfactant solution. The polyelectrolyte-like behavior of the dissolved polymers. *J. Polym. Sci.*, *33*, 337–52.
- Jacobsen, C., Adler-Nissen, J., & Meyer, A. S. (1999a). Effect of ascorbic acid on iron release from the emulsifier interface and on the oxidative flavor deterioration in fish oil enriched mayonnaise. *Journal of Agriculture and Food Chemistry*, *47*, 4917–4926.
- Jacobsen, C., Hartvigsen, K., Lund, P., Meyer, A. S., Adler-Nissen, J., Holstborg, J., & Hølmer, G. (1999b). Oxidation in fish-oil-enriched mayonnaise 1. Assessment of propyl gallate as an antioxidant by discriminant partial least squares regression analysis. *Eur. Food Res. Technol.*, *210*, 13–30.

- Jacobsen, C., Hartvigsen, K., Lund, P., Adler-Nissen, J., Hølmer, G., & Meyer, A. S. (2000a). Oxidation in fish-oil-enriched mayonnaise 2. Assessment of the efficacy of different tocopherol antioxidant systems by discriminant partial least squares regression analysis. *Eur. Food Res. Technol.*, *210*, 242–257.
- Jacobsen, C., Hartvigsen, K., Lund, P., Thomsen, M. K., Skibsted, L. H., Adler-Nissen, J., Hølmer, G., & Meyer, A. S. (2000b). Oxidation in fish oil-enriched mayonnaise 3. Assessment of the influence of the emulsion structure on oxidation by discriminant partial least squares regression analysis. *European Food Research and Technology*, *211*(2), 86-98.
- Jacobsen, C., Timm, M., & Meyer, A. S. (2001). Oxidation in fish oil enriched mayonnaise: Ascorbic acid and low pH increase oxidative deterioration. *J. Agric. Food Chem.*, *49*, 3947–3956.
- Jacobsen, C., Let, M. B., Nielsen, N. S., & Meyer, A. S. (2008). Antioxidant strategies for preventing oxidative flavour deterioration of foods enriched with n-3 polyunsaturated lipids: a comparative evaluation. *Trends Food Sci. Technol.*, *19*, 76–93.
- Jacobsen, C. (2010). Challenges when developing omega-3 enriched foods. *Conference Chevreul*, *17*, 251–258.
- Judde, A., Villeneuve, P., Rossignol-Castera, A., & Guillou, A. L. (2003). Antioxidant effect of soy lecithins on vegetable oil stability and their synergism with tocopherols. *J. Am. Oil Chem. Soc.*, *80*, 1209–1215.
- Karadağ, A., Hermund, D. B., Jensen, L. H. S., Andersen, U., Jónsdóttir, R., Kristinsson, H. G., Alasalvar, C., & Jacobsen, C. (2017). Oxidative stability and microstructure of 5% fish-oil-enriched granola bars added natural antioxidants derived from brown alga *Fucus vesiculosus*. *European Journal of Lipid Science and Technology* *119* (4), pp. 1500578.
- Khajeh, M. (2010). Application of Factorial Design and Box–Behnken Matrix in the Optimization of a Magnetic Nanoparticles Procedure for Copper Determination in Water and Biological Samples. *Biol. Trace Elem. Res.*, *135*, 355–363.
- Kim, G. N., Jang, H. D., & Kim, C. I. (2007). Antioxidant capacity of caseinophosphopeptides prepared from sodium caseinate using Alcalase. *Food Chemistry*, *104* (4), 1359–1365.
- Kittipongpittaya, K., Panya, A., Phonsatta, N., & Decker, E. A. (2016). Effects of Environmental pH on Antioxidant Interactions between Rosmarinic Acid and α -Tocopherol in Oil-in-Water (O/W) Emulsions. *J. Agric. Food Chem.*, *64*, 6575–6583.

- Köhler, P. (2001). Synthesis and characterization of DATEM components. *LWT-Food Sci. Technol.* 34, 359–366.
- Kulås, E., Olsen., & Ackman, R. G. (2003). Oxidation of fish lipids and its inhibition with tocopherols. In *Lipid Oxidation Pathways*, Kamal-Eldin, A. (Ed.), pp.37-84. AOCS Press: Champaign, IL, USA.
- Laguerre, M., Lopez Giraldo, L. J., Lecomte, J., Figueroa- Espinoza, M. C., Barea, B., Weiss, J., Decker, E. A., & Villeneuve, P. (2009). Chain length affects antioxidant properties of chlorogenate esters in emulsion: The cutoff theory behind the polar paradox. *J. Agric. Food Chem.*, 57, 11335–11342.
- Laguerre, M., López Giraldo, L. J., Lecomte, J., Figueroa-Espinoza, M. C., Barea, B., Weiss, J., Decker, E. A., & Villeneuve, P. (2010). Relationship between Hydrophobicity and Antioxidant Ability of "Phenolipids" in Emulsion: A Parabolic Effect of the Chain Length of Rosmarinate Esters. *Journal of Agricultural and Food Chemistry*, 58 (5), 2869–2876.
- Laguerre, M., Bily, A., Roller, M., & Birtic, S. (2017). Mass Transport Phenomena in Lipid Oxidation and Antioxidation. *Annu. Rev. Food Sci. Technol.*, 8, 391–411.
- Larson-Smith, K., Jackson, A., & Pozzo, D. C. (2012). SANS and SAXS Analysis of Charged Nanoparticle Adsorption at Oil–Water Interfaces. *Langmuir*, 28, 2493–2501.
- Let, M. B., Jacobsen, C., & Meyer, A. S. (2007). Lipid oxidation in milk, yoghurt, and salad dressing enriched with neat fish oil or pre emulsified fish oil. *Journal of Agricultural and Food Chemistry*, 55, 7802-7809.
- Lopez-Rubio, A., & Gilbert, E. P. (2009). Neutron scattering: A natural tool for food science and technology research. *Trends in Food Science & Technology*, 20 (11-12), 576-586.
- Lu, F. S. H., Nielsen, N. S., Baron, C. P., Diehl, B. W. K., & Jacobsen, C. (2013). Impact of primary amine group from aminophospholipids and amino acids on marine phospholipids stability: Nonenzymatic browning and lipid oxidation. *Food Chem.*, 141 (2), 879–888.
- Mackie, A. R., Gunning, A. P., Wilde, P. J., & Morris, V. J. (2000). Orogenic displacement of protein from the oil/water interface. *Langmuir*, 16, 2242–47.

- Mackie, A. R., Gunning, A. P., Ridout, M. J., Wilde, P. J., & Morris, V. J. (2001). Orogenic displacement in mixed β -lactoglobulin/ β -casein films at the air/water interface. *Langmuir*, *17*, 6593–98.
- Mackie, A. R. (2004). Structure of adsorbed layers of mixtures of proteins and surfactants. *Current Opinion in Colloid Interface Science*, *9*, 357–61.
- Mao, L. K., Yang, J., Xu, D. X., Yuan, F., & Gao, Y. X. (2010). Effects of homogenization models and emulsifiers on the physicochemical properties of b-Carotene nanoemulsions. *Journal of Dispersion Science and Technology*, *31*, 986–993.
- Matsumura, & Matsumiya, 2012. Chapter 5: Proteins–Peptides as Emulsifying Agents in *Food Proteins and Peptides: Chemistry, functionality, interactions and commercialization* (Ed. Navam S. Hettiarachchy). CRC Press, Boca Raton, FL, USA.
- McClements, D. J., Decker, E. A. (2000). Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of Food Science*, *65*, 1270-1282.
- McClements, D. J. (2005). Food emulsions: principles, practice, and techniques. Second Edition. CRC Press: Boca Raton, Florida, USA.
- McClements, D. J., & Decker, E. (2018). Interfacial antioxidants: A review of natural and synthetic emulsifiers and coemulsifiers that can inhibit lipid oxidation. *J. Agric. Food Chem.*, *66*, 20–35.
- McClements, D. J., & Jafari, S. M. (2018). Improving emulsion formation, stability and performance using mixed emulsifiers: A review. *Advances in Colloid and Interface Science*, *251*, 55–79.
- Mei, L., McClements, D. J., Wu, J., & Decker, E. A. (1998). Iron-catalyzed lipid oxidation in emulsion as affected by surfactant, pH and NaCl. *Food Chemistry*, *61*(3), 307–12.
- Narita, Y., Iwai, K., Fukunaga, T., & Nakagiri, O. (2012). Inhibitory activity of chlorogenic acids in decaffeinated green coffee beans against porcine pancreas lipase and effect of a decaffeinated green bean extract on an emulsion of olive oil. *Biosci. Biotechnol. Biochem.*, *76* (12), 2329-2331.
- Nichols, P. D., McManus, A., Krail, K., Sinclair, A. J., & Miller, M. (2014). Recent advances in omega-3: Health benefits, sources, products and bioavailability. *Nutrients*, *6*, 3727–3733.

- Nielsen, N. S., Debnath, D., & Jacobsen, C. (2007). Oxidative stability of fish oil enriched drinking yoghurt. *International Dairy Journal*, *17*, 1478–1485.
- Pallandre, S., Decker, E. A., & McClements, D. J. (2007). Improvement of stability of oil-in-water emulsions containing caseinate-coated droplets by addition of sodium alginate. *J. Food Sci.*, *72*, Nr.9.
- Pegi, A., Julijana, K., Slavko, O., Janez, S., & Marjeta, S. (2003). The Effect of Lipophilicity of Spin-Labeled Compounds on Their Distribution in Solid Lipid Nanoparticle Dispersions Studied by Electron Paramagnetic Resonance. *Journal of Pharmaceutical Sciences*, *92*, 58–66.
- Philips, G. O., & Williams, P. A. (1995). Chapter 11: The use of hydrocolloids to improve food texture, in *Texture in Foods, Volume 1: Semi-Solid Foods*, McKenna, B.M., Ed., CRC Press, Boca Raton, FL.
- Pokorný, J. (2001). Chapter 1: Introduction. In *Antioxidants in Food*. J. Pokorný, N. Yanishlieva, M. Gordon (Eds.) Woodhead Publishing Ltd and CRC Press LLC.
- Porter, W. L. (1980). Recent trends in food applications of antioxidants. In *Autoxidation in food and biological systems*, Simic, M. G., Karel, M. (Eds.) New York: Plenum Press.
- Pugnaloni, L. A., Dickinson, E., Ettelaie, R., Mackie, A. R., & Wilde, P. J. (2004). Competitive adsorption of proteins and low-molecular weight surfactants: computer simulation and microscopic imaging. *Adv. Colloid Interface Sci.*, *107* (1), 27–49.
- Reische, D. W., Lillard, D. A., & Eitenmiller, R. R. (2002). Antioxidants. In *Food Lipids: Chemistry, Nutrition, and Biotechnology*. 2nd rev. and expanded edn. Akoh, C. C., & Min, D. B. (Eds.) New York, Marcel Dekker.
- Richards, M. P., Chaiyasit, W., McClements, D. J., & Decker, A. E. (2002). Ability of Surfactant Micelles to Alter the Partitioning of Phenolic Antioxidants in Oil-in-Water Emulsions. *J. Agric. Food Chem.*, *50*, 1254–1259.
- Ries, D., Ye, A., Haisman, D., & Singh, H. (2010). Antioxidant properties of caseins and whey proteins in model oil-in-water emulsions. *International Dairy Journal*, *20*, 72-78.
- Salvia-Trujillo, L., Decker, E. A., & McClements, D. J. (2016). Influence of an anionic polysaccharide on the physical and oxidative stability of omega-3 nanoemulsions: Antioxidant effects of alginate. *Food Hydrocolloids*, *52*, 690-698.

- Samdani, K. G., McClements, D. J., & Decker, E. A. (2018). Impact of Phospholipids and Tocopherols on the Oxidative Stability of Soybean Oil-in-Water Emulsions. *Journal of Agricultural and Food Chemistry*, *66*, 3939–3948
- Schaich, K. M. (2013). Chapter 1: Challenges in Elucidating Lipid Oxidation Mechanisms When, Where, and How Do Products Arise? In *Lipid Oxidation Challenges in Food Systems*. A. Logan, U. Nienaber, and X. Pan. (Eds.) by AOCS Press, Urbana, IL.
- Schwarz, K., Huang, S. W., German, J. B., Tiersch, B., Hartmann, J., Frankel, E. N. (2000). Activities of antioxidants are affected by colloidal properties of oil-in-water and water-in-oil emulsions and bulk oils. *J. Agric. Food Chem.*, *48*, 4874–82.
- Shahidi, F., & Zhong, Y. (2011). Revisiting the polar paradox theory: a critical overview. *J. Agric. Food Chem.* *59*, 3499–504.
- Shimada, K., Okada, H., Matsuo, K., & Yoshioka, S. (1996). Involvement of chelating action and viscosity in the antioxidative effect of xanthan in an oil/water emulsion. *Biosci. Biotech. Biochem.*, *60* (1), 125-127.
- Silvestre, M. P. C., Chaiyasit, W., Brannan, R. G., McClements, D. J., & Decker, E. A. (2000). Ability of surfactant headgroup size to alter lipid and antioxidant oxidation in oil-in-water emulsions. *J. Agric. Food Chem.*, *48* (6), 2057–2061.
- Singh, H. (2011). Aspects of milk-protein-stabilised emulsions. *Food Hydrocolloids*, *25*, 1938-1944.
- Sims, R., Fioriti, J., & Trumbetas, J. (1979). Effect of sugars and sugar alcohols on autoxidation of safflower oil in emulsions. *Journal of the American Oil Chemists' Society*, *56*(8), 742-745.
- Song, C., Shieh, C., Wu, Y., Kalueff, A., Gaikwad, S., & Su, K. (2016). The role of omega-3 polyunsaturated fatty acids eicosapentaenoic and docosahexaenoic acids in the treatment of major depression and Alzheimer's disease: Acting separately or synergistically? *Prog. Lipid Res.*, *62*, 41–54.
- Sosa-Herreraa, M. G., Berlib, C. L. A., Martinez-Padilla, L. P. (2008). Physicochemical and rheological properties of oil-in-water emulsions prepared with sodium caseinate/gellan gum mixtures. *Food Hydrocoll.*, *22*, 934–942.

- Southward, C. R., & Walker, N. J. (1980). The manufacture and industrial use of casein. *New Zealand Journal of Dairy Science and Technology*, 15 (3), 201-217.
- Sørensen, A.-D. M., Haahr, A.-M., Becker, E. M., Skibsted, L. H., Bergenstahl, B., Nilsson, L., & Jacobsen, C. (2008). Interactions between Iron, Phenolic Compounds, Emulsifiers, and pH in Omega-3-Enriched Oil-in-Water Emulsions. *J. Agric. Food Chem.*, 56, 1740–1750.
- Sørensen, A.-D. M., Nielsen, N. S., & Jacobsen, C. (2010a). Oxidative stability of fish oil-enriched mayonnaise-based salads. *Eur. J. Lipid Sci. Technol.*, 112, 476–487.
- Sørensen, A.-D. M., Nielsen, N. S., Hyldig, G., & Jacobsen, C. (2010b). Influence of emulsifier type on lipid oxidation in fish oil-enriched light mayonnaise. *European Journal of Lipid Science and Technology*, 112(9), 1012–1023.
- Sørensen, A.-D. M., Nielsen, N. S., Decker, E. A., Let, M. B., Xu, X., & Jacobsen, C. (2011). The Efficacy of Compounds with Different Polarities as Antioxidants in Emulsions with Omega-3 Lipids. *J. Am. Oil. Chem. Soc.*, 88, 489–502
- Sørensen, A.-D. M., Durand, E., Laguerre, M., Bayrasy, C., Lecomte, J., Villeneuve, P., & Jacobsen, C. (2014). Antioxidant properties and efficacies of synthesized alkyl caffeates, ferulates and coumarates. *J. Agric. Food Chem.*, 62, 12553–12562.
- Stevenson, E. M., Horne, O. S., & Leaver, J. (1997). Displacement of native and thiolated β -casein from oil-water Interfaces - effects of heating, ageing and oil phase. *Food Hydrocolloids*, 11, 3-6.
- Venkateshwarlu, G., Let, M. B., Meyer, A. S., & Jacobsen, C. (2004). Modeling the sensory impact of defined combinations of volatile lipid oxidation products on fishy and metallic off-flavors. *Journal of Agricultural and Food Chemistry*, 52, 1635-1641.
- van Aken, G. A. (2003). Competitive adsorption of protein and surfactants in highly concentrated emulsions: effect on coalescence mechanisms. *Colloids Surf. A.*, 213 (2–3), 209–19.
- van Nieuwenhuyzen, W., & Tomás, M. C. (2008). Update on vegetable lecithin and phospholipid technologies. *European Journal of Lipid Science and Technology*, 110 (5), 472-486.
- Venkateshwarlu, G., Let, M. B., Meyer, A. S. & Jacobsen, C. (2004). Modeling the sensory impact of defined combinations of volatile lipid oxidation products on fishy and metallic off-flavors. *Journal of Agricultural and Food Chemistry*, 52, 1635-1641.

- Walstra, P. (1993). Principles of emulsion formation. *Chemical Engineering Science*, 48 (2), 333-349.
- Waninge, R., Walstra, P., Bastiaans, J., Nieuwenhuijse, H., Nylander, T., Paulsson, M., & Bergenståhl, N. (2005). Competitive Adsorption between α -Casein or α -Lactoglobulin and Model Milk Membrane Lipids at Oil-Water Interfaces. *J. Agric. Food Chem.*, 53, 716-724.
- Wang, F. C., & Marangoni, A. G. (2016) Advances in the application of food emulsifier a-gel phases: Saturated monoglycerides, polyglycerol fatty acid esters, and their derivatives. *Journal of Colloid and Interface Science*, 483, 394–403.
- Wilde, P., Mackie, A., Husband, F., Gunning, P., & Morris, V. (2004). Proteins and emulsifiers at liquid interfaces. *Adv. Colloid Interface Sci.*, 108–109, 63–71.
- Wikimedia commons, schematic diagram of colloid mill. (2019, January 6). Retrieved from https://commons.wikimedia.org/wiki/File:Schematic_diagram_of_colloid_mill.jpg.
- Wikipedia, phosphatidylcholine molecular structure. (2019, January 5). Retrieved from <https://en.wikipedia.org/wiki/Phosphatidylcholine>.
- Wysoczanski, T., Sokoła-Wysoczanska, E., Pekala, J., Lochynski, S., Czyz, K., Bodkowski, R., Herbinger, G., Patkowska-Sokola, B., & Librowski, T. (2016). Omega-3 fatty acids and their role in central nervous system – A review. *Curr. Med. Chem.*, 23, 816–831.
- Xue, J., & Zhong, Q. X. (2014). Thyme oil nanoemulsions coemulsified by sodium caseinate and lecithin. *Journal of Agricultural and Food Chemistry*, 62, 9900–7.
- Yucel, U., Elias, R. J., & Coupland, J. N. (2012). Solute distribution and stability in emulsion-based delivery systems: An EPR study. *J. Colloid Interface Sci.*, 377, 105-113.
- Zhou, L., & Elias, R. J. (2012). Factors influencing the antioxidant and prooxidant activity of polyphenols in oil-in-water emulsions. *J. Agric. Food Chem.*, 60, 2906–2915.
- Zhou, L., & Elias, R. J. (2013). Chapter 9: Understanding Antioxidant and Prooxidant Mechanisms. In *Lipid Oxidation Challenges in Food Systems*. A. Logan, U. Nienaber, and X. Pan. (Eds.) by AOCS Press, Urbana, IL.

APPENDICES

A.1. Comparison of colloid mill vs. Stephan mixer and effect on physical and oxidative stability

This study was performed to study the effects of the type of homogenizer on physical and oxidative stability of the high fat oil-in-water emulsions.

1. Emulsion production and storage experiment

Emulsion formula used for this experiment consisted of 60% wt fish oil, 2.8% wt total emulsifier, and 37.2 % wt water. In these emulsions, two types of emulsifiers were used; CAS + DATEM or CAS + PC. The ratio between two emulsifiers were 2; CAS:DATEM or CAS:PC were 2:1. Both emulsifiers were dispersed in the water and oil was added slowly into the aqueous phase during homogenization. Total amount of emulsion produced was 500 g. Emulsions were produced using Stephan mixer by adding the oil into the aqueous phase in 3 min at 1200 rpm. After that, emulsions were mixed for additional 2 x 2 min (Horn et al., 2012a). Emulsions produced with colloid mill were objected to premixing using an ultra-turrax at 13000 rpm for 3 min. After that, obtained pre-emulsion was passed through 0.159 mm gap (angle = 180°) in the colloid mill at 15000 rpm 3 times. After production, emulsions were stored for 15 days at room temperature in darkness. Samples were collected on days 0, 2, 5, 10, and 15 for the analyses of physical and oxidative parameters.

2. Materials and methods

Physical parameters such as creaming index, droplet size, and apparent viscosity as well as oxidation parameters such as peroxide value, tocopherol content, and volatile secondary oxidation products were analysed according to the methods described in Chapter 5.

3. Experimental design

The aim of the experimental design was to investigate if the homogenizer type and the surfactants (DATEM and PC) had an effect on physical and oxidative stability of 60% fish oil-in-water emulsions (Table A.1.1).

Table A.1.1. Experimental design

Emulsion Code	Emulsifiers	Homogenizers
E1	CAS+DATEM	Stephan mixer
E2	CAS+DATEM	Ultra Turrax + Colloid Mill
E3	CAS+PC	Stephan mixer
E4	CAS+PC	Ultra Turrax + Colloid Mill

(CAS: sodium caseinate, DATEM: Diacetyl Tartaric Acid Ester of Mono- and Diglycerides, PC: phosphatidylcholine)

4. Results

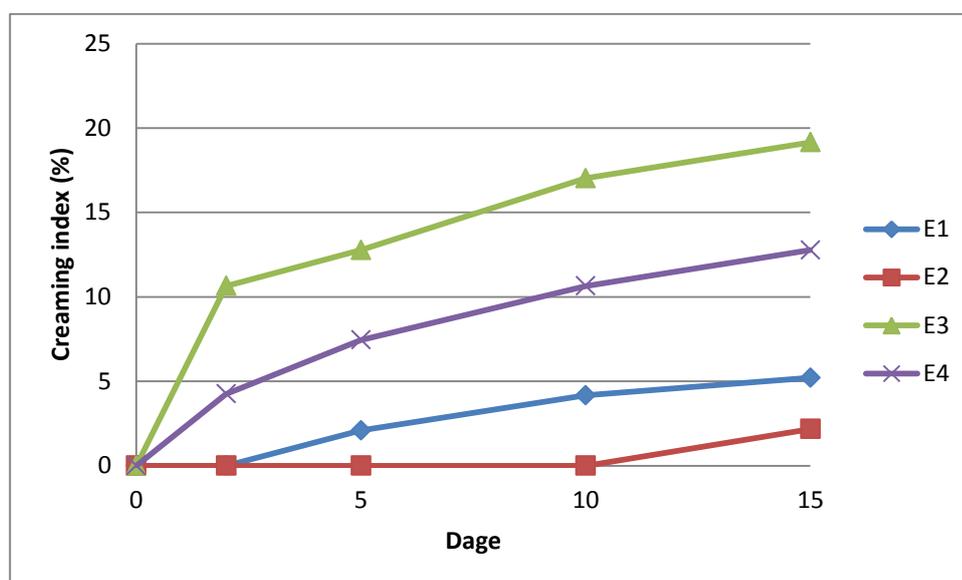


Figure A.1.1. Creaming index of the emulsions during 15 days of storage

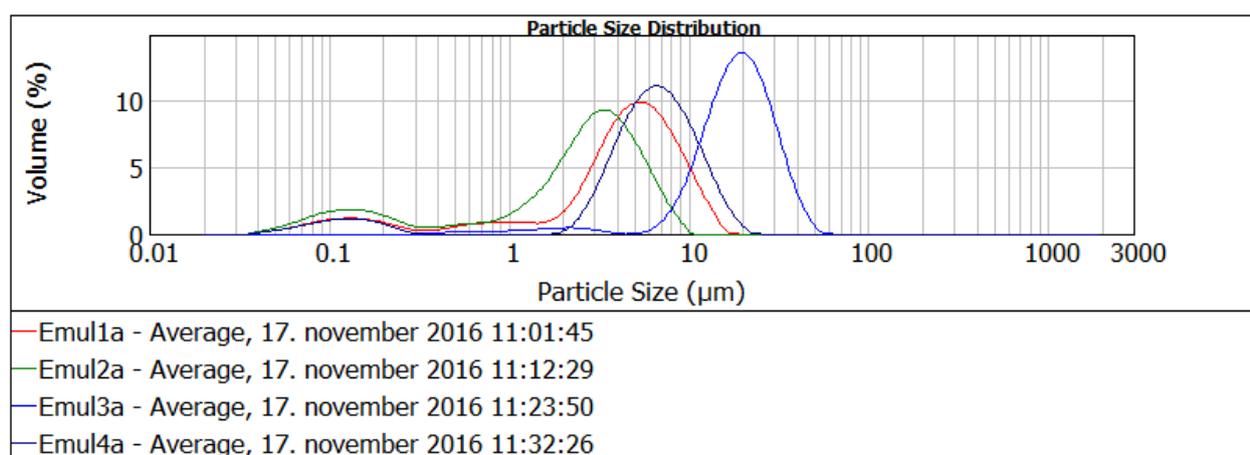


Figure A.1.2. Droplet size distribution of the emulsions at day 1.

Table A.1.2. Viscosity of the emulsions on days 1 and 15.

Viscosity (Pa·s)	Day 1		Day 15	
	Average	Std. Dev.	Average	Std. Dev.
E1	0.31	0.02	0.39	0.01
E2	0.43	0.02	0.49	0.05
E3	0.12	0.00	0.12	0.00
E4	0.17	0.00	0.19	0.00

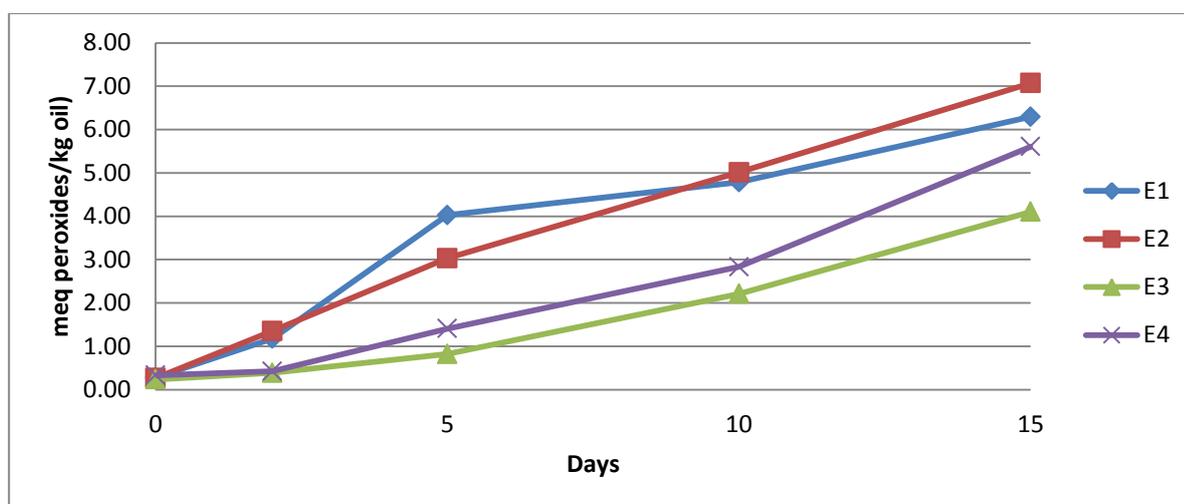


Figure A.1.3. Peroxide value of the emulsions during 15 days of storage.

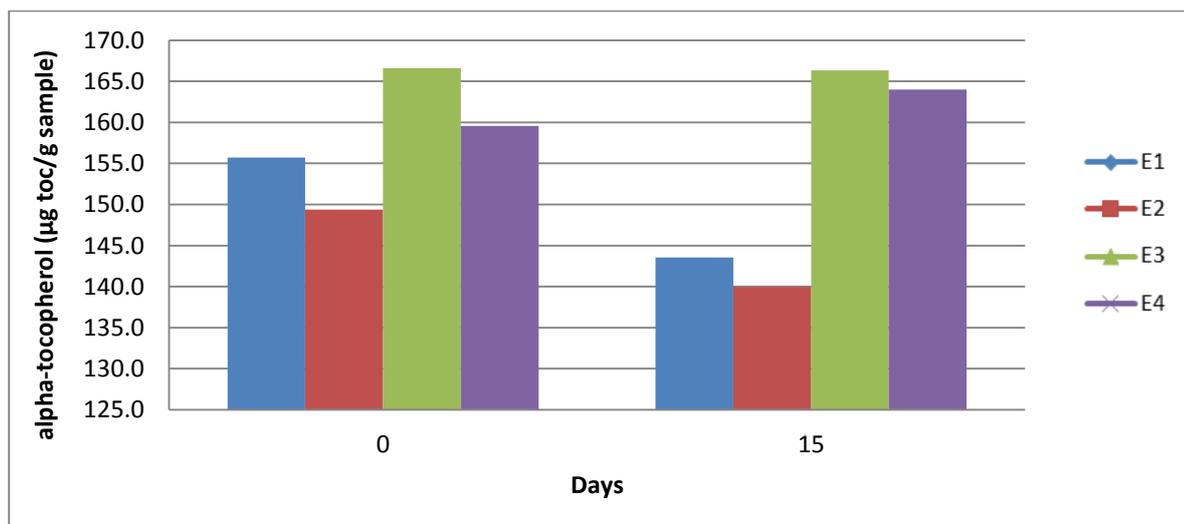


Figure A.1.4. Alpha-tocopherol concentration of the emulsions at day 0 and 15.

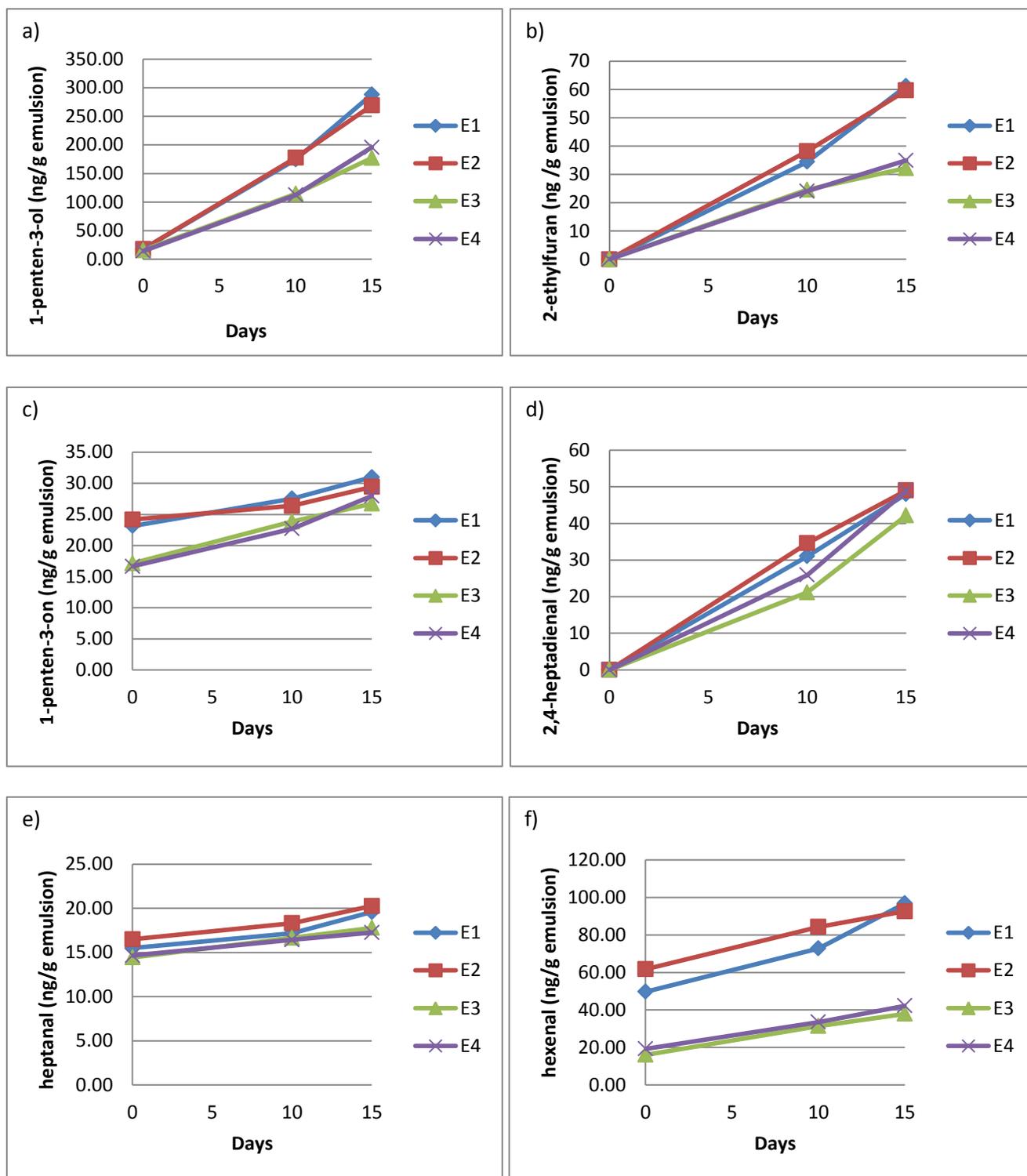


Figure A.1.5. Volatile compound formation in the emulsions during 15 days of storage; a) 1-penten-3-ol, b) 2-ethylfuran, c) 1-penten-3-on, d) (*E,E*)- 2,4-heptadienal, e) heptanal, and f) hexenal.

5. Conclusions

Outcomes of this study were summarized as following;

- Emulsions produced with the combination of CAS and DATEM were more resistant towards creaming. Colloid mill provided better creaming stability compared to Stephan mixer.
- Smaller droplet sizes were obtained for the emulsions produced with CAS and DATEM compared to CAS and PC. On the other hand, Stephan mixer produced larger droplets compared to colloid mill.
- Viscosity of the emulsions produced with CAS and DATEM were higher compared to emulsions with CAS and PC. Emulsions produced with colloid mill resulted in higher viscosity compared to emulsions with Stephan mixer.
- Oxidative stability of the emulsions produced with CAS and PC were higher compared to emulsions produced with CAS and DATEM. Formation of primary and secondary (volatile) oxidation products and consumption of alpha-tocopherol were both lower in the presence of PC as a surfactant, which indicated that the PC showed better antioxidant activity compared to DATEM in the 60% fish oil-in-water emulsions. Even though the difference was not high, emulsions produced with Stephan mixer provided better oxidative stability compared to colloid mill. This could be related to the lower creaming stability of the emulsions produced with Stephan mixer compared to colloid mill.
- In summary, both surfactant and homogenizer types resulted in different physical and oxidative stabilities.

A.2. Distribution of labelled DATEMs in high fat emulsions studied by EPR spectroscopy

This study was conducted in order to understand the distribution of emulsifiers in the different phases of high fat (70%) fish oil-in-water emulsions such as water phase, oil phase as well as interface.

1. Sample preparation, emulsion production and sampling

In order to run the EPR spectroscopy for investigating the distribution of the modified DATEMs in 70% oil-in-water emulsions, tailor-made spin labelled-DATEMs were provided by our collaborative partners. Labelled DATEMs were produced with various alkyl chains (C10, C12, and C18). Molecular structures of these labelled-DATEMs are shown in Figure A.2.1.

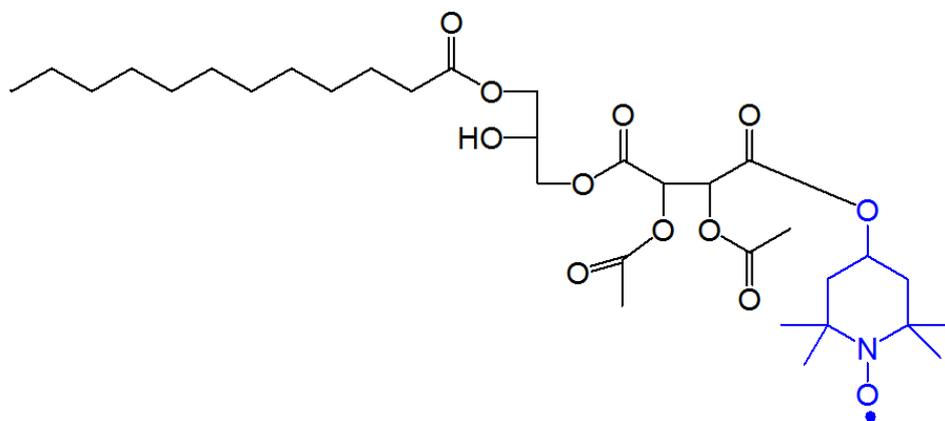


Figure A.2.1. Molecular structure of spin labelled DATEM C12. Blue part of the molecule (nitroxide) gives the signal in EPR spectroscopy.

Labelled DATEMs were solubilized in ethanol to achieve final concentration of 50 μM . These solutions were added into different mediums such as only water, CAS solution, commercial DATEM solution, aqueous phase of the emulsion containing CAS and commercial DATEM, and tetradecane-in-water emulsions in order to determine the distribution of these labelled DATEMs in different phases such as lipid, interface and aqueous. TEMPOL is the nitroxide radical, which gives signal in EPR, see Figure 2.3 in chapter 2 for the molecular structure and spectrum obtained from 50 μM TEMPOL in water. Labelled DATEMs were compared with TEMPOL due to the fact that the labelled DATEMs were labelled using TEMPOL. For this reason, TEMPOL was used as a control. Emulsions were produced in batches of 10 g using hand-held ultra-turrax (POLYTRON PE 1200g, at 1000 rpm for 7 min), under nitrogen flow as shown in Figure A.2.2.

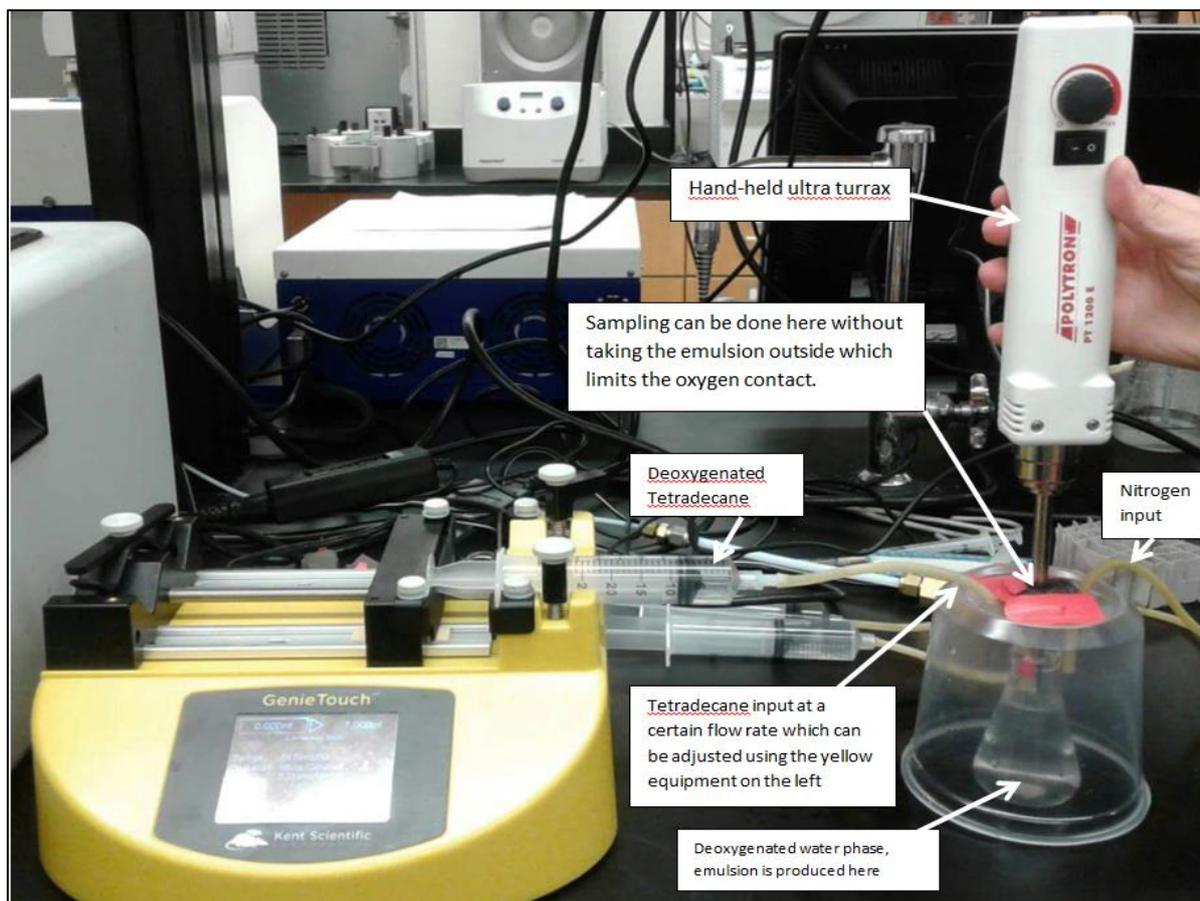


Figure A.2.2. Emulsion production system using a hand-held ultra-turrax under nitrogen.

2. Materials and methods

Tetradecane was used as a model system for the fish oil due to susceptibility of fish oil to lipid oxidation and lipid radicals give signal in EPR, hydrocarbons are usually employed in EPR studies.

Labelled DATEMs were solubilized in ethanol in order to make sure that they are totally dissolved in water, CAS and/or DATEM solutions, and 70% tetradecane-in-water emulsions. The concentration of labelled DATEMs was 50 μ M in the final system.

All samples were objected to deoxygenation due to the presence of oxygen is known to affect EPR patterns such as resulting in signal intensity losses and line broadening (Yucel et al., 2012). Samples were deoxygenated using a 3 L/min nitrogen flow. Water solutions were deoxygenated for 15 min. Aqueous-based samples such as CAS and/or DATEM solutions and emulsions were deoxygenated under humidified nitrogen flow for 1 h. The reason for the humidification of the nitrogen is to prevent the water evaporation. Non-aqueous systems such as oil were deoxygenated for 1 h under non-humidified nitrogen flow. Measurements were done using Miniscope 300 S.

3. Results

TEMPOL in water gave the same spectra during its storage for 6 days as shown in Figure A.2.3. This indicates that the TEMPOL was stable and not degraded during 6 days of storage at room temperature.

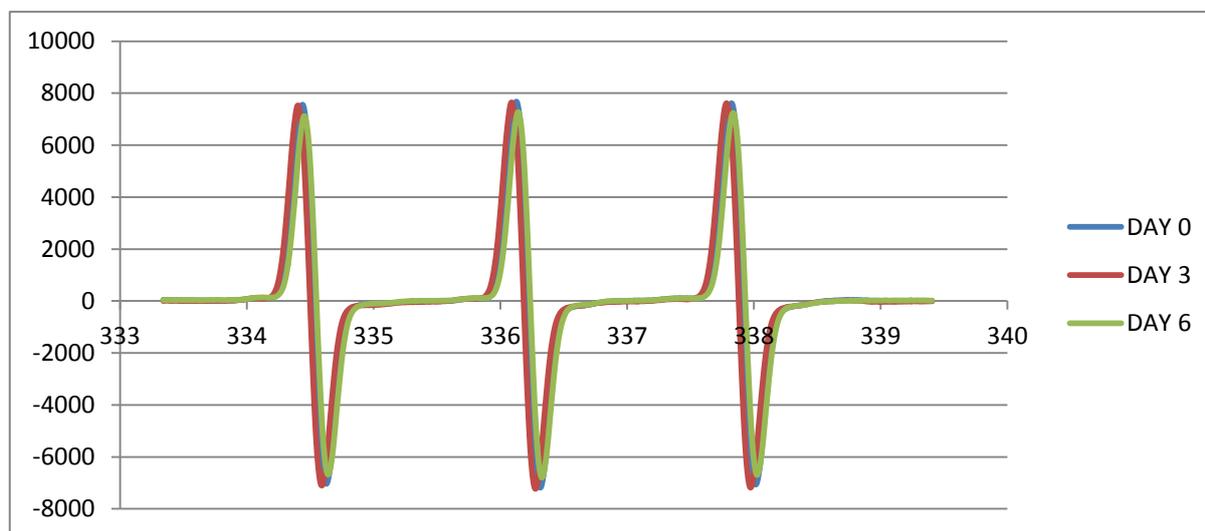


Figure A.2.3. EPR spectra of 50 μM TEMPOL in water.

3.1. Decrease in intensity of TEMPOL and labelled DATEMs

Intensity of the labelled compounds was measured according to the double integral of the spectrum as shown in the Figure A.2.4.

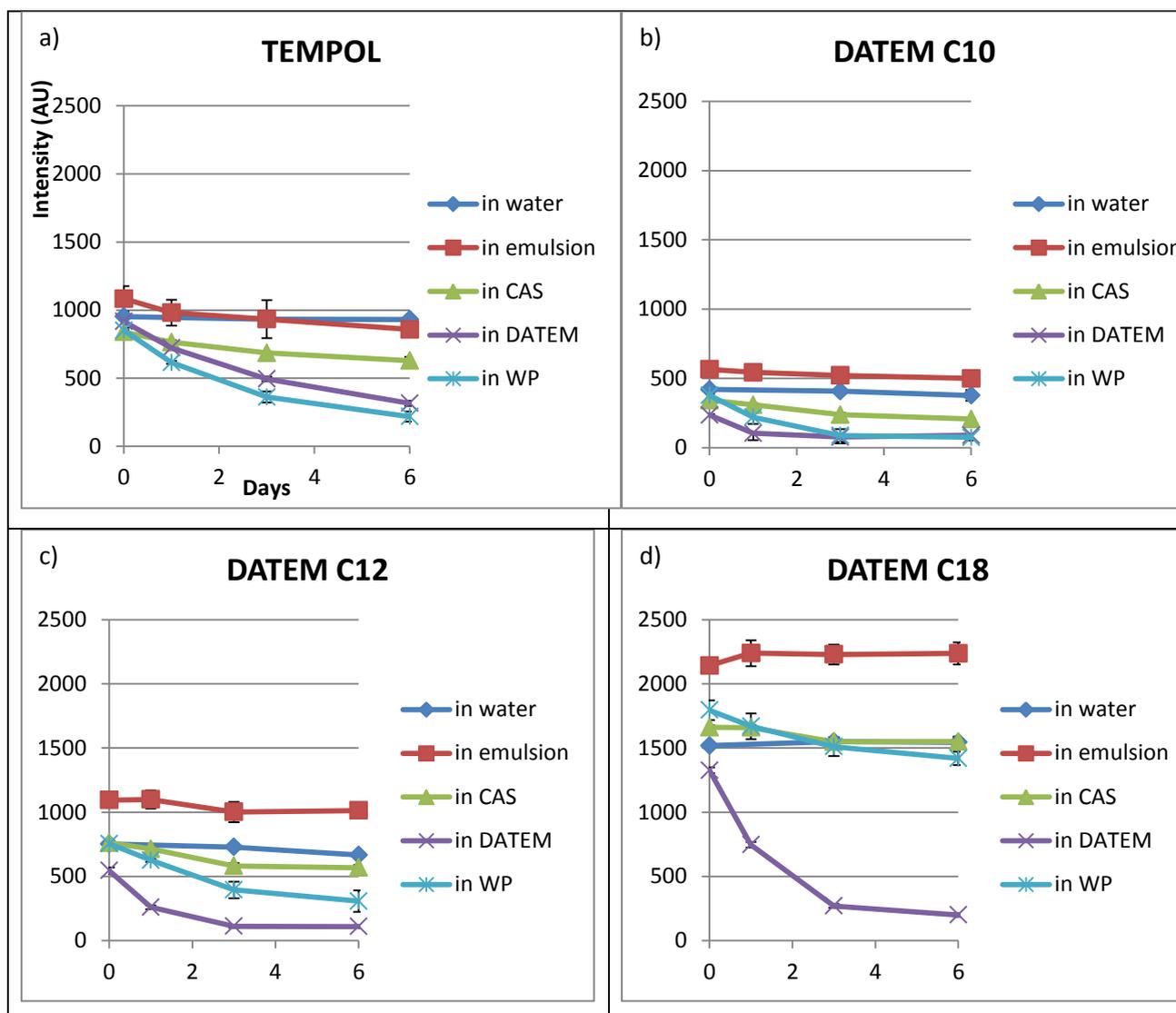


Figure A.2.4. Stability of a) TEMPOL and b-d) labelled DATEMs in different mediums; in water, emulsion, CAS solution, DATEM solution, and aqueous phase. Intensity (AU) is calculated by the double integration of the spectra obtained from TEMPOL or labelled DATEMs in different mediums

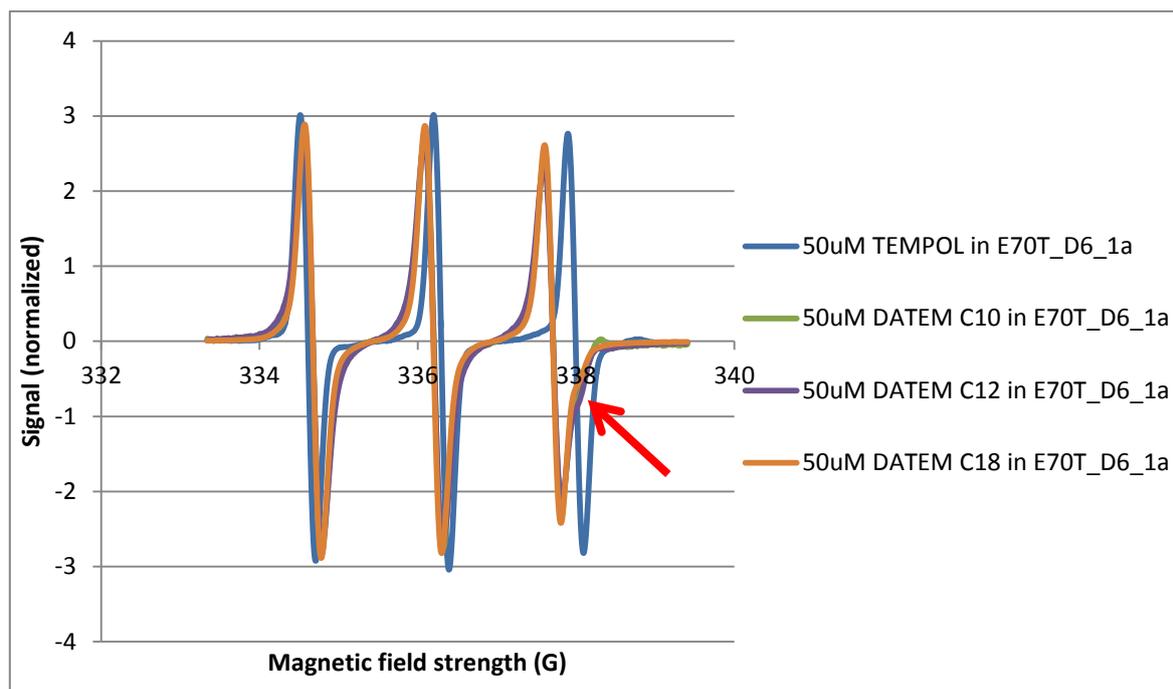


Figure A.2.5. EPR spectra of TEMPOL and labelled DATEMs in emulsion on day 6.

Outcomes of the study:

- Equivalent molar concentrations of labelled DATEMs (with different chain lengths) gave different intensity
- Labelled DATEMs are largely distributed/located in the oil phase of the 70% tetradecane-in-water emulsions
- Stability of labelled DATEMs was high when they were in emulsion and water, whereas their stability was lower in emulsifier (CAS and/or DATEM) solutions.
- It was very clear to see that the TEMPOL had a higher a_N value compared to labelled DATEMs, which had higher lipophilicity (Figure A.2.5).
- A clear trend was not observed for the distribution of the labelled DATEMs with different chain lengths depending on their lipophilicity in the different phases of the emulsion such as aqueous phase, lipid phase, and oil-water interface. However, when looked at the red arrow in Figure A.2.5, it is possible to see a slight difference in the distribution of the DATEM_C12 and DATEM_C18, indicating that the DATEM_C12 had higher amount of compound distributed in the water phase compared to DATEM_C18.

A.3. Mayonnaises enriched with high fat n-3 delivery oil-in-water emulsions

In this study, impact of modified DATEM with alkyl chain lengths and covalently attached caffeic acid on physical and oxidative stability of the mayonnaise samples were investigated.

Delivery emulsion preparation and the experimental plan on how they were mixed with mayonnaise samples are shared in section 5.4, chapter 5. Mayonnaises were produced according to the recipe below, which was described by Meyer and Jacobsen (1996). Mayonnaises included 80% rapeseed oil, 4% egg yolk, 0.3 % salt, 1% sugar, 0.1% potassium sorbate, 0.2% Grindsted FF DC stabilizer, 4% estragon vinegar (7%), 1.2% lemon juice, and 9.2% distilled water (Table A.3.1). Mayonnaise with fish oil was produced by replacing 20% of the rapeseed oil with fish oil, which results in 16% fish oil in the final mayonnaise. Mayonnaise samples were mixed with delivery emulsions as described in Table 5.4, in chapter 5.

Table A.3.1. Ingredients and amounts for the production of mayonnaise.

Ingredients	Amounts (g)
Rapeseed oil*	800
Egg yolk w. 3% salt	40
Salt (NaCl)	3
Sugar	10
Potassium sorbate	1
Grindsted FF DC stabilizer	2
Estragon vinegar 7%	40
Lemon juice	12
Water	92

*Fish oil added mayonnaise was produced by replacing 20% of the rapeseed oil with fish oil (160 g fish oil + 640 g rapeseed oil)

1. Materials and Methods

1.1. Materials

Cod liver oil was supplied by Vesteraalens A/S (Sortland, Norway) and stored at -40 °C until use. Peroxide value of the cod liver oil was 0.08 ± 0.00 meq peroxide/kg oil. The fatty acid (% w/w) content of the cod liver oil was as follows: C14:0 (4.0), C16:0 (9.2), C16:1 n-7 (8.3), C18:0 (2.2), C18:1 n-9 (15.8), C18:1 n-7 (4.1), C18:2 n-6 (2.5), C18:3 n-3 (0.2), C20:1 n-9 (11.4), C20:5 n-3 (8.8), C22:1 n-11 (5.4), and C22:6 n-3 (11.4). Alpha-, beta-, gamma-, and delta tocopherol contents were 141 ± 9 , 0 ± 0 , 95 ± 5 , 42 ± 2 µg toc/g oil, respectively. Rapeseed oil was provided by AAK Sweden AB and peroxide value was 0.14 ± 0.01 meq peroxide/kg oil. The fatty acid (% w/w)

content of the rapeseed oil was as follows: C16:0 (4.5), C18:1 n-9 (60), C18:1 n-7 (2.5), C18:2 n-6 (19), C18:3 n-6 (9.4), C20:1 n-9 (1.5). Alpha-, beta-, gamma-, and delta-tocopherol contents of the rapeseed oil were 162 ± 1 , 65 ± 3 , 263 ± 13 , 0 ± 0 $\mu\text{g toc/g oil}$, respectively. Sodium caseinate, CAS (Miprodan 30) was provided by Arla Foods Ingredients a.m.b.a (Viby J, Denmark). Arla reported a protein content of 92% in sodium caseinate for Miprodan 30. DATEM (PANODAN AB 100 VEG-FS MB, PD 244-18.3 EN) was provided by Danisco (Brabrand, Denmark). Peroxide value of the DATEM was 0.05 meq peroxides/kg sample. Caffeic acid was purchased from Sigma-Aldrich. Modified DATEMs with caffeic acid and different alkyl chains C12 or C14 were synthesized as described in a previous study (Anankanbil et al., 2017). The fatty acid (% w/w) content of the DATEM_C12 and DATEM_C14 was as follows: C12:0 (98.77%) and C14:0 (81.72%), respectively. All other chemicals and solvents used were of analytical grade.

1.2. Methods for characterization of emulsions

1.2.1. Zeta potential

Zeta potential of the samples were determined using Zetasizer Nano 2S (Malvern Instruments, Ltd.) in order to obtain the surface charge of the oil droplets of the mayonnaise samples. Each sample (0.32 g) was diluted in distilled water (40 g) and vortexed before measuring. Samples were placed in DTS-1070 disposable folded capillary cell (Malvern Instruments, Ltd., United Kingdom). The zeta potential range was set to -100 to +50 mV and measurements were done at 25°C on the samples collected on day 3. Measurements were carried out in duplicate.

1.2.2. Droplet size

Mayonnaises (1 g) were solubilized in 9 g of SDS buffer (10mM NaH₂PO₄, 5mM SDS, pH 7) and the solution was whirly mixed for 0.5 min. After that the samples were placed in ultrasonic bath (30°C, 20 min). This mixing procedure was repeated once more. The particle size of the mayonnaises was determined using laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) on days 7 and 28 according to the method described by Jacobsen et al. (1999). Results were presented as the surface weighted (D[3,2]) and volume weighted (D[4,3]) mean diameter, which were calculated according to equations 1 and 2, respectively:

$$D[3,2] = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \quad (\text{Equation 1})$$

$$D[4,3] = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \quad (\text{Equation 2})$$

where n is the number of droplet with a specific diameter and d is the diameter. Measurements were carried out as duplicates.

1.2.3. Apparent viscosity

Apparent viscosity of the mayonnaise samples was measured using a stress-controlled rheometer (Stresstech, Reologica Instruments AB, Lund, Sweden) on days 7 and 28. Rheometer was equipped with both upper and lower plate serrated (P30 serrated polycarbonate plate) and operated at 2 mm gap. Mayonnaise samples were placed in between the serrated plates and measured over a shear stress range from 0.0125 to 200 Pa in 20 logarithmic steps, 11s delay time and 11s integration time at 25°C. Results were calculated on a specific shear rate (1.3 s^{-1}) for each emulsion in Pascal second (Pa·s). Samples were measured in triplicate.

1.2.4. Relative protein content in the aqueous phase and the surface load of proteins

Separation of the aqueous phase of the mayonnaise was performed according to the method described by Jacobsen, Meyer, & Adler-Nissen (1998). Mayonnaise sample (~20 g) was centrifuged for 10 min at 25,400g and 10 °C (Sorvall RC-6 PLUS, Thermo Fisher Scientific, Osterode, Germany; rotor SS-34). Supernatant (oil phase) was removed by the use of a pipette. The rest was mixed with distilled water (1:1) and then subjected to ultracentrifugation (Beckman Ultracentrifuge L8-60M, Fullerton, CA; rotor 21102) for 1 h at 106,979g and 15 °C. Relative protein content in the aqueous phase was determined using Dumas method (Elementar, Mt. Laurel, NJ, USA). Around 1 g of aqueous phase was placed in the sample tray and further steps were automated including sample combustion in a chamber at a high temperature (900°C) in the presence of oxygen. Crude protein content was estimated by using a conversion factor (6.25). Protein concentration is presented as percent of aqueous phase. Measurements were carried out in duplicate.

The surface load of proteins (Γ , mg/m²) was calculated according to the equation described by Zhu et al. (2018), equation 3:

$$\Gamma = \frac{V_C(C_{INI} - C_{SER})}{S V_{OIL}} = \frac{(1-\Phi)d_{32}}{6\Phi} (C_{INI} - C_{SER}) \quad (\text{Equation 3})$$

where V_C and V_{OIL} are the volume of the aqueous and oil phase (mL), S is the surface area of the emulsion droplets (m²), Φ is the oil phase volume fraction, C_{INI} is the initial concentration of the protein in the aqueous phase (mg/L), and C_{SER} is the non-adsorbed protein concentration in the aqueous phase after emulsification (mg/L).

1.3. Methods for lipid oxidation measurements of mayonnaise samples

1.3.1. Primary oxidation products—peroxide value (PV)

Primary oxidation products of the mayonnaise samples were determined according to the Bligh and Dyer method with slight changes (Bligh & Dyer, 1959). Lipids were extracted using 5 g of mayonnaise sample and a reduced amount of solvent (30.0 mL of methanol and chloroform, 1:1) was used. As described by Shantha and Decker (1994), PV was subsequently measured on the lipid extracts by colorimetric determination of iron thiocyanate on a spectrophotometer (Shimadzu, UV mini 1240, Kyoto, Japan) at 500 nm. Measurements were carried out in duplicate.

1.3.2. Tocopherol content - HPLC

Tocopherol content of mayonnaises was determined by HPLC (Agilent 1100 Series; Column: Waters Spherisorb 3 μm Silica; 4.6 \times 150 mm). Tocopherols were determined according to the official AOCS method (1998) using lipid extracts (see section 1.3.1) that were further evaporated and re-dissolved in heptane. Measurements were carried out in duplicate.

1.3.3. Secondary oxidation products—Dynamic Head Space GC-MS

Volatile secondary oxidation products were analyzed according to the method described by Yesiltas et al (2018). Volatile acids in mayonnaises were removed by KOH during the headspace collection as described by Hartvigsen, Lund, Hansen, and Holmer (2000). S-shaped tubes filled with KOH were used. Volatile compounds were trapped on Tenax GR tubes and separated in a gas chromatograph (Agilent Technologies, 6890N Network GC System, DE, USA) on a 30 m DB 1701 fused silica capillary column (0.25 mm i.d., 1 μm film thickness; Agilent Technologies, J&W GC Columns, CA, USA). The individual compounds were analyzed by mass-spectrometry (Agilent 5973 Network Mass Selective Detector, Agilent Technologies, 70 eV; mass to charge ratio scan between 30 and 250) and identified by MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard). The volatile compounds (2-ethyl-furan, 1-penten-3-one, 1-penten-3-ol, (*E*)-2-pentenal, hexanal, (*E*)-2-hexenal, (*Z*)-4-heptenal, 2-pentyl-furan, (*E*)-2-heptenal, benzaldehyde, (*E,E*)-2,4-heptadienal, nonanal and (*E,Z*)-2,6-nonadienal) were selected for quantification and analyzed in all samples. Calibration curve was prepared by injecting standards into a mayonnaise produced with only rapeseed oil. Then volatiles were collected the same way as the mayonnaise samples. This was carried out in order to maintain similar release conditions for standard volatile

compounds and volatiles in the mayonnaise samples. Analysis were performed in triplicate and the results were given in ng/g of emulsion.

1.4. Sensory evaluation

Sensory evaluation was conducted on samples freshly made and stored at room temperature in lightproof containers for 28 days. The sensory panel consisted of 8 assessors from DTU sensory panel. All panel members passed tests of their capability to use their senses and expressing a response. The sensory sessions: the first sessions were used to develop a series of attributes to describe the odour, texture and appearances of the samples. The sensory attributes that were selected for profiling are described in table A.3.2. Furthermore, the panel was trained in using a scale from 0 to 15 for describing the intensity of the attributes using different samples ensuring that all experimental conditions that could be foreseen were represented. Thereby, it was ensured that assessors were able to use the whole scale for each attribute and that all relevant attribute are trained. At each session, panellists were served the samples in random order. Data was collected using FIZZ Network (Version 2.0, Biosystems, France). The sensory profiling data were analysed using Panel Check V1.4.0. For analysis of the performance of the assessors a two way ANOVA was used.

Table A.3.2. Sensory attributes of mayonnaise samples

Type	Attribute	Description
Odour	Vinegar-sourish	Vinegar, wine vinegar, lemon
	Rancid	
	Sweet	
	Fish oil	Fresh fish oil
	Estragon	Dried herb, hay
	Green	Cucumber, pepper, green, grass
	Oily	
Appearance	Colour	From white to yellow
	Shininess	From matt to shiny
	Firmness	From fluid to firm
	Broken	From not at all to the sample is totally separated
	Smooth	From grainy to homogenous and smoot
Texture	Adhesiveness	How much it stick to the spoon
	Firmness	From soft to firm (high viscosity)

1.5. Statistical analysis and principle component analysis (PCA)

Statgraphics XVII (Statpoint Technologies, Inc., Virginia, USA) was employed for the analysis of variance (ANOVA) using Fisher's least significant difference test. The significance was evaluated statistically at the confidence level $1-\alpha = 95\%$.

2. Results

Physical parameters of the emulsions were presented in Table A.3.3.

Table A.3.3. Droplet size, viscosity, zeta potential, protein in the aqueous phase, and protein surface load results of emulsions. Refer to Table 5.4 for the descriptions of sample codes

Sample code	D[3,2] (μm) (Day 7)	D[4,3] (μm) (Day 7)	Apparent viscosity (Pa·s at 1.3 s^{-1}) (Day 7)	Zeta potential (mV) (Day 3)	Protein in the aqueous phase (g) (Day 4)	Protein surface load (mg/m^2) (Day 4)
1_MAYO_FO	1.0 ± 0.2^a	4.8 ± 0.2^a	$83.08 \pm 0.55^{e*}$	(-) 30.3 ± 1.2^a	4.26 ± 0.43^d	2.02 ± 0.03^a
2_MAYO_CAS	$1.4 \pm 0.1^{b*}$	$9.3 \pm 0.2^{c*}$	$57.81 \pm 2.73^{d*}$	(-) 31.0 ± 1.5^a	3.67 ± 0.16^{cd}	4.05 ± 0.02^e
3_MAYO_DATEM	1.1 ± 0.1^a	5.5 ± 0.2^b	50.16 ± 1.37^c	(-) 51.0 ± 2.1^c	2.67 ± 0.13^{abc}	2.73 ± 0.01^c
4_MAYO_DATEM_C12	- §	- §	- §	(-) 44.0 ± 0.9^b	3.20 ± 0.87^{bc}	- §
5_MAYO_DATEM_C14	1.4 ± 0.1^b	13.5 ± 0.7^d	13.77 ± 0.79^a	(-) 44.8 ± 1.6^b	2.41 ± 0.98^{ab}	3.58 ± 0.10^d
6_MAYO_DATEM_CA	$1.0 \pm 0.1^{a*}$	5.9 ± 0.1^b	38.99 ± 0.63^b	(-) 55.4 ± 1.0^d	1.86 ± 0.33^a	2.53 ± 0.02^b

*Significant increases observed in droplet size and decrease viscosity between days 7 and 28 of storage at $p < 0.05$. Both D[3,2] and D[4,3] significantly decreased for 5_MAYO_DATEM_C14.

^{a-c}Letters indicate the significant differences between samples for the same physical parameter.

§This parameter was not measured/ calculated, because the mayonnaise sample was broken.

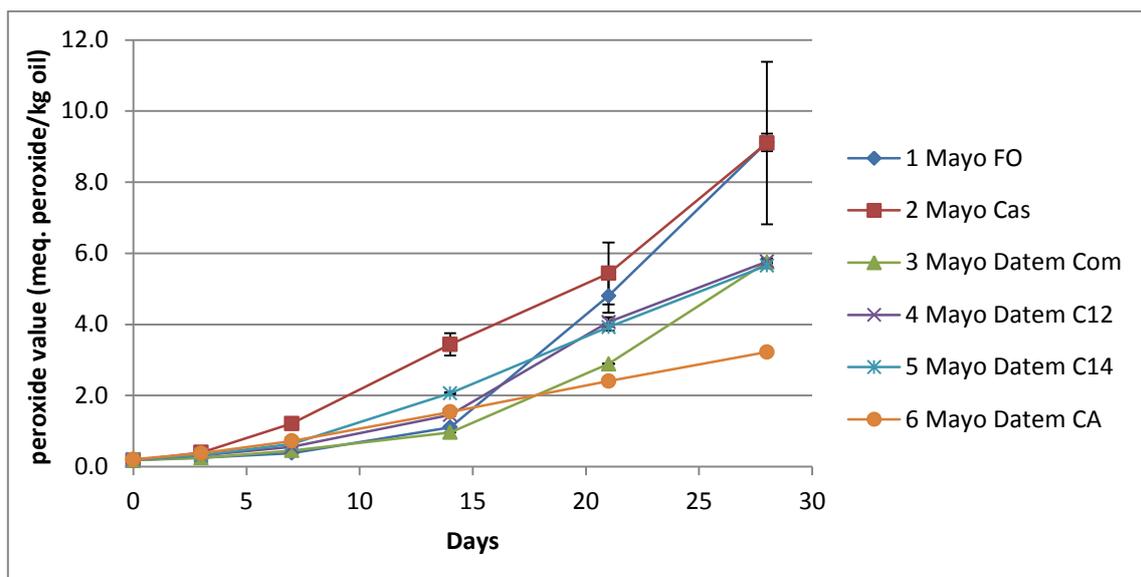


Figure A.3.1. Formation of primary oxidation products in mayonnaise samples during 28 days of storage.

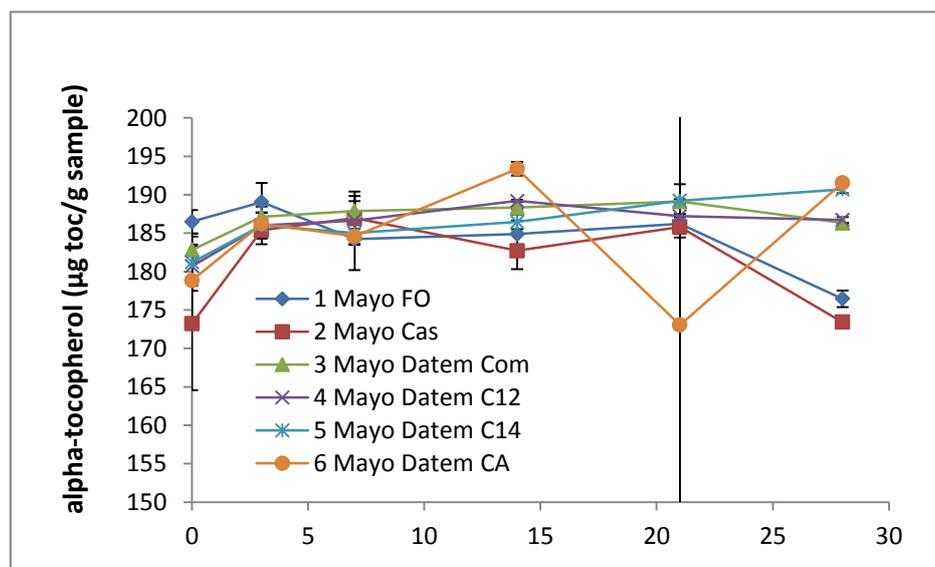


Figure A.3.2. Consumption of alpha-tocopherol in mayonnaise samples during 28 days of storage

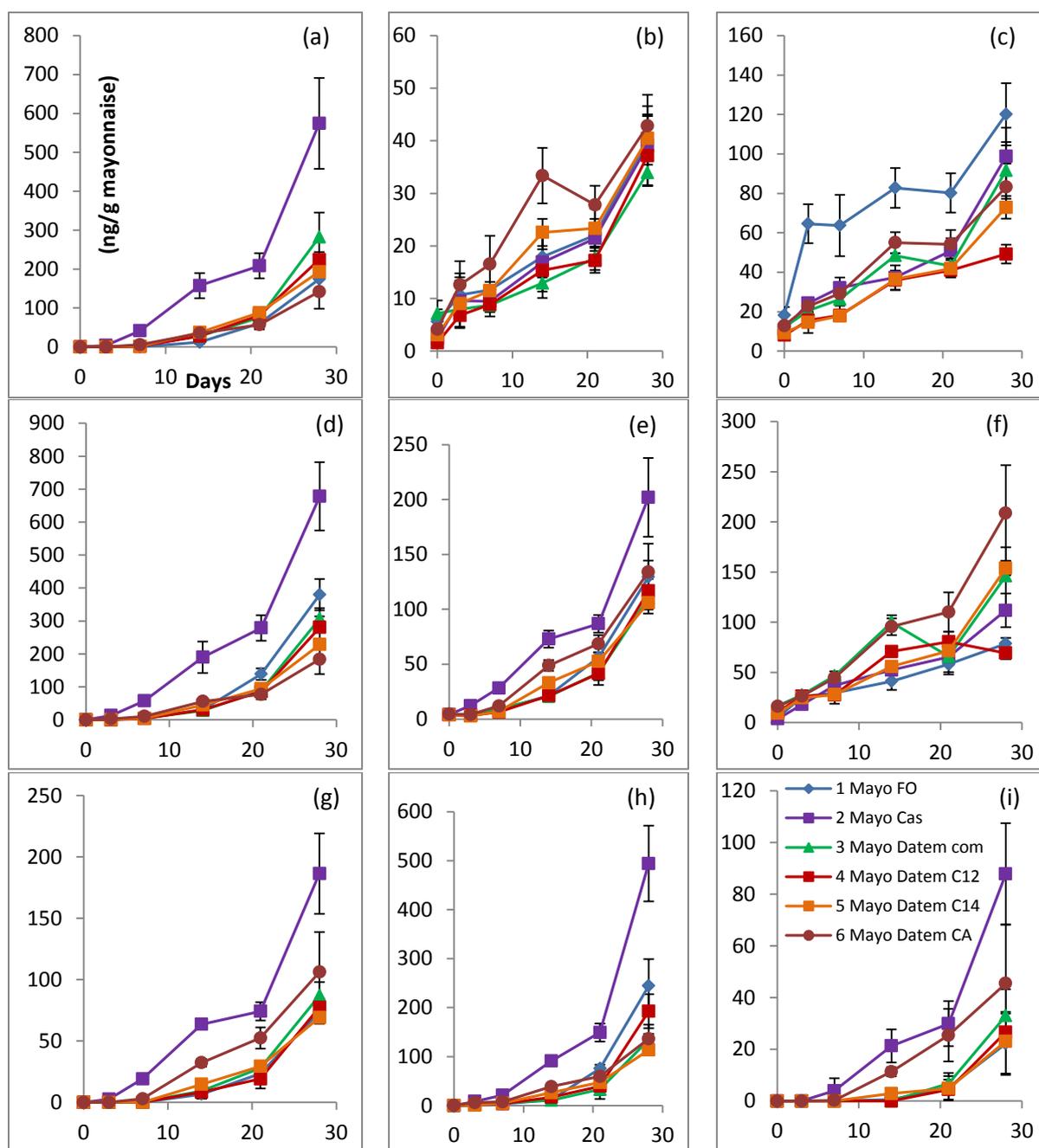


Figure A.3.3. Secondary volatile oxidation products; a) 2-ethylfuran, b) 2-butenal, c) pentanal, d) 1-penten-3-ol, e) 2-pentenal, f) hexanal, g) 2-hexenal, h) 2,4-heptadienal, and i) 2,6-nonadienal formed in emulsion samples during 28 days of storage

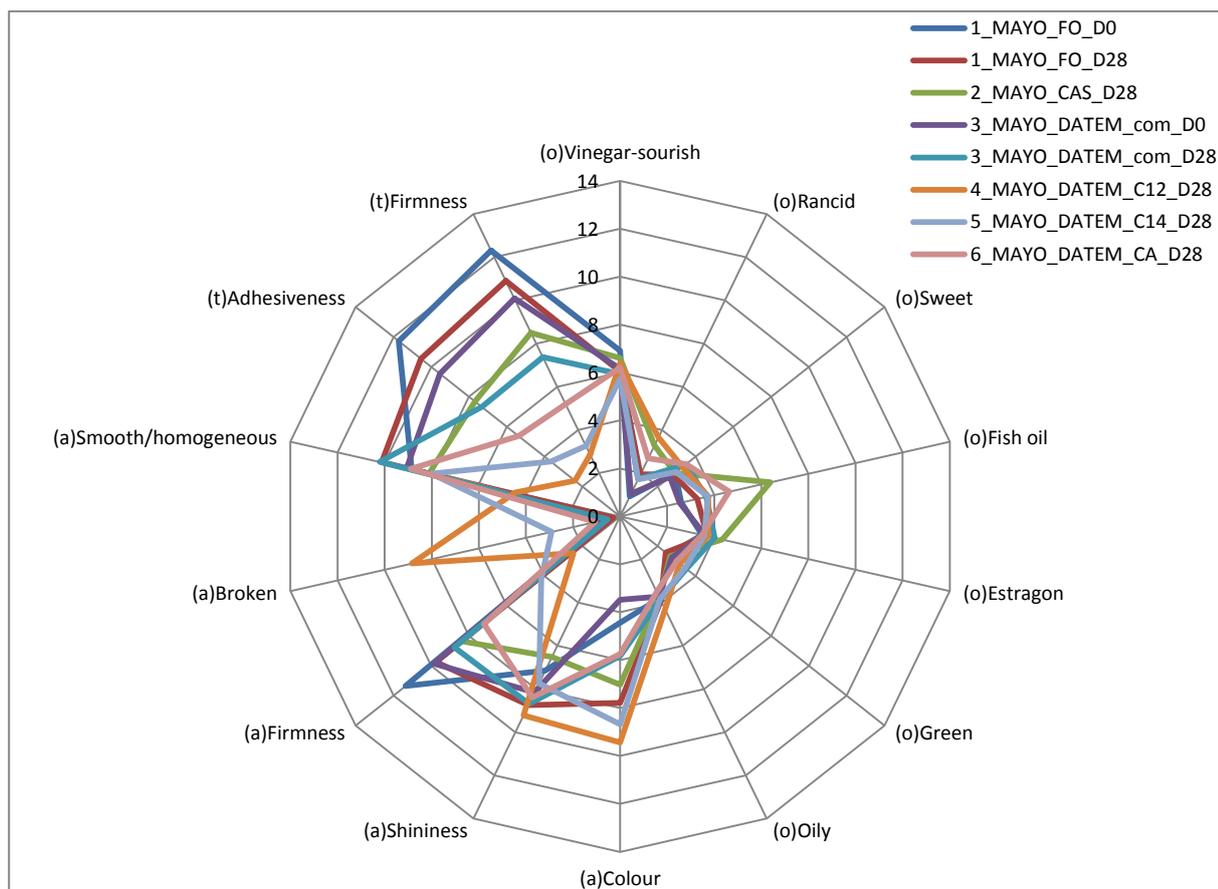


Figure A.3.4. Sensory attributes of mayonnaise samples determined by descriptive profiling

3. Conclusions

- Mayonnaise samples with added neat fish oil had significantly smaller droplet size compared to other mayonnaises with delivery emulsions. Mayonnaises enriched with n-3 delivery emulsions produced with CAS and commercial DATEM provided smaller droplets compared to mayonnaises enriched with n-3 delivery emulsions produced with only CAS. Mayonnaises enriched with n-3 delivery emulsions produced with CAS, commercial DATEM, and DATEM_C14 provided the largest droplets compared to the rest of the mayonnaises.
- As expected, viscosity of the mayonnaise samples were negatively correlated with droplet size, indicating that smaller droplet sizes led to higher viscosity. Therefore, viscosity was the highest for mayonnaise with neat fish oil, whereas the lowest was obtained for mayonnaise enriched with n-3 delivery emulsion produced with DATEM_C14. However, this trend was not valid when mayonnaises enriched with n-3 delivery emulsion produced with CAS and commercial DATEM was compared to mayonnaise enriched with n-3 delivery emulsion produced with only CAS; even though the droplet size was almost 2-fold higher for 2_MAYO_CAS compared to

3_MAYO_DATEM or 6_MAYO_DATEM_CA, its viscosity (57.81 Pa·s) was still significantly higher than their viscosities (50.16 and 38.99 Pa·s).

- Mayonnaises with added neat fish oil and enriched with n-3 delivery emulsion produced with only CAS had the lowest negative droplet surface charge compared to the rest of the mayonnaises. Mayonnaises enriched with n-3 delivery emulsions produced with the addition of modified DATEMs had significantly lower negative droplet surface charge compared to mayonnaises enriched with n-3 delivery emulsions produced with the addition of commercial DATEM. It was even observed that the addition of free caffeic acid in these delivery emulsions resulted in increased droplet surface charge for 6_MAYO_DATEM_CA.
- Protein content in the aqueous phases of the mayonnaise samples showed that the highest non-adsorbed protein was for mayonnaise with added neat fish oil and mayonnaise enriched with n-3 delivery emulsion produced with only CAS. Mayonnaises enriched with n-3 delivery emulsions produced with any kind of DATEMs had similar and significantly lower amount of non-adsorbed protein content compared to 1_MAYO_FO and 2_MAYO_CAS, except for 4_MAYO_DATEM_C12. This was due to its broken mayonnaise structure, which indicated that some of the protein left the oil-water interface due to coalescence of oil droplets.
- Protein surface load was also calculated, which showed that 2_MAYO_CAS had the highest protein adsorbed at the oil-water interface. Mayonnaise enriched with n-3 delivery emulsion produced with the addition of DATEM_C14 had the second highest protein surface load. Mayonnaises enriched with n-3 delivery emulsion produced with the addition of commercial DATEM followed 5_MAYO_DATEM_C14. Finally, mayonnaise with added neat fish oil had the lowest protein surface load compared to the rest of the mayonnaise samples.
- Primary oxidation product formation was the highest for the mayonnaise samples produced with added neat fish oil and enriched with n-3 delivery emulsions produced with only CAS at the end of the 28 days of storage. Mayonnaise enriched with n-3 delivery emulsions produced with CAS and commercial DATEM plus added caffeic acid had the lowest PV at day 28. Alpha-tocopherol content of these two mayonnaises (1_MAYO_FO and 2_MAYO_CAS), which oxidized the most, was consumed the most as well.
- Formation of 2-ethylfuran, 1-penten-3-ol, 2-pentenal, 2-hexenal, 2,4-heptadienal, and 2,6-nonadienal was the highest for 2_MAYO_CAS. 1_MAYO_FO had also high formation of pentanal, 1-penten-3-ol, and 2,4-heptadienal. 6_MAYO_DATEM_CA showed prooxidant effect in the formation of 2-butenal and hexanal, whereas had the lowest formation of 1-penten-3-ol.

4_MAYO_DATEM_C12 and 5_MAYO_DATEM_C14 showed high stability in terms of volatile compound formation; however, 4_MAYO_DATEM_C12 had physical stability issues, which needs to be borne in mind.

- Sensory evaluation results supported the results obtained from PV and volatile compound formation by reporting that the fish oil odour was the highest for 3_MAYO_CAS at day 28 (Figure A.3.4). It was followed by 6_MAYO_DATEM_CA. However, 1_MAYO_FO was the lowest which fish oil odour was detected at day 28, which contradicts with the formation of pentanal, 1-penten-3-ol, and 2,4-heptadienal. In the literature, these volatile compounds may give pungent, glue, green, grassy, apple, sweet, fishy, burnt, rotten apples, nasty, green, rancid hazel nuts; however, sensory analysis results for 1_MAYO_FO did not report the detection of green, fishy, and sweet odour characteristics, which were selected as the sensory attributes for mayonnaise samples.
- Panelists also reported that 4_MAYO_DATEM_C12 was broken by giving lower number on the scale for firmness and higher numbers for the attributes such as colour, shininess, and broken. 5_MAYO_DATEM_C14 was also found as broken (given number 3 out of 14). Moreover, panellists also observed that the textural attributes of mayonnaise samples stored for 28 days lost firmness and adhesiveness for the samples 1_MAYO_FO and 3_MAYO_DATEM_com, which were the only samples included in the evaluation as day 0 and 28.

PAPER I

Yesiltas, B., García-Moreno, P. J., Sørensen, A. M., Jacobsen, C., **2017.**

Physical and oxidative stability of high fat fish oil-in-water emulsions stabilized with combinations of sodium caseinate and sodium alginate.

European Journal of Lipid Science and Technology, 119, 1600484.

Short Communication

Physical and oxidative stability of high fat fish oil-in-water emulsions stabilized with combinations of sodium caseinate and sodium alginate

Betül Yesiltas , Pedro J. García-Moreno, Ann-Dorit M. Sørensen and Charlotte Jacobsen

Division of Food Technology, National Food Institute, Technical University of Denmark, Lyngby, Denmark

A systematic study was carried out in order to evaluate the physical and oxidative stability of high fat omega-3 delivery fish oil-in-water emulsions stabilized with combinations of sodium caseinate (NaCas) and sodium alginate (NaAlg). The influence of three factors related to emulsion composition (fish oil content: 50, 60, and 70%; total amount of NaCas and NaAlg: 1.4, 2.1 and 2.8%; and ratio NaCas: NaAlg: 0.4, 1.2, and 2) on physical (droplet size, viscosity, and zeta potential) and oxidative (primary and secondary oxidation products) parameters was evaluated. It was possible to produce emulsions with a combination of NaCas and NaAlg, except when the ratio between NaAlg and aqueous phase was high (0.047 or 0.054). Viscosity of the emulsions significantly increased with increasing fish oil and total stabilizer content. Zeta potential was significantly affected by total stabilizer content. The content of primary oxidation products in the emulsions was very low (0.93 meq peroxides/kg oil). Secondary oxidation products were detected in small amounts (<60 ng/g emulsion). Even though the optimum formulation concerning physical parameters was suggested as 61.8% fish oil content, 1.4% total stabilizer, and 1.2 ratio NaCas:NaAlg by Box-Behnken's design, the formulae 70%-1.4%-1.2 was decided due to high fish oil content's decreasing effect on droplet size and peroxide value.

Practical applications: Physically and oxidatively stable high fat (50–70%) omega-3 delivery fish oil-in-water emulsions are of high interest to food industry for the production of omega-3 fortified products. Our results show the feasibility to stabilize high fat delivery fish oil-in-water emulsions using combinations of NaCas and NaAlg. As these emulsions had high amount of fish oil, food products can be enriched with smaller amounts of high fat emulsions when compared to low fat delivery emulsions. This results in minor changes of the product's original structure. Examples for enrichment of food products with omega-3 are dressings, cream cheese, yoghurt, and mayonnaise.

Keywords: 50–70% o/w Emulsion / Emulsifier / Lipid oxidation / Omega-3 / Stabilizer

Received: November 21, 2016 / Revised: June 27, 2017 / Accepted: July 3, 2017

DOI: 10.1002/ejlt.201600484

1 Introduction

Long chain (LC) omega-3 polyunsaturated fatty acids (PUFAs) have been found to be beneficial to human health

Correspondence: Dr. Charlotte Jacobsen, National Food Institute, Technical University of Denmark, Søtofts Plads, Building 221, 2800 Kgs. Lyngby, Denmark

E-mail: chja@food.dtu.dk

Fax: +45 45 88 47 74

Abbreviations: LC, long chain; MRO, multiple response optimization; NaAlg, sodium alginate; NaCas, sodium caseinate; PCA, principal component analysis; PUFAs, polyunsaturated fatty acids; PV, peroxide value; RSM, response surface methodology

in several respects such as preventing cardiovascular diseases, improving immune system, and mental health [1–3]. As a consequence and due to the low consumption of fish, food products enriched with omega-3 fatty acids have been developed (e.g., milk, mayonnaise, yoghurt, dressings, and cream cheese). However, these LC omega-3 PUFAs are highly prone to oxidation due to the high content of bis-allylic hydrogens in their structure. The production of fish oil delivery emulsions could be an efficient strategy to protect omega-3 fatty acids against oxidation when incorporated into food matrices. In addition to preventing lipid oxidation, maintaining the physical stability of emulsions is another important parameter, which needs to be addressed due to its direct effect on overall quality and

shelf life of the final product [4, 5]. A large number of studies have been performed on physical and oxidative stability of low fat (5–30%) emulsions; however, only a few studies have been reported on high fat (50–70%) emulsions. The advantage of using a high fat emulsion for enrichment of food products is its lower water and higher fat content compared to low fat emulsions, which means that lower amounts of emulsions would be required for enrichment. Therefore, high fat omega-3 delivery emulsions are gaining increasing interest by the food industry, particularly for highly viscous food products such as mayonnaise, dressings, and cream cheese, etc., which have similar structure as high fat emulsions. Challenges are to keep them physically and oxidatively stable.

Physical and oxidative stability of emulsions are affected by charge density, ionic strength, temperature, pH, emulsifier, or surfactant concentration as well as homogenization techniques and conditions [6–11]. Particularly, the type of emulsifiers and thickening agents employed has a great influence on emulsion stability. They determine the properties of the interface, which is the place where oxidation is initiated and they increase the viscosity of the emulsions' aqueous phase slowing the creaming of oil droplets [12, 13]. In this regard, protein and polysaccharide combinations have been widely used in food industry in order to stabilize low-fat emulsion based food systems [14].

There are two different methodologies to produce emulsions stabilized with combinations of proteins and polysaccharides, leading to the so called “mixed emulsion” and “bilayered emulsion” [15]. Mixed emulsion is prepared by dissolving both protein and polysaccharide in the buffer and then oil is emulsified within the aqueous phase. Bilayered emulsion is produced by emulsification only with protein followed by addition of polysaccharide into the emulsion. The polysaccharides are oppositely charged than the protein so that they may adsorb at the interface creating a second layer. It was stated in previous studies that bilayered emulsion has a major problem with flocculation which promotes creaming instability; hence, these authors found mixed emulsion to be the best alternative [6, 15]. On the other hand, bilayered emulsions are more appropriate for nanoencapsulation of nutritious compounds inside the protein and polysaccharide layers.

Some studies have shown that the use of polysaccharide in protein stabilized mixed oil-in-water emulsions increased their physical stability [11, 15–18]. The main functionality of polysaccharides in food emulsions is their ability to thicken the system, which reduces the creaming rate and enhances the textural properties of the emulsion [4]. In this study, mixed emulsion technique was applied and sodium alginate was used as a thickener. It was also reported that the amount of polysaccharides dissolved in the water phase in the emulsion has a critical importance due to promoting the occurrence of depletion or bridging flocculation and coalescence [5].

Previous studies on high fat fish oil-in-water emulsions employed whey protein isolate, NaCas, milk phospholipids, or soy lecithin as emulsifier [9]. These authors found that emulsions prepared at pH 7 and stabilized with NaCas were more oxidatively stable compared to the other emulsions and to neat fish oil. This is mainly explained by the metal chelating activity of NaCas and to its flexible structure, which allowed a better coverage of the droplets. Combinations of sodium caseinate and sodium alginate have already been studied for the stabilization of low fat emulsions. Pallandre *et al.* aimed at obtaining a double-interfacial layer by using caseinate and alginate for the stabilization of 1 wt% corn oil-in-water emulsions [19].

However, to the best of the authors' knowledge, stabilization of high fat fish-oil-in-water emulsions with combinations of NaCas and NaAlg has not yet been reported.

Therefore, this work aimed to investigate the physical and oxidative stability of high fat omega-3 delivery fish oil-in-water emulsions stabilized with combinations of NaCas and NaAlg. A systematic study was carried out in order to evaluate the influence of fish oil content, total content of NaCas plus NaAlg and ratio between NaCas and NaAlg on both physical (droplet size, zeta potential, and viscosity) and oxidation (peroxide value and volatiles content) parameters.

2 Materials and methods

2.1 Materials

Cod liver oil was provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway), and stored at -40°C until use. Fatty acid composition, peroxide value, and tocopherol composition of the cod liver oil was previously reported by García-Moreno *et al.* [20]. Sodium caseinate (Miprodan 30) was donated by Arla Foods Ingredients amba (Viby J, Denmark). Arla reported a protein content of 92% in sodium caseinate for Miprodan 30. Sodium alginate (Grindsted[®] Alginate FD 170) was provided by DuPont (Brabrand, Denmark).

2.2 Emulsion preparation and sampling

Prior to emulsification, both NaCas and NaAlg were dissolved in distilled water and left for mixing on a magnetic stirrer over night at 4°C . All aqueous phases were adjusted to pH 7 with 2M HCl or 2M NaOH. Emulsions were produced in 500 g batches in a Stephan Universal mixer (Stephan, UMC5, 1995, Hameln, Germany) equipped with an emulsification blade as described by Horn *et al.* [9]. The three experimental factors considered for this study were fish oil content, total content of NaCas plus NaAlg, and ratio between NaCas and NaAlg since they are expected to affect the physical and oxidative stability of the emulsions by determining the composition

of the interface. A Box–Behnken design including three central points was executed, in which each input variable was set at three levels: 50, 60, and 70% for fish oil content; 1.4, 2.1, and 2.8% for total amount of NaCas + NaAlg; and 0.4, 1.2, and 2 as ratio between NaCas and NaAlg (Table 1).

Emulsions were stored in 100 mL blue cap bottles at room temperature for up to 4 week with sampling points at day 0 or 1, day 3, week 1–4. The viscosity and droplet size were measured on day 1, week 1–4, and zeta potential was measured on day 4. Peroxide value was measured at all sampling points. Volatile oxidation products (GC-MS) were measured at day 0 and week 4.

2.3 Characterization of emulsions

2.3.1 Droplet size

Droplet size of the emulsions was measured by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) using the method described by Let et al. [21] and Horn et al. [9]. Results were given as the volume weighted mean diameter $D[4,3] = \sum n_i d_i^4 / \sum n_i d_i^3$. Measurements were carried out in duplicate.

2.3.2 Zeta potential

Surface charge of the emulsion droplets was determined by the zeta potential measured with a Zetasizer Nano 2S (Malvern Instruments, Ltd.) at 25°C. Each sample (0.32 g

sample) was diluted in distilled water (40 g of distilled water) before measuring and the zeta potential range was set to –100 to +50 mV. Measurements were carried out in duplicate.

2.3.3 Apparent viscosity measurements

Viscosity was measured using a stress-controlled rheometer (Stresstech, Reologica Instruments AB, Lund, Sweden) equipped with a CC25 standard bob cup system in a temperature vessel. Measurements were done at 25°C over a shear stress range from 0.0125 to 500 Pa. The apparent viscosity was obtained at a shear rate of 20 s^{-1} and was expressed in Pa · s. Viscosities were measured twice on each emulsion.

2.4 Lipid oxidation measurements of emulsions

2.4.1 Primary oxidation products—peroxide value (PV)

For determination of primary oxidation products, a lipid extract was prepared according to the method described by Bligh and Dyer [22] using 5–10 g of emulsion for each extraction and a reduced amount of solvent (30 mL of methanol and chloroform, 1:1). PV was subsequently measured on the lipid extracts by colorimetric determination of iron thiocyanate at 500 nm, as described by Shantha and Decker [23]. Measurements were carried out in duplicate.

Table 1. Experimental design, droplet size, viscosity, and zeta potential results for the emulsions

Emulsion code	Fish oil, %	NaCas + NaAlg, %	NaCas:NaAlg, ratio	Droplet size D[4,3] (μm) (day 1)	Viscosity (Pa · s @ 20 s ⁻¹) (week 4)	Zeta potential (mV) (day 4)
50-1.4-1.2	50	1.4	1.2	17.9 ± 0.0	0.4 ± 0.0	–81.3 ± 1.7
50-2.1-0.4	50	2.1	0.4	7.8 ± 0.0	3.3 ± 0.1	–91.2 ± 1.6
50-2.1-2	50	2.1	2	14.7 ± 0.1	0.5 ± 0.2	–84.7 ± 2.2
50-2.8-1.2	50	2.8	1.2	9.4 ± 0.0	4.1 ± 0.6	–85.3 ± 2.8
60-1.4-0.4	60	1.4	0.4	9.7 ± 0.3	2.8 ± 0.0	–93.2 ± 3.9
60-1.4-2	60	1.4	2	14.5 ± 0.0	0.9 ± 0.4	–89.3 ± 2.2
60-2.1-1.2	60	2.1	1.2	7.7 ± 0.0	5.7 ± 0.2	–88.4 ± 2.3
60-2.1-1.2	60	2.1	1.2	8.9 ± 1.3	5.2 ± 0.2	–89.0 ± 1.3
60-2.1-1.2	60	2.1	1.2	7.9 ± 0.2	5.3 ± 0.1	–89.8 ± 1.7
60-2.8-0.4	60	2.8	0.4	28.8 ± 10.6	22.9 ^a	–82.3 ± 2.3
60-2.8-2	60	2.8	2	6.6 ± 0.0	7.9 ± 0.6	–87.9 ± 3.2
70-1.4-1.2	70	1.4	1.2	7.5 ± 0.0	6.5 ± 0.1	–97.3 ± 1.5
70-2.1-0.4	70	2.1	0.4	82.8 ± 5.2	22.9 ^a	–75.8 ± 1.6
70-2.1-2	70	2.1	2	7.5 ± 1.4	20.9 ± 0.6	–90.1 ± 0.6
70-2.8-1.2	70	2.8	1.2	39.7 ± 3.3	22.9 ^a	–72.2 ± 1.1

All samples were analyzed in duplicates.

^a These samples did not form an emulsion structure, therefore, 22.9 Pa · s was given as a value which is 10% higher than the highest measured value (20.9 Pa · s).

2.4.2 Secondary oxidation products—dynamic head space GC-MS

Volatile secondary oxidation products were analyzed according to the method described by Jacobsen et al. [24]. Approximately 1 g of emulsion was mixed with 1 mL antifoam and 5 mL distilled water in a 100 mL purge bottle. The bottle was heated in a water bath at 60°C while purging with nitrogen (flow 150 mL/min, 30 min). Volatile compounds were trapped on Tenax GR tubes. The volatiles were separated in a gas chromatograph (Agilent Technologies, 6890N Network GC System) on a 30 m DB 1701 fused silica capillary column (0.25 mm i.d., 1 µm film thickness; Agilent Technologies, J&W GC Columns, USA). The oven program had an initial temperature of 45°C for 5 min, increasing with 1.5 °C/min until 55°C, with 2.5°C/min until 90°C, and with 12.0°C/min until 220°C, where the temperature was held for 4 min. The individual compounds were analyzed by mass-spectrometry (Agilent 5973 Network Mass Selective Detector, Agilent Technologies, electron ionization mode, 70 eV; mass to charge ratio scan between 30 and 250). The individual compounds were identified by both MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard) and quantified through calibration curves. The external standards employed were 2-ethylfuran, 1-penten-3-ol, 1-penten-3-one, 2-pentenal, hexanal, (E)-2-hexenal, heptanal, octanal and (E,E)-2,4-heptadienal and nonanal. Measurements were made in triplicate in each sample.

2.5 Statistical analysis

Statgraphics XVII (Statpoint Technologies, Inc., Virginia, USA) was used to generate the statistical analysis and the regression models for the physical parameters of the emulsions. Firstly, the output variables (Y : viscosity at week 4, droplet size at day 1, and zeta potential at day 4) were related to the input variables (X : total fish oil content, total content of NaCas plus NaAlg, and ratio between NaCas and NaAlg) by second degree polynomials as follows, Eq. (1):

$$Y = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i < j} b_{ij} X_i X_j \quad (1)$$

where the coefficients b_i and b_{ii} are related to the linear and quadratic effects, respectively, of each input factor on the output variable and the cross-product coefficients b_{ij} represent the interactions between two input variables. Secondly, the analysis of variance (ANOVA) was carried out. The significance of all terms in the models was judged statistically by computing the p -value at a confidence level $1-\alpha = 95\%$. The regression coefficients were then used to generate contour maps and to find the optimal values of the input variables, which maximize the quality of the emulsions

in terms of physical stability, by using the response surface method (RSM) [25].

Multiple response optimization (MRO) was applied in order to determine the values of the experimental factors that led to desired characteristics for more than one output response simultaneously. Desirability function was constructed based on values of response variables and then maximized. Desirability function $d(y)$ expresses the desirability of a response value equal to y on a scale of 0–1. Parameter s defines the shape of the function. Response could be maximized, minimized, or targeted to a specific value. In this study, response variables were minimized, thus the function is defined by Eq. (2);

$$d = \begin{cases} 1 & \left(\frac{y - \text{high}}{\text{low} - \text{high}} \right)^s, y < \text{low}; \text{low} \leq y \leq \text{high}; y > \text{high} \\ 0 & \end{cases} \quad (2)$$

To be able to use MRO, RSM was applied first in Statgraphics XVII for each output variable and then the information was retrieved to perform multiple optimization.

Samples with the codes 70-2.8-1.2, 70-2.1-0.4, and 60-2.8-0.4 (Table 1) did not form an emulsion. Droplet size and zeta potential for these samples were still measured by shaking the samples before running the analysis. On the other hand, viscosity was not determined for these samples. Thus, in order to have all the data to create a model, values being 10% higher than the highest measured viscosity value were given to these samples since minimum viscosity was desired.

Principal component analysis (PCA) was done by Latentix 2.12 (LatentiX, Copenhagen, Denmark). The PCA was carried out with the emulsions as objects and viscosity, droplet size, zeta potential, and peroxide values as variables. Data were mean centered to make the variables contribute equally to the model, and the PCA model was validated systematically segmented, according to replicates of the emulsions.

3 Results and discussion

3.1 Production of emulsions

Emulsions 70-2.8-1.2, 70-2.1-0.4, and 60-2.8-0.4 did not lead to formation of an emulsion, since the two phases were totally separated right after production. This might be due to the high ratio between the amount of NaAlg and water phase where both stabilizers were dissolved. This ratio can be calculated from the compositional data in Table 1. It was found that emulsions did not form when the ratio of NaAlg to water phase was 0.047 or 0.054. On the other hand, the remaining emulsions (12 out of 15) were successfully produced when the ratio of NaAlg to water was between 0.012 and 0.031. The reason behind these findings could be both depletion flocculation and bridging flocculation when

high amounts of NaAlg were used. Depletion flocculation may have occurred since an osmotic attractive force between droplets was generated which removed the polymer molecules present around oil droplets. Bridging flocculation might have been caused due to a rearrangement of an alginate molecule which adsorbs at more than one oil droplet [5, 19]. Besides, high amount of NaAlg led to a highly viscous water phase, which might have hampered the diffusion of both emulsifier and stabilizer to the droplet interface and thus affected the stabilization of the oil droplets [19, 26, 27]. High viscosity of the aqueous phase might make it harder to detach and adsorb at the water/oil interface and result in droplet aggregation during production.

The other emulsions were stable right after production. NaCas is known as a surface active emulsifier and NaAlg is mainly used as a thickening agent for increasing the viscosity of aqueous phase [28, 29]. This explains that the use of both molecules together in the right concentrations help to produce physically stable emulsions.

3.2 Characterization of emulsions

Droplet size, viscosity, and zeta potential results are shown in Table 1 together with the experimental design.

3.2.1 Droplet size

Most of the emulsions were stable during 4 weeks of storage, without a significant increase in droplet size. Therefore, results at day 1 (Table 1) are representative for the rest of the sampling points. Emulsions showed monomodal distributions with droplet sizes ($D[4,3]$) which ranged between 6.6 and 17.9 μm except for the emulsions with the following codes 70-2.8-1.2, 70-2.1-0.4, and 60-2.8-0.4, which did not

form an emulsion structure (Table 1). The ones that did not form an emulsion structure had bimodal distributions with much larger droplet sizes up to 127 μm due to a severe coalescence of oil phase (data not shown). Emulsions with codes 50-1.4-1.2 and 50-2.1-2 had the highest droplet sizes values among all emulsions and were also creaming after 4 weeks of storage (20.7 and 20.9%, respectively). The rest of emulsions were physically stable in terms of creaming (data not shown).

A high coefficient of correlation ($R^2 = 88.88$) was obtained for the model obtained for droplet size, indicating a good correlation between predicted and measured values.

ANOVA analysis indicated that droplet size significantly decreased when the ratio of NaCas to NaAlg and the interaction between fish oil content and the ratio between NaCas and NaAlg increased. Total stabilizer content did not have a significant effect on droplet size. Fish oil content also had a significant effect on droplet size, with a positive correlation (Table 2). However, raw data show that droplet size of emulsions significantly decreased when increasing fish oil (see droplet sizes for emulsions 50-1.4-1.2 and 70-1.4-1.2 or 50-2.1-2, and 70-2.1-2 in Table 1). Likewise, Hadnede et al. [30] also reported that an increase in specific surface area occurred with the increasing oil concentration when the emulsions were stabilized with 20% of emulsifier content.

Since the model fitted the experimental data well, RSM was employed to obtain the experimental factors related to emulsion composition, which minimized droplet size. This optimum value is shown in Fig. 1a and corresponds to 68.3% fish oil content, 1.4% total stabilizer, and 2 as the ratio of NaCas to NaAlg. A minimum droplet size was desired since it implies higher physical stability of the emulsion.

Table 2. Polynomial coefficients and p -values for the response variables (droplet size, viscosity, and zeta potential)

	Droplet size, $D[4,3]$ (μm)			Viscosity ($\text{Pa} \cdot \text{s}$)			Zeta potential (mV)		
	Coefficient	Correlation	p -value	Coefficient	Correlation	p -value	Coefficient	Correlation	p -value
Constant									
A: fish oil content, %	-13.1225	+	0.0403	-3.9689	+	0.0009	-5.2888	+	0.4081
B: NaCas + NaAlg, %	-54.4286	+	0.3218	-11.2089	+	0.0037	-61.0893	+	0.0065
C: ratio NaCas to NaAlg	135.4060	-	0.0431	-4.9922	-	0.0635	46.2969	-	0.3783
AA	0.1187	+	0.0987	0.0316	+	0.1214	0.0331	+	0.0708
AB	1.4571	+	0.1298	0.4582	+	0.1061	1.0393	+	0.0034
AC	-2.5687	-	0.0147	0.0222	+	0.9175	-0.6500	-	0.0134
BB	-2.9252	-	0.8165	-0.1913	-	0.9581	2.1684	+	0.4958
BC	-12.0536	-	0.2842	-5.8527	-	0.1005	-3.3929	-	0.2301
CC	12.7604	+	0.2222	5.2207	+	0.1059	-0.5664	-	0.8122
R^2		88.88			95.09			93.41	

Statistical significance was considered at $p < 0.05$.

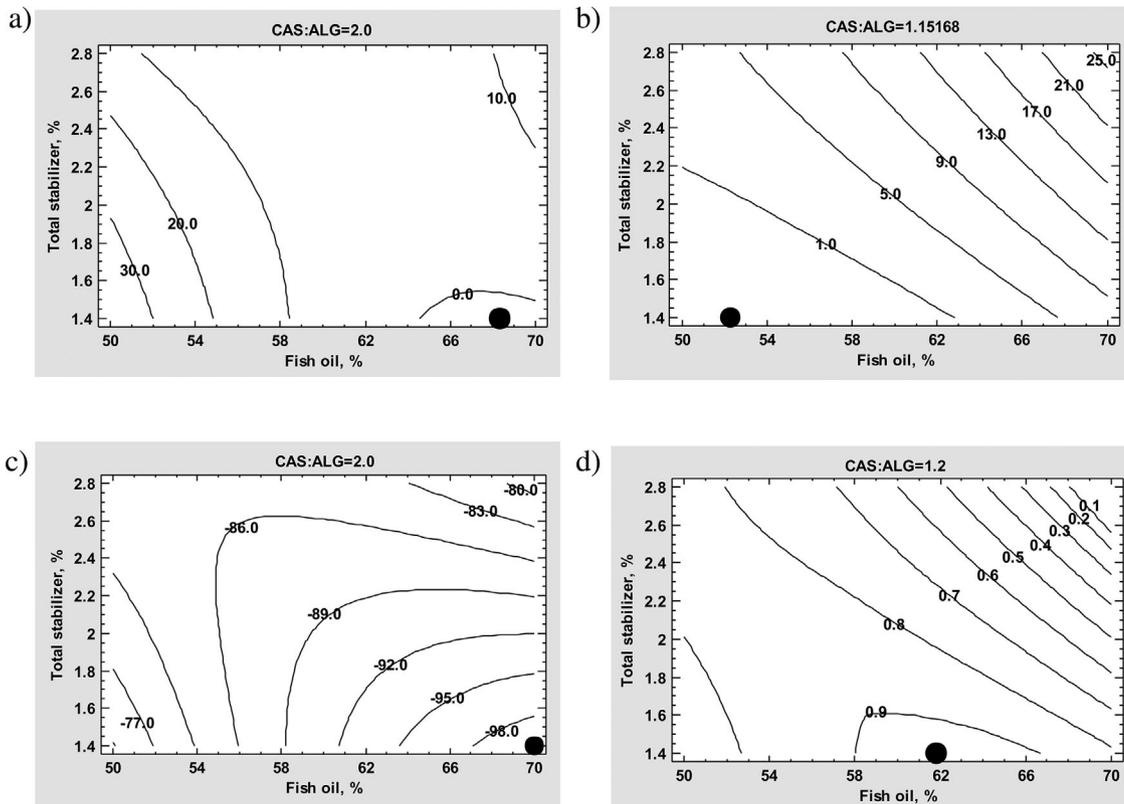


Figure 1. Contour plots of estimated response surface for output variables; (a) droplet size ($D_{4,3}$ in μm), (b) apparent viscosity at 20 s^{-1} in $\text{Pa}\cdot\text{s}$; (c) zeta potential in mV ; and (d) desirability – estimated multiple response surface for viscosity, droplet size, and zeta potential. Contour plots were created by fixing one of the factors, which had the least significant effect on the physical parameters. CAS:ALG ratio was fixed at its calculated optimum value which was suggested to obtain the most desired value for the specific physical parameter. Data are shown at the optimum values of CAS:ALG. Optimum recipes were shown with a black dot (●) on the graphs.

3.2.2 Apparent viscosity

Viscosity was measured at different time points during the storage of the emulsions. However, only at week 4 (last time point) all emulsions were measured at the same shear rate (20 s^{-1}), whereas at earlier time points the emulsions were measured at different shear rates. Thus, results at week 4, which show the rheological behavior of the stable and unstable emulsions at the end of the storage, are shown in Table 1 for comparison among emulsions. Apparent viscosities of the emulsions varied from 0.4 to $20.9\text{ Pa}\cdot\text{s}$ (Table 1) and all the emulsions were non-Newtonian and showed shear thinning behavior. The ANOVA analysis showed that the viscosity of emulsions increased significantly with increasing total fish oil and total stabilizer content, whereas the ratio NaCas:NaAlg was not significant (Table 2). Nonetheless, it was observed for 50% o/w emulsions with a total emulsifier content of 2.1% that increasing the ratio between NaCas and NaAlg from 0.4 to 2.0 led to a lower viscosity due to the thickening effect of NaAlg, as also pointed out by Antonov et al. [29].

The model obtained for viscosity had a high coefficient of correlation ($R^2 = 95.09$). Using RSM, a minimum for viscosity was found when fish oil content was 52.2%, total stabilizer was 1.4%, and ratio of NaCas to NaAlg was 1.2 (Fig. 1b). A minimum viscosity was pursued since the impact of delivery emulsion would be minimized on textural properties of the final product. When optimum values for viscosity were compared with droplet size, both have the same total stabilizer content as 1.4%; however, fish oil content and ratio between stabilizers differs.

3.2.3 Zeta potential

Both NaCas and NaAlg have negative charge at pH 7 [28, 29]. Zeta potential values of emulsions were all negatively charged and ranged between -97.3 and -72.2 mV as shown in Table 1. Zeta potential became significantly less negative with increasing total fish oil and total stabilizer contents (Table 2). The interaction between fish oil content and the ratio between NaCas and NaAlg had a significant negative effect on zeta potential, which means that zeta potential became less negative when the interaction of

these two input variables decreased. NaAlg was more negatively charged compared to NaCas, which confirms that the increase in the NaCas and NaAlg ratio decreased zeta potential (not significant) meaning that the charge will be less negative at the water/oil interface.

The model obtained had a high coefficient of correlation ($R^2 = 93.41$). In order to obtain more negative zeta potential, which denotes a higher repulsion within droplets, optimum values for fish oil content, total stabilizer, and ratio of NaCas to NaAlg were calculated as 70%, 1.4%, and 2, respectively (Fig. 1c).

3.2.4 Multiple response optimization of physical parameters

Each output variable was set to have the equal impact and weight when the desirability function was calculated. MRO provided joint optimization results for all physical parameters including droplet size, viscosity, and zeta potential as 61.8% for fish oil content, 1.4% for total stabilizer, and 1.2 for the ratio of NaCas to NaAlg (Fig. 1d). Optimum total stabilizer amount was found the same for the individual optimization of each physical parameter as 1.4%, which was also confirmed by MRO. Optimum fish oil content varied for each output variable and multiple optimization gave 61.8% as the optimum, which is close to the middle condition (60%). The optimum ratio between NaCas and NaAlg was 2 for droplet size and zeta potential, whereas it was 1.2 for viscosity. MRO also suggested 1.2 as optimum NaCas:NaAlg ratio for the simultaneous optimization of the three physical parameters.

3.3 Lipid oxidation in the emulsions

3.3.1 Peroxide value

Peroxide values (PV) were very low (<0.93 meq peroxides/kg oil) and reached their top values in week 2 for most of the emulsions, except emulsion 50-1.4-1.2 and 50-2.8-1.2 for which PV continued to increase during the 3rd and 4th weeks, respectively (Fig. 2). The decrease after week 2 could

be explained by formation of secondary oxidation products. Increase in fish oil content provided better oxidative stability when 70-1.4-1.2 (0.32 ± 0.05 meq peroxides/kg oil) and 50-1.4-1.2 (0.71 ± 0.07 meq peroxides/kg oil) or 70-2.1-2 (0.18 ± 0.01 meq peroxides/kg oil) and 50-2.1-2 (0.35 ± 0.09 meq peroxides/kg oil) were compared after their storage of 4 weeks (Fig. 2). This might be due to the better physical stability observed when the fish oil content was increased which promoted smaller droplet size and higher viscosity. Number of lipid molecules per droplet decreases when the droplet size becomes smaller which could limit propagation chain reactions [27]. For all emulsions, low PVs might be explained by the metal chelating properties of NaCas [10, 11, 25, 31] and radical scavenging and metal chelating activities of NaAlg [32]. Horn et al. [9] showed that emulsions stabilized with 2.8% of NaCas at pH 7.0 had 3.9 meq peroxides/kg oil after 42 days of storage. Comparison of the results by Horn et al. [9] with the present data, showed that emulsions stabilized with different combinations of NaCas and NaAlg had a lower PV (<0.93 meq peroxides/kg oil) when compared to 70% fish oil-in-water emulsion stabilized only with NaCas (2.8%) after 28 days of storage (≈ 2 meq peroxides/kg oil). This might be explained with the synergistic effect of both stabilizers on improving oxidative stability as well as physical stability.

3.3.2 Volatile compounds

2-ethylfuran, 1-penten-3-one, 1-penten-3-ol, hexanal, heptanal, (E,E)-2,4-heptadienal and nonanal are volatile secondary oxidation products that were identified in emulsions 70-1.4-1.2, 50-2.8-1.2, 50-2.1-0.4, 70-2.1-2, 60-1.4-0.4, 60-1.4-2, and the three center points of the experimental design (60-2.1-1.2). These emulsions were selected since they were physically stable in terms of creaming until the end of the storage (data not shown). However, the amounts of these volatile oxidation products were quite low (data not shown), indicating together with the PV results that the emulsions were oxidatively stable. Figure 3 shows the volatile compounds (1-penten-3-ol, heptanal and nonanal), which were present in higher amounts compared to other

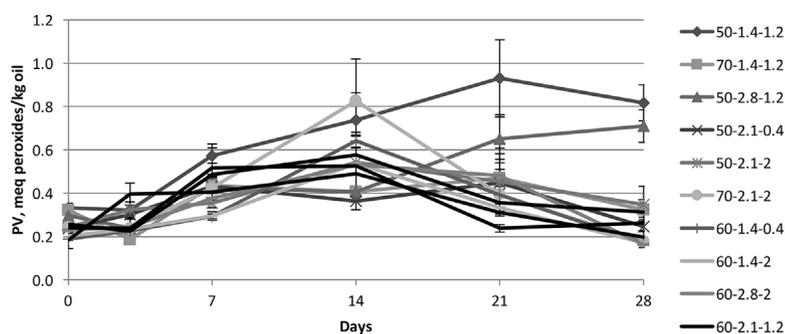


Figure 2. Peroxide value of emulsions during their storage time for 4 weeks.

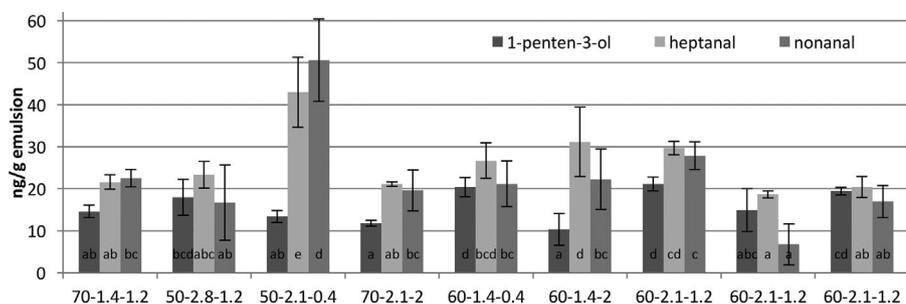


Figure 3. Volatile compounds (1-penten-3-ol, heptanal and nonanal) formed after 4 weeks of storage.

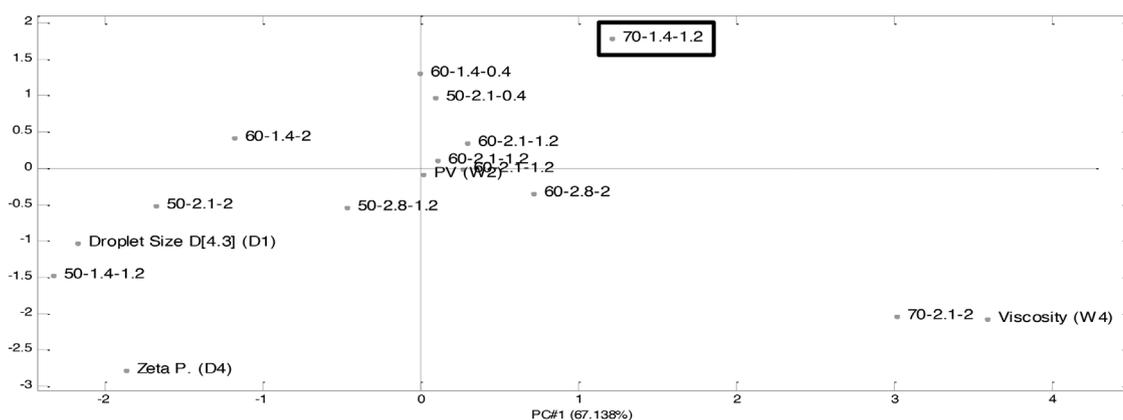


Figure 4. PCA bi-plot plotted using the results from Table 1.

volatile compounds in the emulsions after 4 weeks of storage at room temperature. Heptanal and nonanal contents were found as 43.0 and 50.6 ng/g emulsion, respectively, for the emulsion 50-2.1-0.4 (0.6% NaCas + 1.5% NaAlg) which had significantly higher values than the other emulsions. These findings are in agreement with Horn *et al.* [9], who reported heptanal content between 40 and 55 ng/g emulsion for 70% fish oil-in-water emulsions stabilized with 2.8 and 1.4% of NaCas at pH 7 and stored during 42 days at room temperature. The formation of other volatiles such as pentanal, (E,Z)-2,4-heptadienal and (E,E)-2,4-heptadienal were also reported [9]. Even though the storage time was shorter for 50-2.1-0.4, heptanal formation was higher compared to 2.8% NaCas and 70% fish oil-in water emulsion. This could be due to the lower fish oil and NaCas contents which caused less physical stability. Lower levels of NaCas could also have decreased the metal chelating ability of NaCas [10, 11, 25, 27, 32].

3.4 Principal component analysis

As seen in Fig. 4, PCA was run excluding 70-2.8-1.2, 70-2.1-0.4, and 60-2.8-0.4 that did not form an emulsion structure.

Peroxide values were very low (<0.93 meq peroxides/kg oil) for all emulsions and had no effect on differentiating the emulsion samples. Volatile compounds were not included in PCA as their concentrations were very low and would not make any difference on separating emulsion samples from each other. As expected, three emulsions with the code 60-2.1-1.2 were placed close to each other since they were the center points of the Box-Behnken's design and had the same formulation.

PCA confirmed that emulsion 50-1.4-1.2, 50-2.1-2 and 60-1.4-2 had large droplets and low viscosity. Thus, these emulsions were found to be less physically stable compared to the other emulsions. Emulsion 70-2.1-2 had the highest viscosity. As seen in the PCA, 70-1.4-1.2 performed as the optimum formulation among all emulsions by giving smaller droplet size, lower viscosity, low PV, and highest negative droplet charge. This formulation was also supported by the MRO results for physical parameters, which was 61.8% for fish oil content, 1.4% for total stabilizer, and 1.2 for the ratio of NaCas to NaAlg; except for the lower fish oil content. Seventy percent of fish oil was selected as the optimum because it provided lower PV values and droplet sizes when total stabilizer and the ratio between NaCas and NaAlg were

kept the same. Based on both RSM and PCA analyses, the optimum formulation (70-1.4-1.2) was selected which was part of the experimental design. Therefore, the formulation was validated by characterization of rheology, size distribution, zeta potential, and oxidation.

4 Conclusions

The use of NaCas and NaAlg in combination allowed the production of emulsions, except when high ratio between NaAlg and aqueous phase was employed. The viscosity of the emulsions was significantly influenced by fish oil and total stabilizer content, whereas droplet size was mainly affected by fish oil content and the ratio between the two stabilizers. Moreover, zeta potential was affected by the interaction between fish oil content and both total stabilizer content and the ratio between the two stabilizers. Emulsions were found oxidatively stable during their storage of 4 weeks which confirms the protecting effect of NaCas and NaAlg as stabilizers. Different optimum formulations were obtained depending on which output variables was used for the modeling. Therefore, the optimum recipe was selected by looking at all the results together with PCA and RSM. The emulsion produced with 70% fish oil content, 1.4% total stabilizer, and 1.2 as the ratio of NaCas to NaAlg would be the optimum for enrichment of food systems with omega-3 fatty acids.

We would like to thank Arla Foods Ingredients amba and Dupont for donating sodium caseinate and sodium alginate, respectively. We would also like to thank Mariutex A/S, Norway for providing cod liver fish oil. Furthermore, we are grateful to Lis Berner and Sevda Ekici Seker for their skillful work in the laboratory. Finally, we would like to thank the Danish Council for Independent Research Technology and Production Sciences for financing the project Mapping and Characterizing of Lipid Oxidation in Emulsified Systems (MAPOX).

The authors have declared no conflict of interest.

References

- [1] Song, C., Shieh, C., Wu, Y., Kalueff, A., et al., The role of omega-3 polyunsaturated fatty acids eicosapentaenoic and docosahexaenoic acids in the treatment of major depression and Alzheimer's disease: Acting separately or synergistically? *Prog. Lipid Res.* 2016, 62, 41–54.
- [2] Wysoczański, T., Sokoła-Wysoczańska, E., Pékala, J., Lochyński, S., et al., Omega-3 fatty acids and their role in central nervous system – A review. *Curr. Med. Chem.* 2016, 23-28, 816–831.
- [3] Nichols, P. D., McManus, A., Krail, K., Sinclair, A. J., Miller, M., Recent advances in omega-3: Health benefits, sources, products and bioavailability. *Nutrients* 2014, 6, 3727–3733.
- [4] Aken G. A., in: Stephen, A. M., Phillips, G. O. (Eds.), *Food Polysaccharides and Their Applications*, CRC Press, Boca Raton, Florida 2006.
- [5] McClements, D. J., *Food Emulsions: Principles, Practice and Techniques*, CRC Press LLC, Boca Raton, Florida 1999.
- [6] Pongsawatmanit, R., Harnsilawat, T., McClements, D. J., Influence of alginate, pH and ultrasound treatment on palm oil-in-water emulsions stabilized by β -lactoglobulin. *Colloid Surf. A: Physicochem. Eng. Aspect* 2006, 287, 59–67.
- [7] Ghosh, A. K., Bandyopadhyay, P., A simple strategy for charge selective biopolymer sensing. *Chem. Commun.* 2011, 47, 8937–8939.
- [8] Ghosh, A. K., Bandyopadhyay, P., in: Karunaratne, D. N. (ed.), *The Complex World of Polysaccharides*, INTECH, Rijeka, Croatia 2012.
- [9] Horn, A. F., Nielsen, N. S., Andersen, U., Søgaard, L. H., et al., Oxidative stability of 70% fish oil-in-water emulsions: Impact of emulsifiers and pH. *Eur. J. Lipid Sci. Technol.* 2011, 113, 1243–1257.
- [10] Horn, A. F., Nielsen, N. S., Jensen, L. S., Horsewell, A., Jacobsen, C., The choice of homogenisation equipment affects lipid oxidation in emulsions. *Food Chem.* 2012, 134, 803–810.
- [11] Sosa-Herrera, M. G., Lozano-Esquivel, I. E., Ponce de León-Ramírez, Y. R., Martínez-Padilla, L. P., Effect of added calcium chloride on the physicochemical and rheological properties of aqueous mixtures of sodium caseinate/sodium alginate and respective oil-in-water emulsions. *Food Hydrocoll.* 2012, 29, 175–184.
- [12] Falkeborg, M., Guo, Z., Dodeceny succinylated alginate (DSA) as a novel dual-function emulsifier for improved fish oil-in-water emulsions. *Food Hydrocoll.* 2015, 46, 10–18.
- [13] McClements, D. J., Decker, E. A., Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *J. Food Sci.* 2000, 65, 1270–1282.
- [14] Schmitt, C., Sanchez, C., Desobry-Banon, S., Hardy, J., Structure and technofunctional properties of protein-polysaccharide complexes: A review. *Crit. Rev. Food Sci. Nutr.* 1998, 38:8, 689–753.
- [15] Dickinson, E., Interfacial structure and stability of food emulsions as affected by protein-polysaccharide interactions. *Soft Matter* 2008, 4, 932–942.
- [16] Sosa-Herrera, M. G., Berlib, C. L. A., Martínez-Padilla, L. P., Physicochemical and rheological properties of oil-in-water emulsions prepared with sodium caseinate/gellan gum mixtures. *Food Hydrocoll.* 2008, 22, 934–942.
- [17] Jourdain, L., Leser, M. E., Schmitt, C., Michel, M., Dickinson, E., Stability of emulsions containing sodium caseinate and dextran sulfate: Relationship to complexation in solution. *Food Hydrocoll.* 2008, 22, 647–659.
- [18] Evans, M., Ratcliffe, I., Williams, P. A., Emulsion stabilisation using polysaccharide-protein complexes. *Curr. Opin. Colloid Interf. Sci.* 2013, 18, 272–282.
- [19] Pallandre, S., Decker, E. A., McClements, D. J., Improvement of stability of oil-in-water emulsions containing caseinate-coated droplets by addition of sodium alginate. *J. Food Sci.* 2007, 72, Nr.9.

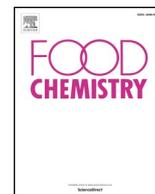
- [20] García-Moreno, P. J., Horn, A. F., Jacobsen, C., Influence of casein – phospholipid combinations as emulsifier on the physical and oxidative stability of fish oil-in-water emulsions. *J. Agric. Food Chem.* 2014, 62, 1142–1152.
- [21] Let, M. B., Jacobsen, C., Meyer, A. S., Lipid oxidation in milk, yoghurt, and salad dressing enriched with neat fish oil or pre-emulsified fish oil. *J. Agric. Food Chem.* 2007, 55, 7802–7809.
- [22] Bligh, E. G., Dyer, W. J., A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 1959, 37, 911–917.
- [23] Shantha, N. C., Decker, E. A., Rapid, sensitive, ironbased spectrophotometric methods for determination of peroxide values of food lipids. *J. AOAC Int.* 1994, 77, 421–424.
- [24] Jacobsen, C., Meyer, S. A., Adler-Nissen, J., Oxidation mechanisms in real food emulsions: Oil-water partition coefficients of selected volatile off-flavor compounds in mayonnaise. *Z Lebensm Unters Forsch A* 1999, 208, 317–327.
- [25] Myers, R. H., Montgomery, D. C., *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*, Wiley, Hoboken, New Jersey 2002.
- [26] Behrend, O., Ax, K., Schubert, H., Influence of continuous phase viscosity on emulsification by ultrasound. *Ultrason. Sonochem.* 2000, 7, 77–85.
- [27] Genot, C., Meynier, A., Riaublanc, A., in: Kamal-Eldin, A. (ed.), *Lipid Oxidation Pathways*, AOCS Press, Champaign, Illinois 2013, Chapter 7.
- [28] Capron, I., Costeux, S., Djabourov, M., Water in water emulsions: Phase separation and rheology of biopolymer solutions. *Rheol. Acta* 2001, 40, 441–456.
- [29] Antonov, Y. A., Van Puyvelde, P., Moldenaers, P., Interfacial tension of aqueous biopolymer mixtures close to the critical point. *Int. J. Biol. Macromol.* 2004 34, 29–35.
- [30] Hadnadev, T. D., Dokic, P., Krstonosic, V., Hadnadev, M., Influence of oil phase concentration on droplet size distribution and stability of oil-in-water emulsions. *Eur. J. Lipid Sci. Technol.* 2013, 115, 313–321.
- [31] Berton-Carabin, C. C., Ropers, M., Genot, C., Lipid oxidation in oil-in-water emulsions: Involvement of the interfacial layer. *Comprehen. Rev. Food Sci. Food Safety* 2014, 13, 945–977.
- [32] Falkeborg, M., Cheong, L., Gianfico, C., Sztukiel, K. M., et al., Alginate oligosaccharides: Enzymatic preparation and antioxidant property evaluation. *Food Chem.* 2014, 164, 185–194.

PAPER II

Yesiltas, B., García-Moreno, P. J., Sørensen, A. M., Akoh, C. C., Jacobsen, C., **2019.**

Physical and oxidative stability of high fat fish oil-in-water emulsions stabilized with sodium caseinate and phosphatidylcholine as emulsifiers.

Food Chemistry, 276, 110–118.



Physical and oxidative stability of high fat fish oil-in-water emulsions stabilized with sodium caseinate and phosphatidylcholine as emulsifiers



Betül Yesiltas^a, Pedro J. García-Moreno^a, Ann-Dorit M. Sørensen^a, Casimir C. Akoh^b,
Charlotte Jacobsen^{a,*}

^a Division of Food Technology, National Food Institute, Technical University of Denmark, Denmark

^b Department of Food Science and Technology, University of Georgia, Athens, GA, USA

ARTICLE INFO

Keywords:

Omega-3
O/w emulsions
Lipid oxidation
Milk protein
Phospholipids
Optimization

ABSTRACT

The physical and oxidative stability of high-fat omega-3 delivery systems such as fish oil-in-water emulsions stabilized with combinations of sodium caseinate (CAS) and phosphatidylcholine (PC) was optimized. The influence of fish oil content (50, 60 and 70%, w/w), amount of total emulsifier CAS + PC (1.4, 2.1 and 2.8%, w/w) and ratio between CAS and PC (0.4, 1.2 and 2) on physical and oxidative parameters was investigated. Creaming and droplet size significantly decreased when the amount of fish oil, total emulsifier and ratio of CAS to PC were increased. Viscosity decreased significantly with decreasing fish oil content, whereas the ratio of CAS to PC did not have a significant influence. Decreasing the ratio of CAS to PC led to emulsions with a significantly lower concentration of 1-penten-3-ol, while no significant effect was found for other volatiles such as (*E*)-2-pentenal, (*E*)-2-hexenal and (*E,E*)-2,4-heptadienal.

1. Introduction

Marine long chain (LC) omega-3 polyunsaturated fatty acids (PUFAs) have been reported to have numerous beneficial effects on health such as decreasing cardiovascular diseases, improving immune system and mental health (Song et al., 2016; Wysoczanski et al., 2016; Nichols, McManus, Krail, Sinclair, & Miller, 2014). LC omega-3 PUFAs are mainly found in fish and fish products. However, the average daily intake of fish products in Western societies does not reach the required levels to obtain positive impacts on health (Papanikolaou, Brooks, Reider, & Fulgoni, 2014). Therefore, omega-3 enriched foods have gained increasing attention from consumers who want to maintain a balanced diet. Accordingly, the food industry has looked for new product opportunities and technologies for producing omega-3 enriched foods. Nevertheless, omega-3 PUFAs are highly prone to oxidation, which negatively affects taste, smell and texture of omega-3 enriched food products and decreases consumers' acceptance (Kolanowski, Jaworska, & Weißbrodt, 2007; Bermudez-Aguirre & Barbosa-Canovas, 2011).

Fish oil-in-water emulsions are one of the delivery systems that have been used for delivering LC omega-3 PUFAs, while providing protection

against oxidation if the emulsion recipe is optimal. Many studies have been performed on physical and oxidative stability of low fat (5–30%) emulsions (McClements, Decker & Weiss, 2007; Waraho, McClements, & Decker, 2011). Nevertheless, only a few studies have been reported on high fat (50–70%) emulsions (Horn et al., 2011; Horn, Nielsen, & Jacobsen, 2012; Yesiltas, García-Moreno, Sørensen, & Jacobsen, 2017). High fat delivery emulsions with high viscosity are preferred for enrichment of food systems with high oil content (e.g., mayonnaise, dressings and cream cheese), since their incorporation will have a reduced impact on the texture of the product. Moreover, a lower amount of high fat emulsions would be required for a certain level of food enrichment compared to low fat emulsions. If viscosity of high fat emulsions can be decreased they may also be applicable to low-viscous products (e.g. milk, soft-drinks, and juices), while reducing possible negative effects on the texture and rheology of the final product.

Emulsifiers, amongst others, are used for decreasing the interfacial energy between oil and water molecules by being adsorbed at the interface, which makes emulsion energetically less unfavorable (McClements, 1999). Understanding the structure, composition and mechanical properties of the interface seems essential for controlling the physico-chemical stability properties of food emulsions. There have

Abbreviations: CAS, sodium caseinate; DHA, docosahexaenoic acid; DHS, dynamic head space; EPA, eicosapentaenoic acid; PC, phosphatidylcholine; PV, peroxide value; RSM, response surface methodology

* Corresponding author at: National Food Institute, Technical University of Denmark, Kemitorvet, Building 204, 2800 Kgs. Lyngby, Denmark.

E-mail addresses: betye@food.dtu.dk (B. Yesiltas), pejeg@food.dtu.dk (P.J. García-Moreno), adms@food.dtu.dk (A.-D.M. Sørensen), cakoh@uga.edu (C.C. Akoh), chja@food.dtu.dk (C. Jacobsen).

<https://doi.org/10.1016/j.foodchem.2018.09.172>

Received 26 April 2018; Received in revised form 5 September 2018; Accepted 29 September 2018

Available online 01 October 2018

0308-8146/ © 2018 Elsevier Ltd. All rights reserved.

Table 1
Experimental design, droplet size and viscosity of emulsions.

Emulsion Code ^a	Fish oil, %, w/w	CAS + PC, %, w/w	CAS:PC, Ratio	Droplet Size D[4,3] (µm)		Viscosity (mPa·s @ 20 s ⁻¹)	
				Day 1	Day 12	Day 1	Day 12
50-1.4-1.2	50	1.4	1.2	20.68	24.02	17.76	17.63
50-2.1-0.4	50	2.1	0.4	21.19	24.63*	18.31	16.19
50-2.1-2	50	2.1	2.0	18.94	20.12*	26.42	24.27
50-2.8-1.2	50	2.8	1.2	15.95	17.66*	30.86	29.28
60-1.4-0.4	60	1.4	0.4	20.80	24.44*	35.74	33.06
60-1.4-2	60	1.4	2.0	18.72	20.05	52.14	47.83
60-2.1-1.2	60	2.1	1.2	16.04	16.07	75.14	69.07
60-2.1-1.2	60	2.1	1.2	15.42	15.74	72.97	68.85
60-2.1-1.2	60	2.1	1.2	16.40	14.90*	71.08	67.30
60-2.8-0.4	60	2.8	0.4	15.47	16.44	78.23	66.32
60-2.8-2	60	2.8	2.0	10.61	10.80	107.07	97.59
70-1.4-1.2	70	1.4	1.2	13.02	13.96	446.24	436.10
70-2.1-0.4	70	2.1	0.4	11.84	12.56*	522.70	418.16*
70-2.1-2	70	2.1	2.0	6.73	8.17*	968.75	903.25
70-2.8-1.2	70	2.8	1.2	6.31	6.62	1132.21	1107.18

n = 2 for the droplet size and viscosity measurements.

Relative standard deviations (RSDs, %) for droplet size were lower than 10% except for the value 24.02 which had RSD as 21%. RSDs for viscosity results were lower than 8.5% except for the values 78.23 and 17.63 which had RSDs as 10.75 and 14.12%, respectively.

* Significant changes in samples between days 1 and 12 (p < 0.05).

^a Code represents amount of fish oil, CAS + PC and CAS:PC, respectively. CAS = sodium caseinate, PC = phosphatidylcholine.

been many studies, which reported that combined use of emulsifiers provided advantages in terms of improving physical and oxidative stability of emulsions compared to single use of an emulsifier (Fang & Dalgleish, 1996; Sünder, Scherze, & Muschiolik, 2001; Gao et al., 2017).

Sodium caseinate (CAS) is a protein product, which is commonly used as an emulsifier in food products. Horn et al. (2012) reported that CAS provides a good physical barrier at the oil-water interface, which prevents prooxidant (e.g. metal ions) permeation from water phase to oil phase. Additionally, CAS showed metal chelating activity both at the oil-water interface and in the water phase (Diaz, Dunn, McClements, & Decker, 2003; Horn, Barouh, Nielsen, Baron, & Jacobsen, 2013). However, high fat emulsions produced with protein-based emulsifiers have high viscosity, which might hamper the usage of emulsions in food enriched applications (Horn et al., 2011). Thus, combined usage of proteins and phospholipids was applied as a strategy for reducing the viscosity of the emulsions (García-Moreno, Horn, & Jacobsen, 2014).

Phosphatidylcholine (PC) is a zwitterionic phospholipid extracted from egg or soybean, which is widely used as an emulsifier in oil-in-water emulsions (García-Moreno et al., 2014; Scuriatti, Tomás, & Wagner, 2003; Sui et al., 2016; Magnusson, Nilsson, & Bergenstål, 2016). Furthermore, findings of previous researchers indicated that PC showed antioxidant activity by donating protons to stabilize radicals, stabilizing peroxy radicals to yield stable compounds (e.g. trimethylammonium oxide) and chelating metals due to the amino group (García-Moreno et al., 2014; Berton-Carabin, Ropers, & Genot, 2014; Bandarra, Campos, Batista, Nunes, & Empis, 1999). Nevertheless, phospholipids as a sole emulsifier have been reported to cause an insufficient physical stability and result in phase separation (Sznitowka, Janicki, Dabrowska, & Zurowska-Pryczkowska, 2001). Therefore, some studies have suggested the use of PC or lecithin (mainly containing PC) together with other emulsifiers such as sodium caseinate or whey protein in order to increase emulsion stability by the formation of a complex interfacial film, which potentially helps to improve the physical and oxidative stability of emulsions (Fang & Dalgleish, 1996; García-Moreno et al., 2014; Sünder et al., 2001; Xue & Zhong, 2014). However, there are no studies that used a systematic approach with a statistical experimental design such as Box-Behnken to study the combined effects of PC and CAS in high fat oil-in-water emulsions.

In this study, we hypothesize that using optimum combinations of

CAS and PC as emulsifiers will allow the obtainment of physically and oxidatively stable high-fat omega-3 delivery emulsions. This is due to the antioxidant capacity that both emulsifiers can exhibit at the interface and in the aqueous phase as well as on the potential formation of an interface with enhanced structural properties. Moreover, the use of CAS and PC combinations, which implies a reduction in the amount of CAS used, may lead to high fat fish oil-in-water emulsions with a reduced viscosity, which is desired for an easy application in a wide range of products. Box-Behnken's experimental design and response surface methodology (RSM) were used to investigate the effects of fish oil content, total CAS and PC content, and the ratio of CAS to PC on the physical and oxidative stability of the high-fat emulsions.

2. Materials and methods

2.1. Materials

Cod liver oil was provided by Maritex A/S, a subsidiary of TINE, BA (Sortland, Norway), and stored at -40 °C until use. The fatty acid (% of total fatty acids) content of the fish oil as determined and supplied by the manufacturer was as follows: C14:0 (3.02), C16:0 (8.91), C16:1n-7 (8.20), C18:0 (1.88), C18:1n-9 (16.00), C18:1n-7 (5.16), C18:2n-6 (1.79), C18:3n-3 (0.84), C20:1n-9 (11.59), C20:5n-3 (9.27), C22:1n-11 (6.06), C22:6n-3 (11.64) and other fatty acids (15.64). Phosphatidylcholine (PC) extracted from soybean (LIPOID S100) was kindly provided by Lipoid GmbH, Germany. Analysis of certificate of the Lipoid S100 reported that 97.1% of the product is phosphatidylcholine (based on dry weight). There is 0.20% DL- α -Tocopherol, which is an antioxidant. Other lipid molecules were also reported as follows: 1.2% lysophosphatidylcholine, 0.5% N-acyl-phosphatidylethanolamine, lower than 0.1% phosphatidylethanolamine, lower than 0.1% phosphatidylinositol, 0.8% non-polar lipids, and 0.3% triglycerides. PC had initial peroxide value (PV) of 1.58 meq/kg oil and its fatty acid (in % of total fatty acids) composition was as follows: 16:0 (13.42), 18:1n-9 (10.97), 18:2n-6 (62.61), 18:3n-6 (6.96), and others (5.51). Initial PV of the fish oil was 0.12 meq peroxide/kg oil. Sodium caseinate (Miprodan 30) was provided by Arla Foods Ingredients a.m.b.a (Viby J, Denmark). Protein content of sodium caseinate was reported as 92% (N x 6.38).

2.2. Emulsion preparation and sampling

Different amounts of CAS and PC (see Table 1) were dissolved/dispersed in distilled water and stirred overnight at 500 rpm at 4 °C. The pH of the aqueous phases was adjusted to 7.0 using 2 M HCl or 2 M NaOH. Emulsions (500 g) were produced using a Stephan Universal mixer (Stephan, UMC5, Hameln, Germany) which was equipped with an emulsification blade as described by Horn et al (2011). Fish oil concentration at three different levels (50, 60 and 70%, w/w of total emulsion), total emulsifier content (CAS + PC) at three different levels (1.4, 2.1 and 2.8%, w/w of total emulsion) and ratio of CAS to PC at three different levels (0.4, 1.2 and 2.0, w/w) were the three factors studied. A Box-Behnken's experimental design was carried out with fifteen emulsions in total, including 3 replicates of the central point (see Table 1). Fe²⁺ (0.03% FeSO₄·7H₂O dissolved in water, corresponding to approx. 100 μM in the final emulsion) and 0.05% sodium azide were added into the emulsions in order to accelerate oxidation and prevent microbial growth, respectively, right after the emulsions were produced. For the storage, each emulsion was divided into portions of 85 g and placed in 100 mL blue cap bottles. There was one bottle for each sampling day. Emulsions were stored for 12 days at room temperature in darkness and sampling was performed on days 0, 2, 5, 8 and 12. Peroxide value and tocopherols were analyzed at all sampling points. All samples for analysis of oxidation parameters were taken from blue capped bottles and put in brown colored bottles where they were stored at –40C until analysis. Emulsions were stirred with a spoon before sampling.

2.3. Characterization of emulsions

2.3.1. Droplet size

Particle size of the emulsions was measured on days 1 and 12 by laser diffraction (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). The method used was described by Let, Jacobsen and Meyer (2007) and Horn et al. (2011). Samples were collected directly from the bottles where the emulsions were stored. There were separate bottles for each sampling day. Right before sampling, emulsion was mixed with a spoon and samples were collected. Volume weighted mean diameter of the samples were calculated according to the Eq. (1):

$$D[4, 3] = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \quad (1)$$

Measurements were carried out in duplicates.

2.3.2. Apparent viscosity

Apparent viscosity was measured on days 1 and 12 using a stress-controlled rheometer (Stresstech, Reologica Instruments AB, Lund, Sweden). CC25 standard bob cup system was used and samples were placed in a vessel which was set at 25 °C. Shear stress range was set from 0.0125 to 50 Pa for each measurement. Apparent viscosity results were obtained at a shear rate of 20 s⁻¹ and expressed in mPa·s. Measurements were carried out in duplicates.

2.3.3. Creaming index

Creaming index (CI) was followed on days 1, 5, 8, 12 in the 100 mL blue cap bottles. CI was calculated using Eq. (2):

$$CI(\%) = (b/a) \times 100 \quad (2)$$

where (a) is the height of total emulsion and (b) is the height of water phase separated at the bottom of the bottle. CI was calculated as in percentage for each emulsion sample for each sampling point without replicates.

2.4. Lipid oxidation in emulsions

2.4.1. Primary oxidation products – peroxide value (PV)

Primary oxidation products were determined by preparing a lipid extract which was previously described by Bligh and Dyer (1959). Five grams of emulsion and a reduced amount of solvent (60 mL of methanol and chloroform, 1:1; v/v) were used for each extraction. PV was subsequently performed on the lipid extracts according to IDF standard method by colorimetric determination of iron thiocyanate on a spectrophotometer (Shimadzu, UV mini 1240, Kyoto, Japan) at 500 nm (Shantha & Decker, 1994). All measurements were carried out in duplicates.

2.4.2. Tocopherol content – high pressure liquid chromatography (HPLC)

Tocopherol contents of emulsions were prepared using lipid extracts (see Section 2.4.1), which were further evaporated and re-dissolved in heptane and then analyzed by HPLC (Agilent 1100 Series, Waldbronn, Germany; Column: Waters Spherisorb 3 μm Silica; 4.6 x 150 mm, MA, USA). Analysis was carried out according to the official AOCS method (1998) in duplicates.

2.4.3. Secondary volatile oxidation products – dynamic headspace (DHS) GC-MS

Secondary volatile oxidation products were evaluated on days 0 and 12 according to the method described by Jacobsen, Meyer and Adler-Nissen (1999). Emulsion sample (4 g) was mixed with 2 mL antifoam and 10 mL distilled water in a 100 mL purge bottle which was heated in a water bath at 60 °C while purging with nitrogen (flow 150 mL/min, 30 min). Volatile compounds present in the emulsion samples were trapped on Tenax GR tubes and separated in a gas chromatograph (Agilent Technologies, 6890N Network GC System, DE, USA) on a 30 m DB 1701 fused silica capillary column (0.25 mm i.d., 1 μm film thickness, Agilent Technologies, J&W GC Columns, CA, USA). The oven program had an initial temperature of 45 °C for 5 min, increasing at 1.5 °C/min until 55 °C, then at 2.5 °C/min until 90 °C, and at 12 °C/min until 220 °C, where the temperature was held for 4 min. Mass spectrometry (Agilent 5973 Network Mass Selective Detector, Agilent Technologies, electron ionization mode, 70 eV; mass to charge ratio scan between 30 and 250) was used for the analysis of each individual volatile compound and identified by MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard). Quantification was performed using calibration curves which were prepared by injecting external standards (2-ethyl-furan, 1-penten-3-one, 1-penten-3-ol, (E)-2-pentenal, hexanal, (E)-2-hexenal, (Z)-4-heptenal, 2-pentyl-furan, (E)-2-heptenal, benzaldehyde, (E,E)-2,4-heptadienal, nonanal and (E,Z)-2,6-nonadienal) directly on the Tenax GR tubes. Measurements were made in triplicate on each sample. Results were expressed in ng/g emulsion.

2.5. Box-Behnken's experimental design, RSM and statistical analysis

A 3-factor Box-Behnken design is a RSM design, which includes sets of runs in which pair of factors is varied between their low (–1) and high (+1) levels, while the other experimental factor is held at the medium (0) level. Three center-points (0, 0, 0) are added to the design in order to check the differences between replicates. In this study, 3 different factors (fish oil content, total content of CAS + PC and the ratio between CAS and PC) were included at 3 different levels, as shown in Table 1.

Statgraphics XVII (Statpoint Technologies, Inc., Virginia, USA) was used to generate the statistical analysis and the regression models for the physical and oxidative parameters of the emulsions. Firstly, the output variables (Y: viscosity at day 1, droplet size at day 1, creaming at day 12, peroxide values at day 12 and volatile compounds at day 12) were related to the input variables (X: fish oil content, total content of CAS plus PC and ratio between CAS and PC) by second degree polynomials as follows, Eq. (3):

$$Y = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i < j}^3 b_{ij} X_i X_j \quad (3)$$

where the coefficients b_i and b_{ii} are related to the linear and quadratic effects, respectively, of each input factor on the output variable and the cross-product coefficients b_{ij} represent the interactions between two input variables. Coefficient b_0 indicates the mean change in viscosity, droplet size and creaming, primary oxidation product formation, etc., when the fish oil content, total emulsifier content, and ratio between emulsifiers are 0. Secondly, the analysis of variance (ANOVA) was carried out using Fisher's least significant difference test. The significance of all terms in the models was judged statistically by computing the p -value at a confidence level $1-\alpha = 95\%$. The quadratic models were then used to generate contour maps and to find the optimal values of the input variables, which maximize the quality of the emulsions in terms of physical and oxidative stability, by using RSM (Myers, Montgomery, & Anderson-Cook, 2002).

3. Results and discussion

3.1. Characterization of emulsions

3.1.1. Droplet size

Droplet size results were significantly affected by the linear effect of all input variables studied (fish oil content, total emulsifier amount and ratio of CAS to PC) as well as the quadratic effect of fish oil content (Table 2). Droplet size at day 1, with $D[4,3]$ values ranging from 6.31 to 21.19 μm , decreased with increasing fish oil content, total emulsifier content and ratio of CAS to PC (Table 1). García-Moreno et al. (2014) reported that 10% fish oil-in-water emulsion stabilized with 0.3% w/w CAS and 0.5% w/w PC had a $D[3,2]$ value of 210 nm. Another study on 20% oil-in-water emulsions stabilized with 0.5% w/w CAS and 0.5% w/w egg-PC emulsifiers showed an average droplet size of 320 nm (Fang & Dalgleish, 1996). The larger droplet sizes observed in our study compared to these studies were most likely due to the total emulsifier content and the ratio between total emulsifier and fish oil content, as well as homogenizer type.

Droplet sizes of 50-2.1-0.4, 50-2.1-2, 50-2.8-1.2, 60-1.4-0.4, 70-2.1-0.4, and 70-2.1-2 significantly increased after 12 days of storage. The rest of the emulsions were stable in terms of droplet size during 12 days storage (Table 1). Droplet size decreased 2–3 folds for emulsions produced with 70% fish oil content compared to 50%, when having the same amount of emulsifier content and ratio of CAS to PC (e.g. emulsions 50-1.4-1.2 vs 70-1.4-1.2 or 50-2.1-2 vs 70-2.1-2) (Table 1). In these emulsions, the decrease in the droplet size could be due to the increase in the dispersed phase volume fraction from 50% to 70%, which increased the viscosity of the whole emulsion. Higher viscosity occurs due to a high volume fraction of dispersed phase, so that droplet packing itself gives rise to higher viscosity. This high volume occupancy of the dispersed phase not only led to a high viscosity, but also allowed the mixer blade to perform better in disruption of the oil droplets during homogenization. Moreover, Hadnadev, Dokic, Krstonosic, & Hadnadev (2013) also reported that increased oil concentration resulted in increased specific surface area at 20% emulsifier concentration. As expected, droplet size decreased when the total amount of emulsifiers were increased while oil content and ratio between CAS to PC were kept constant (see values for emulsions 50-1.4-1.2 and 50-2.8-1.2, 60-1.4-2 and 60-2.8-2, or 70-1.4-1.2 and 70-2.8-1.2 in Table 1). This was due to the higher amount of emulsifier available during the emulsion production, which resulted in smaller droplets. The difference between droplet sizes of 50-1.4-1.2 and 70-2.8-1.2 was even larger ($D[4,3]$ values of 20.68 and 6.31 μm , respectively), which supports the mutual effect of increasing both fish oil (from 50 to 70%) and total emulsifier (from 1.4 to 2.8%) contents.

Ratio of CAS to PC also had a significant effect on decreasing droplet size, with a negative correlation (Table 2). This may be attributed to a

superior emulsifying ability of CAS. Moreover, the higher amount of CAS led to smaller droplet sizes due to the higher amount of CAS available during the droplet disruption in the mixer. Results indicated that, when the ratio of CAS to PC was increased, the decrease in droplet size was more pronounced when fish oil content was increased (Table 1). For instance, a reduction in $D[4,3]$ from 21.19 to 18.94 μm was observed for samples 50-2.1-0.4 and 50-2.1-2, whereas a more pronounced reduction in $D[4,3]$ (from 11.84 to 6.73 μm) was found for samples 70-2.1-0.4 and 70-2.1-2 (Table 1). As explained above, this added effect was also attributed to higher viscosity of the system when the amount of dispersed phase was increased.

The obtained quadratic model adequately explained the experimental data ($R^2 = 0.9896$, Table 2). Thus, optimization was carried out using this model in order to find the optimal values for the input variables that minimized droplet size. A smaller droplet size is more favorable in high fat emulsions as it increases the physical stability leading to less creaming compared to larger droplet sizes. The optimal recipe suggested by RSM was 70% fish oil, 2.8% total emulsifier and a ratio of CAS to PC of 2 (Table 3, Supplementary Fig. 1a), which correlates well with the significant effect of each input variable and their negative correlation with droplet size.

3.1.2. Apparent viscosity

All emulsions were non-Newtonian and showed shear thinning behavior. Emulsions were stable in terms of viscosity, showing similar values of apparent viscosity at 20 s^{-1} on day 1 and 12. There was only one emulsion (70-2.1-0.4) that had a significant decrease in viscosity during 12 days of storage (Table 1). At day 1, apparent viscosity of samples was significantly influenced by the linear and quadratic effects of fish oil content and the interaction between fish oil content and total emulsifier. On the other hand, linear and quadratic effects of total emulsifier content and the ratio between emulsifiers as well as the other interactions between factors did not have a significant effect on viscosity (Table 2). Apparent viscosity of samples increased with increasing fish oil content. Emulsions prepared with 50 and 60% of fish oil had apparent viscosities from 17.76 to 107.07 mPa·s, while emulsions with 70% fish oil content gave apparent viscosities between 446.24 and 1132.21 mPa·s on day 1 (Table 1). This might be due to the lower droplet size of emulsions containing higher amount of oil. Smaller droplets led to more friction between oil droplets, which were caused by expanded surface-to-volume ratio of the dispersed phase. It resulted in less mobility of the droplets in the emulsion and therefore higher viscosity compared to emulsions having larger droplets (Pal, 1996).

Emulsions with high viscosity are preferred in order to reduce creaming, as a consequence of a reduced mobility of oil droplets in the water phase. However, high viscosity might hamper applications of delivery emulsions in liquid-based food systems (e.g. milk, soft-drinks). Therefore, optimal level of viscosity needs to be set depending on the food matrix to which the emulsion will be added.

Optimization of viscosity was carried out by means of the quadratic model obtained, which explained the experimental data to a large extent ($R^2 = 0.9637$, Table 2). However, lack-of-fit test indicated that the selected model did not adequately describe the observed data (p -value 0.0015). According to RSM results, the optimum recipe to obtain minimum viscosity was 59% of fish oil content, 1.4% total emulsifier and 0.4 ratio of CAS to PC (Table 3, Supplementary Fig. 1b). To maximize viscosity, RSM suggested fish oil content, total emulsifier content and ratio of CAS to PC as 70%, 2.8% and 2, respectively.

3.1.3. Creaming

Increasing fish oil content, total emulsifier content and the ratio of CAS to PC significantly increased creaming stability (Fig. 1, Table 2). Particularly, the effect of fish oil content on creaming was highly significant ($p < 0.000$). Emulsions produced with 50% fish oil showed creaming between 25 and 30% already at the first day of the storage, whereas emulsions with 60% and 70% fish oil contents had 5–17% and

Table 2
Polynomial coefficients and p-values for the response variables (creaming, droplet size, viscosity, peroxide value and volatile compounds).

	Creaming			Droplet size			Viscosity			Peroxide value		
	Coefficient	Correlation	p-value	Coefficient	Correlation	p-value	Coefficient	Correlation	p-value	Coefficient	Correlation	p-value
Constant	55.7292			-27.5523			13.2255			-12.9505		
A: fish oil content, %	0.2875	-	0.0000	1.9830	-	0.0000	-0.4191	+	0.0003	0.3504	-	0.9566
B: CAS + PC, %	0.8929	-	0.0008	2.3107	-	0.0001	-1.3875	+	0.0602	5.8232	-	0.4903
C: ratio CAS to PC	0.1562	-	0.0056	3.6125	-	0.0015	-0.7172	+	0.1863	2.3047	+	0.9628
AA	-0.0108	-	0.0113	-0.0184	-	0.0072	0.0032	+	0.0029	-0.0019	-	0.4267
AB	-0.1786	-	0.0054	-0.0707	-	0.2762	0.0239	+	0.0337	-0.0446	-	0.2080
AC	-0.0937	-	0.0372	-0.0894	-	0.1378	0.0137	+	0.1150	-0.0200	-	0.4925
BB	1.3605	+	0.0611	-0.2432	-	0.7888	0.0196	+	0.8795	-0.7440	-	0.1661
BC	1.7857	+	0.0132	-1.2411	-	0.1469	0.0089	+	0.9343	-0.1518	-	0.7104
CC	0.2604	+	0.5736	0.8841	+	0.2373	-0.0202	-	0.8383	-0.3236	-	0.3997
R ²		0.9992			0.9896			0.9637			0.5863	
Lack-of-fit		0.6148			0.2160			0.0015			0.1201	
Standard Error of Estimation		0.5774			0.4957			0.0058			0.1955	
Mean absolute error		0.2556			0.4218			0.0542			0.2191	

	1-penten-3-ol			(E)-2-pentenal			(E)-2-hexenal			(E,E)-2,4-heptadienal		
	Coefficient	Correlation	p-value	Coefficient	Correlation	p-value	Coefficient	Correlation	p-value	Coefficient	Correlation	p-value
Constant	-80.0401			30.8216			21.8762			92.2761		
A: fish oil content, %	-1.0169	+	0.0033	-1.3090	+	0.013	-1.8046	+	0.0042	-3.6504	+	0.0458
B: CAS + PC, %	92.4518	+	0.4557	15.0393	-	0.3016	32.6768	-	0.854	25.4196	-	0.9745
C: ratio CAS to PC	82.0484	+	0.0210	11.3938	+	0.5014	14.7484	+	0.2565	-0.7484	+	0.1304
AA	0.0434	+	0.4324	0.0169	+	0.2717	0.0234	+	0.2046	0.0351	+	0.3488
AB	-0.8511	-	0.2773	-0.1221	-	0.5445	-0.1918	-	0.4238	-0.1446	-	0.7692
AC	-0.4950	-	0.4547	-0.0944	-	0.5909	-0.0491	-	0.8092	0.2897	+	0.5099
BB	-8.3427	-	0.4582	-2.2270	-	0.4617	-4.9541	-	0.1908	-3.3274	-	0.6519
BC	-1.9643	-	0.8309	0.0937	+	0.9697	-0.5536	-	0.8486	-2.3973	-	0.6982
CC	-14.1178	-	0.1359	-2.1191	-	0.3675	-3.7070	-	0.1994	-2.6569	-	0.6384
R ²		0.9020			0.8016			0.8725			0.7176	
Lack-of-fit		0.0240			0.0657			0.0892			0.0364	
Standard Error of Estimation		1.9583			0.8757			1.1985			1.6139	
Mean absolute error		4.7712			1.2983			1.5783			3.1751	

	Alpha-tocopherol			Delta-tocopherol			Gamma-tocopherol		
	Coefficient	Correlation	p-value	Coefficient	Correlation	p-value	Coefficient	Correlation	p-value
Constant	135.0240			15.3823			5.8010		
A: fish oil content, %	-1.5838	+	0.0022	0.7300	+	0.0048	0.2925	+	0.0037
B: CAS + PC, %	-3.8750	+	0.1741	-8.0357	-	0.1472	-2.9286	-	0.1103
C: ratio CAS to PC	-9.9531	-	0.0091	2.0625	-	0.5037	1.5625	-	0.3647
AA	0.0190	+	0.2323	-0.0030	-	0.7460	-0.0009	-	0.7800
AB	0.4321	+	0.1065	0.2000	+	0.2089	0.0786	+	0.1970
AC	0.0156	+	0.9181	-0.0156	-	0.8853	-0.0062	-	0.8784
BB	-3.1718	-	0.2993	-0.9609	-	0.6144	-0.3486	-	0.6270
BC	-5.4018	-	0.1065	-1.4286	-	0.4056	-0.9375	-	0.2106
CC	4.3685	+	0.1297	0.5534	+	0.7001	0.2018	+	0.7092
R ²		0.9764			0.9757			0.9735	
Lack-of-fit		0.2019			0.4995			0.3602	
Standard Error of Estimation		2.1502			1.5308			0.5774	
Mean absolute error		1.6489			0.7756			0.3522	

Coefficients were regression coefficients obtained from regression models. The regression equation of the fitted model can be formed using Eq. (3).

Correlation ('-' or '+') were given based on the estimated effects and interactions, which involves estimating the average or main effect of each experimental factor and interactions between the factors.

The lack-of-fit test is designed to determine whether the selected model is adequate to describe the observed data. The test is performed by comparing the variability of the current model residuals to the variability between observations at replicate settings of the factors. If the p-value for lack-of-fit in the ANOVA table is greater or equal to 0.05, the model appears to be adequate for the observed data at the 95.0% confidence level. Otherwise, model does not describe the observed data.

0–3% creaming at day 1, respectively (Fig. 1). Likewise, our previous study of high fat fish oil-in-water emulsions stabilized with combinations of CAS and alginate also showed that emulsions containing 70% oil resulted in less creaming than emulsions with 50 or 60% oil (Yesiltas et al., 2017).

It was observed that emulsions produced with 0.4 ratio of CAS to PC had reduced stability to creaming compared to the ratio 2.0 when emulsions had the same fish oil and total emulsifier contents (Fig. 1). This suggested that the higher the CAS amount, the better the creaming stability. This may be due to the ability of CAS to form a thick

interfacial layer with a negative surface charge (at pH 7), which decreased droplet coalescence (Elias, Kellerby, & Decker, 2008). Additionally, creaming was more intense for the emulsions with 1.4% emulsifier content in comparison with 2.8% when the fish oil content and the ratio of CAS to PC were kept at the same levels (Fig. 1). Among all emulsions produced, only 70-2.1-2 and 70-2.8-1.2 did not show any creaming, which was presumably due to the fact that they were packed with oil droplets and thus the strong steric and electrostatic repulsion dominated the emulsion stability, thereby limiting the creaming (Fig. 1).

Table 3

Optimum recipes for each output variable given by RSM analysis (Optimal recipes were obtained in order to minimize each output variable, only tocopherols were maximized).

	Fish oil (% w/w)	Total emulsifier, (CAS + PC) (% w/w)	Ratio of CAS to PC
Droplet size	70	2.8	2.00
Viscosity	59	1.4	0.40
Creaming Index	70	2.8	2.00
Peroxide value	70	2.8	2.00
Alpha-tocopherol	70	2.8	0.40
Delta-tocopherol	70	2.8	0.40
Gamma-tocopherol	70	2.8	0.42
2-Ethyl-furan	70	2.8	2.00
1-Penten-3-one	70	2.8	2.00
1-Penten-3-ol	50	1.4	0.42
(E)-2-Pentalenal	50	2.8	0.40
Hexanal	70	1.9	2.00
(E)-2-Hexenal	50	1.4	0.41
(Z)-4-Heptenal	56	1.4	0.40
2-Pentyl-furan	70	2.8	2.00
(E)-2-Heptenal	70	2.8	2.00
Benzaldehyde	50	1.4	0.41
(E,E)-2,4-Heptadienal	53	1.4	0.40
Nonanal	58	2.0	2.00
(E,Z)-2,6-Nonadienal	50	1.4	0.92

Table 2 shows that the proposed quadratic model explained the variability of creaming data to a large extent ($R^2 = 0.9992$). Observed vs. predicted values were also plotted and shared in Supplementary Fig. 1c. Thus, it was used for obtaining the optimal values of the input variables that minimized creaming. In this regard, the optimal recipe given by RSM was 70% fish oil content, 2.8% total emulsifier and a ratio of 2 for CAS to PC (Table 3, Supplementary Fig. 1c). This also showed the positive effect of having a high ratio of CAS to PC against creaming.

3.2. Lipid oxidation measurements of emulsions

3.2.1. Primary oxidation products – peroxide value (PV)

All CAS + PC emulsions were found to be quite oxidatively stable with the highest PV after 12 days of storage of 5.07 meq peroxides/kg oil (Fig. 2). Likewise, Horn et al. (2012) showed that 70% fish oil-in-water emulsions emulsified with 2.8% CAS had 0.5 meq peroxides/kg oil PV at the end of one week of storage. Compared with PV results obtained after one week of storage in the current study showed that, at this time point PVs were approximately 2–3 meq peroxides/kg oil. This finding indicates that partly replacing CAS with PC did not reduce the formation of hydroperoxides compared to emulsions produced with only CAS. The advantage of having CAS in terms of increasing oxidative stability in the emulsions could be due to its ability to bind transition

metal ions both in water phase and at the interface (Horn et al., 2012; Elias et al., 2008).

It is noteworthy that most of the emulsions had severe creaming instability (Fig. 1), which might have physically limited the oxidation of the emulsions. Therefore, care should be taken when samples are compared. Emulsions with 50% fish oil content had 25% creaming at the end of the 1st day of storage meaning that the half of the water phase was already separated. In the upper part of the bottle for the same volume, creamed emulsion had a lower amount of water phase. Thereby, a lower total amount of metal ions/prooxidants was available for interaction in the proximity of oil droplets, compared to a non-creamed emulsion. Due to the close packing, diffusion of metal ions would also be more difficult. Thus, formation of oxidation products presumably was less than expected compared to an emulsion without creaming. However, droplets in the cream layer are located closer to the air; therefore, they are expected to be more exposed to oxygen diffusion, compared to droplets in a non-creamed emulsion. Also, there is a high possibility for the prooxidants/oxidation products to be involved in droplet-droplet exchange in a creamed emulsion compared to non-creamed one. These factors could thus have favored oxidation for creamed emulsions; however, this was not the case. On the other hand, emulsions with 70% fish oil content had low or no creaming, which might have favored the contact of prooxidants in the water phase with the lipid droplets. Hence, the factors that could reduce lipid oxidation in creamed emulsions seemed more important.

Only emulsions 70-2.8-1.2 and 70-2.1-2 did not have any creaming during storage, with 70-2.8-1.2 presenting a lower PV compared to 70-2.1-2. The lower PV found in 70-2.8-1.2 could be explained by a different content of CAS and PC and the antioxidant properties of these emulsifiers. Emulsion 70-2.8-1.2 had higher CAS and PC content (7.64 g CAS + 6.36 g PC in 500 g of emulsion) compared to emulsion 70-2.1-2 (7.00 g CAS + 3.50 g PC in 500 g of emulsion). CAS exhibits metal chelating activity and can trap metal ions present in the aqueous phase. CAS also hinders the access of metals to the water-oil interface by creating a thick interface (Horn et al., 2012; Elias et al., 2008). Moreover, PC might have retarded lipid oxidation by its metal chelating and radical scavenging activities, regenerating tocopherols and converting hydroperoxides into stable compounds (García-Moreno et al., 2014; Pan, Tikekar, & Nitin, 2013).

The experimental data was not explained well with the obtained quadratic model ($R^2 = 0.5863$, Table 2). Nevertheless, according to RSM results, 70% fish oil content, 2.8% total emulsifier and 2 as the ratio of CAS to PC led to a minimum formation of primary oxidation products (Table 3, Supplementary Fig. 1d).

3.2.2. Tocopherol content

The initial alpha-, beta-, gamma- and delta-tocopherol contents of the cod liver oil were 250 ± 1.9 , 0 ± 0 , 118 ± 1.2 and 48 ± 0.9 mg/kg,

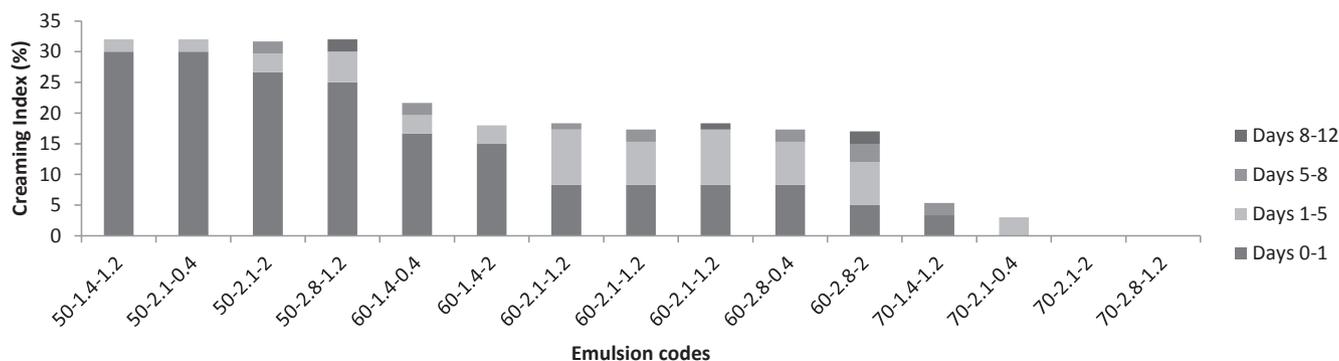


Fig. 1. Creaming index of emulsions during 12 days of storage. (Emulsion codes represent amount of fish oil – CAS + PC – CAS:PC, respectively. CAS: sodium caseinate, PC: phosphatidylcholine. $n = 1$).

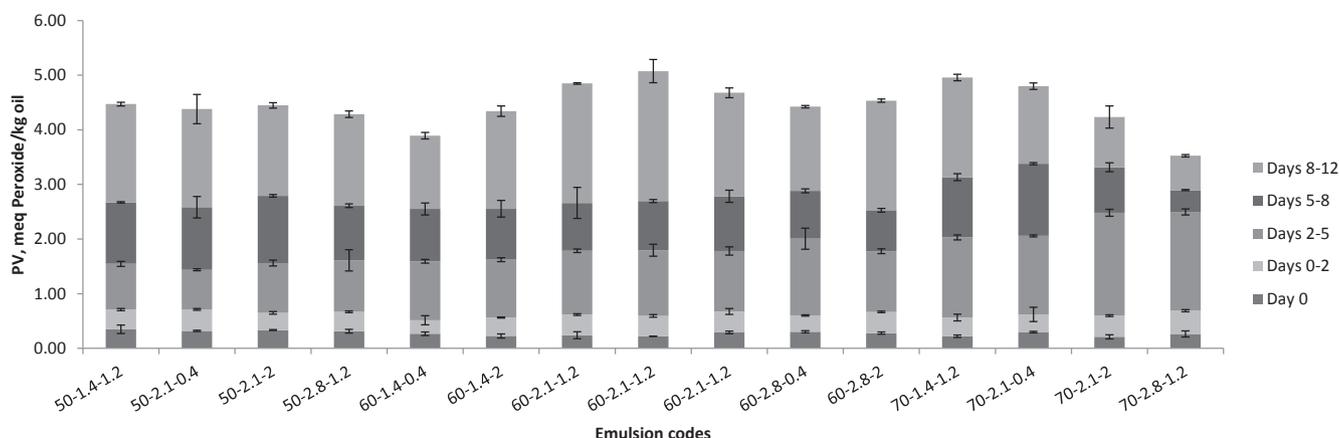


Fig. 2. Formation of primary oxidation products in the emulsions during 12 days of storage. Emulsion codes represent amount of fish oil – CAS + PC – CAS:PC, respectively. CAS: sodium caseinate, PC: phosphatidylcholine. $n = 2$.

respectively. For the emulsions, alpha-, gamma- and delta-tocopherols were quantified and were in the range of 103.2 ± 9.0 – 152.3 ± 3.1 , 17.9 ± 1.6 – 24.9 ± 0.5 , 44.7 ± 3.6 – 61.7 ± 0.9 mg/kg emulsion, respectively on day 0 (see Supplementary Fig. 2). There was a significant decrease in delta-tocopherol for all the emulsion samples, when day 0 and day 12 results were compared, except for emulsions 50-1.4-1.2, 50-2.1-2, 60-1.4-0.4 and 60-1.4-2 ($p < 0.05$). Alpha- and gamma-tocopherol contents of emulsions did not decrease significantly from day 0 to 12, apart from emulsions 70-2.8-1.2, 60-2.1-1.2 and 50-2.1-0.4. Emulsions 70-2.8-1.2 had a significant decrease in alpha-tocopherol, whereas 60-2.1-1.2 had a significant decrease in both alpha- and gamma-tocopherols, and 50-2.1-0.4 had a significant decrease in gamma-tocopherol. Emulsion 70-2.8-1.2 had the highest PV at day 5 and emulsion 60-2.1-1.2 had one of the highest PV at day 12. Therefore, the formation of primary oxidation products during storage for these two emulsions may have been higher if tocopherols had not been consumed during storage as a consequence of their antioxidant activity.

RSM results suggested similar optimal values for different types of tocopherols in order to obtain higher levels of tocopherols on day 12 data (Supplementary Fig. 1e–g). As shown in Table 3, optimal recipes for higher tocopherol levels were obtained for 70% fish oil content, 2.8% total emulsifier content and 0.40–0.42 as the ratio of CAS to PC. Higher levels of alpha-tocopherol was significantly affected by the fish oil content and the ratio of CAS to PC; increasing fish oil content and PC increased the amount of alpha-tocopherol (Table 2). On the other hand, delta- and gamma-tocopherols were only increased significantly by the increasing amount of fish oil content (Table 2). As tocopherols are

antioxidants and were consumed during the storage of the emulsions, it is preferable to track the changes as an indicator of oxidation and antioxidant.

3.2.3. Secondary oxidation products – dynamic head space (DHS) GC–MS

Formation of volatile compounds was quantified on days 0 and 12. Fig. 3 shows the concentration of 1-penten-3-ol, (*E*)-2-pentenal, (*E*)-2-hexenal and (*E,E*)-2,4-heptadienal in emulsions. These volatiles were selected due to their higher concentration in the emulsions compared to other volatile compounds and also because they originate from oxidation of omega-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Genot, Meynier, & Riaublanc, 2003). Similarly to the findings for PV, emulsions with 50% fish oil content had lower contents of volatile compounds compared to emulsions produced with 70% fish oil content (Fig. 3), which presumably was also affected by their creaming index as discussed under Section 3.2.1.

For emulsions without creaming, emulsion 70-2.8-1.2 had significantly lower content of secondary volatile oxidation products compared to emulsion 70-2.1-2, except for (*E*)-2-pentenal (Fig. 3). This could be due to the higher content of emulsifiers in emulsion 70-2.8-1.2 (7.64 g CAS + 6.36 g PC) compared to 70-2.1-2 (7.00 g CAS + 3.50 g PC) which was basically related to CAS and PC's antioxidant activities (similar reasons as explained in detail in Section 3.2.1 for PV). When 70-2.1-0.4 and 70-2.1-2 were compared, 70-2.1-0.4 having higher amount of PC showed better oxidative stability in terms of formation of (*E,E*)-2,4-heptadienal and 1-penten-3-ol (Fig. 3). A previous study showed that 70% fish oil-in-water emulsion stabilized with 2.8% CAS

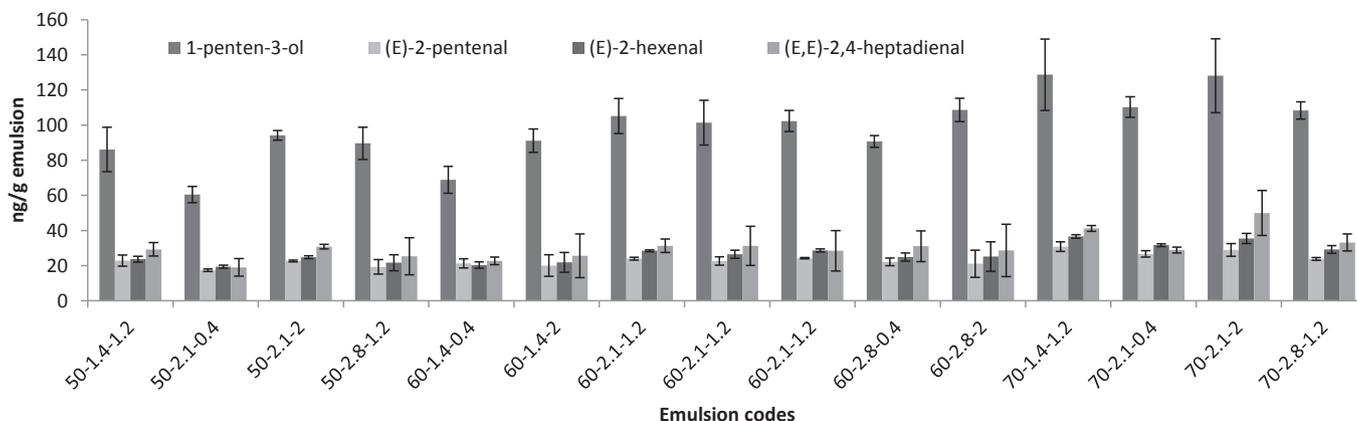


Fig. 3. Content of secondary volatile oxidation products during 12 days of storage. Emulsion codes represent amount of fish oil – CAS + PC – CAS:PC, respectively. CAS: sodium caseinate, PC: phosphatidylcholine. $n = 3$.

had 92 ng/g (*E,E*)-2,4-heptadienal formed after 7 days of storage (Horn et al., 2012), whereas a lower formation of (*E,E*)-2,4-heptadienal (33.2 ng/g) after 12 days of storage was found in this study when replacing 45% of CAS with PC in 70% fish oil-in-water emulsions. This could be due to the improved interfacial properties provided by PC because of its interaction with CAS at the interface and also to the radical scavenging and metal chelating activity of PC (García-Moreno et al., 2014; Berton-Carabin et al., 2014; Bandarra et al., 1999).

When emulsions 70-1.4-1.2 (5% creaming) and 70-2.8-1.2 (no creaming) were compared, oxidative stability was found to be higher for the one with higher total emulsifier content (Fig. 3). This might be due to a better coverage of the oil-water interface with higher amount of CAS and PC by forming a thicker interface, which is expected to limit the diffusion of prooxidants (e.g. metal ions) from the water phase to the oil phase. Moreover, antioxidant properties of adsorbed CAS and PC at the interface and presence of unadsorbed CAS and PC in the water phase might have increased the oxidative stability of emulsion with higher total emulsifier content.

RSM results provided different optimal values for different types of volatile compounds (Table 3, Supplementary Fig. 1h–s). Therefore, it is not possible to select one optimal recipe for decreasing the formation of volatile compounds. However, optimal recipes to minimize the content of 1-penten-3-ol, (*E*)-2-pentenal, (*E*)-2-hexenal and (*E,E*)-2,4-heptadienal, which were formed in higher concentrations compared to other volatile compounds, were suggested to have low fish oil content (50–53%) and low ratio of CAS to PC (0.40–0.42). Therefore, these suggested optimal recipes may have led to the conclusion that low fish oil and high PC content prevent the formation of the most abundant volatile compounds in the emulsions, providing a higher oxidative stability for emulsions stabilized with combinations of CAS and PC. However, this cannot be the case since the emulsions prepared by low fish oil and high PC content were not physically stable and severe creaming was observed during storage limiting oxidation as previously explained. Hence, only emulsions without creaming can be considered for selection of the optimal recipe due to higher physical stability.

4. Conclusion

In conclusion, results confirmed the hypothesis that it was possible to produce physically and oxidatively stable high fat fish oil-in-water emulsion stabilized with combinations of CAS and PC. Creaming was dramatically affected by fish oil content. Emulsions showed significantly less creaming, smaller droplet size with increasing fish oil content, total emulsifier content and ratio of CAS to PC. Viscosity increased significantly with increased fish oil content. RSM results showed that viscosity could be decreased/increased by decreasing/increasing the ratio of CAS to PC, respectively. Among the physically stable emulsions, 70-2.8-1.2 showed the lowest peroxide value and formation of volatile compounds. Therefore, it was considered as the optimal formula as it had the smallest droplet size, did not cream and oxidative stability was acceptable. This study also showed that the substitution of some of the CAS with PC increased oxidative stability of the emulsions while maintaining the physical stability again confirming the original hypothesis.

Acknowledgments

We would like to thank Lipoid GmbH, Arla Foods Ingredients amba and Maritex A/S Norway for donating phosphatidylcholine, sodium caseinate and cod liver fish oil, respectively. Furthermore, we are grateful to Inge Holmberg and Thi Thu Trang Vu for their skillful work in the laboratory. Finally, we would like to thank the Danish Council for Independent Research Technology and Production Sciences for financing the project Mapping and Characterizing of Lipid Oxidation in Emulsified Systems (MAPOX, DFF-4184-0123A).

Conflict of interest

The authors have declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2018.09.172>.

References

- AOCS Official Method Ce 8-89 (1998). *Determination of tocopherols and tocotrienols in vegetable oils and fats by HPLC*. Champaign, IL, USA: AOCS.
- Bandarra, N. M., Campos, R. M., Batista, I., Nunes, M. L., & Empis, J. M. (1999). Antioxidant synergy of α -tocopherol and phospholipids. *Journal of the American Oil Chemists' Society*, 76, 905–913.
- Bermudez-Aguirre, D., & Barbosa-Canovas, G. (2011). Quality of selected cheeses fortified with vegetable and animal sources of omega-3. *LWT – Food Science and Technology*, 7, 1577–1584.
- Berton-Carabin, C. C., Ropers, M. H., & Genot, C. (2014). Lipid oxidation in oil-in-water emulsions: involvement of the interfacial layer. *Comprehensive Reviews in Food Science and Food Safety*, 13(5), 945–977.
- Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37, 911–917.
- Diaz, M., Dunn, C. M., McClements, D. J., & Decker, E. A. (2003). Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 51, 2365–2370.
- Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant Activity of Proteins and Peptides. *Critical Reviews in Food Science and Nutrition*, 48, 430–441.
- Fang, Y., & Dalgleish, D. G. (1996). Comparison of the effects of three different phosphatidylcholines on casein-stabilized oil-in-water emulsions. *Journal of the American Oil Chemists' Society*, 73(4), 437–442.
- Gao, Z., Huang, Y., Zhao, J., Yao, X., Zhang, K., Fang, Y., ... Yang, H. (2017). Edible Pickering emulsion stabilized by protein fibrils: Part 2. Effect of dipalmitoyl phosphatidylcholine (DPPC). *Food Hydrocolloids*, 71, 245–251.
- García-Moreno, P. J., Horn, A. F., & Jacobsen, C. (2014). Influence of casein-phospholipid combinations as emulsifier on the physical and oxidative stability of fish oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 62(5), 1142–1152.
- Genot, C., Meynier, A., & Riaublanc, A. (2003). Chapter 7: Lipid oxidation in emulsions. In A. Kamal-Eldin (Ed.). *Lipid Oxidation Pathways*(1st ed.). New York: AOCS Publishing.
- Hadnadev, T. D., Dokic, P., Krstonosic, V., & Hadnadev, M. (2013). Influence of oil phase concentration on droplet size distribution and stability of oil-in-water emulsions. *European Journal of Lipid Science and Technology*, 115, 313–321.
- Horn, A. F., Barouh, N., Nielsen, N. S., Baron, C. P., & Jacobsen, C. (2013). Homogenization pressure and temperature affect protein partitioning and oxidative stability of emulsions. *Journal of the American Oil Chemists' Society*, 90, 1541–1550.
- Horn, A. F., Nielsen, N. S., Andersen, U., Søgaard, L. H., Horswell, A., & Jacobsen, C. (2011). Oxidative stability of 70% fish oil-in-water emulsions: Impact of emulsifiers and pH. *European Journal of Lipid Science and Technology*, 113, 1243–1257.
- Horn, A. F., Nielsen, N. S., & Jacobsen, C. (2012). Iron-mediated lipid oxidation in 70% fish oil-in-water emulsions: Effect of emulsifier type and pH. *International Journal of Food Science and Technology*, 47, 1097–1108.
- Jacobsen, C., Meyer, S. A., & Adler-Nissen, J. (1999). Oxidation mechanisms in real food emulsions: Oil-water partition coefficients of selected volatile off-flavor compounds in mayonnaise. *Zeitschrift Fur Lebensmittel-Untersuchung Und -Forschung*, 208, 317–327.
- Kolanowski, W., Jaworska, D., & Weißbrodt, J. (2007). Importance of instrumental and sensory analysis in the assessment of oxidative deterioration of omega-3 long-chain polyunsaturated fatty acid-rich foods. *Journal of the Science of Food and Agriculture*, 87, 181–191.
- Let, M. B., Jacobsen, C., & Meyer, A. S. (2007). Lipid oxidation in milk, yoghurt, and salad dressing enriched with neat fish oil or pre emulsified fish oil. *Journal of Agricultural and Food Chemistry*, 55, 7802–7809.
- Magnusson, E., Nilsson, L., & Bergenstahl, B. (2016). Effect of the dispersed state of phospholipids on emulsification—Part 1. Phosphatidylcholine. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 506, 794–803.
- McClements, J. D. (1999). Chapter 5: Interfacial properties and their characterization. *Food emulsions: Principles, practice and techniques* (pp. 136). (1st ed.). Boca Raton, Florida: CRC Press LCC.
- McClements, D., Decker, E., & Weiss, J. (2007). Emulsion-based delivery systems for lipophilic bioactive components. *Journal of Food Science*, 72(8), R109–R124.
- Myers, R. H., Montgomery, D. C., & Anderson-Cook, C. M. (2002). *Process and product optimization using designed experiments Response surface methodology* (3rd ed.). Hoboken, New Jersey: Wiley.
- Nichols, P. D., McManus, A., Krail, K., Sinclair, A. J., & Miller, M. (2014). Recent advances in omega-3: Health benefits, sources, products and bioavailability. *Nutrients*, 6, 3727–3733.
- Pal, R. (1996). Effect of Droplet Size on the Rheology of Emulsions. *American Institute of Chemical Engineers Journal*, 42(11), 3181–3190.
- Pan, Y., Tikekar, R. V., & Nitin, N. (2013). Effect of antioxidant properties of lecithin emulsifier on oxidative stability of encapsulated bioactive compounds. *International*

- Journal of Pharmaceutics*, 450, 129–137.
- Papanikolaou, Y., Brooks, J., Reider, C., & Fulgoni, V. L., III (2014). U.S. adults are not meeting recommended levels for fish and omega-3 fatty acid intake: Results of an analysis using observational data from NHANES 2003–2008. *Nutrition Journal*, 13, 31.
- Scuriatti, M. P., Tomás, M. C., & Wagner, J. R. (2003). Influence of soybean protein isolates–phosphatidylcholine interaction on the stability of oil-in-water emulsions. *Journal of the American Oil Chemists Society*, 80(11), 1093–1100.
- Shantha, N. C., & Decker, E. A. (1994). Rapid, sensitive, ironbased spectrophotometric methods for determination of peroxide values of food lipids. *Journal of AOAC International*, 77, 421–424.
- Song, C., Shieh, C., Wu, Y., Kalueff, A., Gaikwad, S., & Su, K.-P. (2016). The role of omega-3 polyunsaturated fatty acids eicosapentaenoic and docosahexaenoic acids in the treatment of major depression and Alzheimer's disease: Acting separately or synergistically? *Progress in Lipid Research*, 62, 41–54.
- Sui, X., Li, Q., Wang, Z., Qi, B., Zou, X., Li, Y., & Jiang, L. (2016). Does the hydrophobic group on sn-2 position of phosphatidylcholine decide its emulsifying ability? *LWT Food Science and Technology*, 74, 255–262.
- Sünder, A., Scherze, I., & Muschiolik, G. (2001). Physico-chemical characteristics of oil-in-water emulsions based on whey protein–phospholipid mixtures. *Colloids and Surfaces B: Biointerfaces*, 21, 75–85.
- Sznitowka, M., Janicki, S., Dabrowska, E., & Zurowska-Pryczkowska, K. (2001). Submicron emulsions as drug carriers. Studies on destabilization potential of various drugs. *European Journal of Pharmaceutical Sciences*, 12, 175–179.
- Waraho, T., McClements, D. J., & Decker, E. A. (2011). Mechanisms of lipid oxidation in food dispersions. *Trends Food Science and Technology*, 22, 3–13.
- Wysoczanski, T., Sokoła-Wysoczanska, E., Pekala, J., Lochynski, S., Czyz, K., Bodkowski, R., ... Librowski, T. (2016). Omega-3 fatty acids and their role in central nervous system: A review. *Current Medicinal Chemistry*, 23–28, 816–831.
- Xue, J., & Zhong, Q. (2014). Thyme oil nanoemulsions coemulsified by sodium caseinate and lecithin. *Journal of Agricultural and Food Chemistry*, 62, 9900–9907.
- Yesiltas, B., García-Moreno, P. J., Sørensen, A.-D. M., & Jacobsen, C. (2017). Physical and oxidative stability of high fat fish oil-in-water emulsions stabilized with combinations of sodium caseinate and sodium alginate. *European Journal of Lipid Science and Technology*, 119, 1600484.

PAPER III

Yesiltas, B., Torkkeli, M., Almasy, L., Dudás, Z., Dalglish, R., Wacha, A. F., García-Moreno, P. J., Sørensen, A-D. M., Jacobsen, C., Knaapila, M., **2018**.

Interfacial structure of 70% fish oil-in-water emulsions stabilized with combinations of sodium caseinate and phosphatidylcholine.

Submitted to *Colloids And Surfaces B: Biointerfaces*.

Total number of words: 5473

Total number of tables/figures: 8

Interfacial structure of 70% fish oil-in-water emulsions stabilized with combinations of sodium caseinate and phosphatidylcholine

Betül Yesiltas,^{1,*} Mika Torkkeli,^{2,*} László Almásy,^{3,4} Zoltán Dudás,³ András Ferenc Wacha,⁵ Robert Dalglish,⁶ Pedro J. García-Moreno,¹ Ann-Dorit M. Sørensen,¹ Charlotte Jacobsen,^{1,*} and Matti Knaapila^{2,*}

¹*Division of Food Technology, National Food Institute, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark,* ²*Department of Physics, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark,* ³*Institute for Solid State Physics and Optics, Wigner Research Centre for Physics, 1525 Budapest, Hungary,* ⁴*State Key Laboratory of Environment-Friendly Energy Materials, Southwest University of Science and Technology, Mianyang 621010, China,* ⁵*Institute of Materials and Environmental Chemistry, Research Centre for Natural Sciences Hungarian Academy of Sciences, 1525 Budapest, Hungary,* ⁶*Rutherford Appleton Laboratory, ISIS Facility, Chilton, OX11 0QX, UK*

*Corresponding authors: betye@food.dtu.dk, mtork@fysik.dtu.dk, chja@food.dtu.dk, matti.knaapila@fysik.dtu.dk

Abstract: We report on the structural evaluation of high fat (70%) fish oil-in-water and deuterated hexadecane-in-water emulsions emulsified with 1.05-1.4 % of sodium caseinate (CAS) and 0.4-1.75 % phosphatidylcholine (PC) studied by the combination of light scattering and small-angle X-ray and neutron scattering (SAXS/SANS). Aqueous CAS forms aggregates having a denser core of about 100 kDa and less dense shell about 400 kDa with the hard sphere diameter of 20.4 nm, while PC appears as multilayers, coherence length spanning from 40 to 100 nm. Employed oils form 12-15 μm sized droplets. We suggest that PC monolayer separates oil and water phases, while 80% CAS particles are loosely bound to the interface but are not forming continuous coverage. The distance between aggregated CAS particles in emulsion is increased compared to CAS aggregates in pure CAS in water system. PC multilayers become larger in the presence of oil-water interface compared to the pure PC mixtures. Bilayers become larger with increasing PC concentration.

Keywords: interfacial structure, emulsifier adsorption, high fat omega-3 delivery emulsions, SAXS, SANS

1. Introduction

Marine long chain (LC) omega-3 polyunsaturated fatty acids (PUFAs) have myriad beneficial effects on health such as decreasing risk of cardiovascular diseases, reducing inflammation and even play an important role in mental health [1, 2, 3, 4]. Fish oil-in-water emulsions can be used as delivery systems to enrich foods with LC omega-3 PUFAs including eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids [5]. High fat omega-3 delivery emulsions have further advantages, when it comes to enrichment of food systems with high viscosity or high fat content (e.g., mayonnaise, dressings, and cream cheese), such as the fact that addition of relatively lower amount of delivery emulsion is necessary for enrichment. Moreover, similar texture/structure of the delivery emulsion and food system provides easiness for mixing these two systems [6, 7].

However, delivery of bioactive compounds comes with its challenges in terms of physical and oxidative stability. LC omega-3 PUFAs are highly prone to oxidation, which results in formation of lipid oxidation products causing undesired sensory properties as well as loss in nutritional profile. Moreover, delivery systems need to be physically stabilized using the right concentration and combination of emulsifiers as well as improving other conditions during emulsion production. Many factors play important roles for the physical and oxidative stability of these emulsions, such as ingredients (type, quality, and amount), pH, temperature (during production and storage), homogenizers (type and conditions), droplet size, viscosity and surface charge [8, 9]. These factors also have an effect on the formation and properties of the oil-water interface in high fat oil-in-water emulsions. Previous research has shown that lipid oxidation in emulsions are initiated at the oil-water interface and, therefore, food researchers and industry are highly interested in characterizing the oil-water interface and understanding the inner dynamics of these systems.

The stabilization against oxidation depends strongly on the oil-water interface of oil-in-water emulsions. The protein-stabilized interfaces are less efficient in protecting emulsified lipids against oxidation than surfactant-stabilized interfaces [10]. Combined use of protein (CAS or whey protein) and surfactants (PC, Tween 60, or lecithin) increases oxidative stability of the fish oil-in-water emulsions [11,12,13,14].

Particular attention has been placed on combination of CAS and surfactants and adsorption and partitioning of these emulsifiers. It is expected that surfactants alter the characteristics of adsorbed CAS. For example, lecithin may adsorb to the available hydrophobic areas at the interface where there is a bare fat surface in between CAS molecules and this prevents aggregation and coalescence of the lipid droplets [12,13]. It is also known that at higher levels of phospholipid concentrations, the lower limit of CAS layer thickness increases from 5 to 6.5 nm while the upper plateau limit decreases from 10 to 8 nm [12]. The presence of egg-PC or 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (DOPC) in the casein emulsions, gives a thicker CAS layer at low CAS concentration (<1%) and a thinner layer at higher concentrations (>1%) [15]. Increase in the layer thickness is attributed to the change in packing of the adsorbed CAS molecules with phospholipids co-adsorbed at the interface, which was explained by the lack of necessity for CAS to extend so far to cover the oil-water interface, instead the adsorbed molecule adopt a more favored structure [15]. Surfactants may not only displace protein from the interface but also bind to proteins and alter their conformation [16,17]. The structure of oil-water interface is not necessarily homogenous, potentially having partly separated domains. Flexible, disordered proteins such as CAS form thicker but less dense interfacial layers [18]. Surfactant molecules with high surface activity may then adsorb to the interface through the gaps in between protein molecules [19].

Investigating structural details of CAS with and without surfactants has significant impact on understanding the ability of controlling lipid oxidation in oil-in-water emulsions, as these affect parameters such as; surface charge, thickness, and structure of oil-water interface. While the colloidal structures of different forms of CAS have been thoroughly studied by neutrons and X-rays [20, 21, 22], similar studies of oil-water interfaces consisting of combination of emulsifiers are less comprehensive.

In this paper, we use a combination of X-ray and neutron scattering to investigate the structure of a specific 70% fish oil-in-water emulsion produced with combined use of CAS and PC emulsifiers. As deuterated fish oil is not obtainable, and as fish oil has strong incoherent scattering background in neutron experiments, some of the experiments were carried out by replacing fish oil by deuterated hexadecane. This choice of hexadecane was motivated by the fact that fish oil is dominated by long chain fatty acids. Furthermore, hexadecane is well described in various oil-in-water emulsions (see, e.g., Rampon *et al.* [23]). Our results suggest that the interface incorporates a thin PC layer while 80% of CAS molecules remain loosely bound to this interface. Presence of oil-water interface promotes PC multilayers and affects period between PC surfactants in the aqueous phase in the vicinity of the interface. At the same time the presence of interface affects the distance between CAS molecules within CAS aggregates. This is the first study where SANS and SAXS techniques are applied in high fat fish oil-in-water emulsions.

2. Materials and Methods

2.1. Materials

Cod liver oil was provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway), and stored at -40°C until use. The fatty acid (% of total fatty acids) content of the fish oil as determined and supplied by the manufacturer was as follows: C14:0 (3.02), C16:0 (8.91), C16:1n-7 (8.20), C18:0 (1.88), C18:1n-9 (16.00), C18:1n-7 (5.16), C18:2n-6 (1.79), C18:3n-3 (0.84), C20:1n-9 (11.59), C20:5n-3 (9.27), C22:1n-11 (6.06), C22:6n-3 (11.64) and other fatty acids (15.64). Sodium caseinate, CAS, (Miprodan 30) was donated by Arla Foods Ingredients amba (Viby J, Denmark). Arla reported a protein content of 92% in CAS for Miprodan 30. PC from soybean (LIPOID S 100) was donated by Lipoid GmbH (Ludwigshafen, Germany). Specifications from Lipoid reported that phosphatidylcholine content was 96.2% for LIPOID S 100. Deuterium oxide (D₂O) (98%D) and deuterated hexadecane (*n*-hexadecane-d₃₄) (98%D) were purchased from Chemtronica (Stockholm, Sweden). Hexadecane (*n*-hexadecane) was purchased from Bie&Berntsena-S (Denmark).

2.2. Sample preparation

Pure compounds (CAS and PC) were dissolved/dispersed in D₂O for SANS and H₂O for SAXS experiments under magnetic stirring (500 rpm) overnight. The concentrations of the CAS and PC samples were ranged between 1-10% and 0.4-6%, respectively.

Aqueous phases of the emulsions were prepared one day before and left overnight on the magnetic stirrer (500 rpm) in order to allow the CAS (1.05%, w/w) and PC (1.75%, w/w) to dissolve in D₂O or H₂O at room temperature [24]. Emulsions (10g) were prepared using hand-held ultraturrax (POLYTRON PT 1200 E). Water phase was mixed for 30 s using hand-held ultraturrax and oil phase

(70%, w/w) was added slowly in 3 min while the aqueous phase was continuously mixed. After adding oil phase, emulsion was mixed for additional 4 min. Measurements were done within 3 days after sample preparation. The details of prepared samples are listed in Tables S1 and S2 in the Supplementary Data.

2.3. Droplet size

Particle size of the emulsions was measured by laser diffraction (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK) using the method described by Yesiltas *et al.* [5]. Emulsion (1 g) was dissolved in 5 g sodium dodecyl sulfonate (SDS) buffer (10 mM NaH₂PO₄, 5 mM SDS), mixed for 30 s and then sonicated for 15 min in a water bath. Droplets of the pretreated emulsions were diluted in recirculating water (3000 rpm), reaching an obscuration of 12–15%. The refractive indices of sunflower oil (1.469) and water (1.330) were used for particle and dispersant.

2.4. Relative protein content in the aqueous phase

Protein determination in the aqueous phase of emulsions was done mainly based on the method described by Jacobsen, Meyer, & Adler-Nissen [25]. Emulsions (~20 g) were centrifuged for 10 min at 25400 g and 10 °C (Sorvall RC-6 PLUS, Thermo Fisher Scientific, Osterode, Germany; rotor SS-34) and the oil phase was removed by the use of a pipette. The rest was mixed with distilled water (1:2) and then subjected to ultracentrifugation (Beckman Ultracentrifuge L8-60M, Fullerton, CA; rotor 21102) for 16 h at 106979 g and 15 °C, and once again the aqueous phase was extracted by the use of a syringe. The aqueous phase was diluted 1:6 in distilled water and protein concentration was determined using a BCA protein assay reagent kit (Pierce, Thermo Scientific, Rockford, IL, USA) by measuring in a spectrophotometer (Shimadzu, UV mini 1240, Kyoto, Japan) at 562 nm. Results were presented as

relative protein content in the aqueous phase, which was obtained by calculating the percentage of protein content in the aqueous phase compared to total protein amount added into the emulsion.

2.5. SAXS

SAXS measurements on the emulsions were done on the CREDO apparatus of the Research Centre for Natural Sciences, Hungarian Academy of Sciences [26, 27]. Cu K α X-rays produced by a GeniX3D Cu ULD integrated beam delivery system (Xenocs SA, Sassenage, France), and the scattered radiation was detected with a Pilatus-300k CMOS hybrid pixel position sensitive detector (Dectris Ltd., Baden, Switzerland) placed 536 mm downstream from the sample. The samples were filled into borosilicate glass capillaries of approx. 1 mm outer diameter and 0.01 mm wall thickness and kept at 25 °C. The q -range was 0.2 to 3 nm⁻¹, as calibrated by SBA15 mesoporous silica. The total exposure time was divided into 5 minute units, which were repeated several times until the desired signal-to-noise ratio was obtained. Each scattering pattern was corrected for sample self-absorption, instrumental background and detector flatness using the standard procedure implemented in the data collection program. The scattering intensity was transformed to absolute units (differential scattering cross section) by measuring a glassy carbon sample of known absolute scattering intensity under the same conditions as our samples and the scattering patterns were statistically filtered to remove artefacts from external radiation.

2.6. SANS

SANS measurements were performed on the Yellow Submarine instrument at the BNC in Budapest (Hungary) and the Larmor Instrument, Rutherford Appleton Laboratory (Chilton, England). At the Yellow Submarine, the wavelengths of 4.38 and 10.23 Å were used at the sample-to-detector distances of 1.15 and 5.25 m leading to the overall q -range from 0.08 to 4 nm⁻¹. Larmor was operated in the conventional SANS mode with the beam centered in the middle of the detector and a wavelength range of 0.09-1.25nm leading to the overall q -range from 0.03 to 7 nm⁻¹. In both cases, samples were filled in Hellma quartz cells of 2 mm path length and placed in the instrument and the measurements done at 25 °C. The measurement times were from 20 to 60 min. Empty cell and H₂O were also measured. The scattering from the cell and solvent were subtracted from the data.

2.7. Analysis of small-angle scattering data

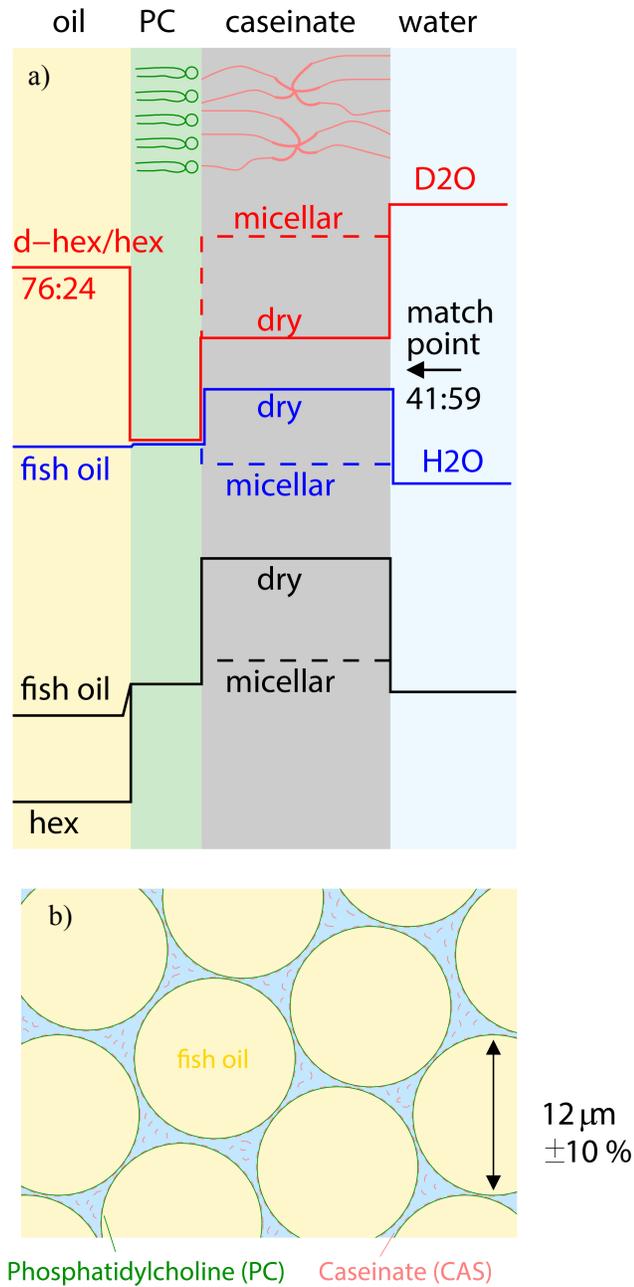


Fig 1. (a) Neutron (above) and X-ray (below) SLDs across the interface. For neutrons, the deuterated case is shown in red and non-deuterated in blue. In case of CAS, contrast may vary between dry protein (solid lines) and micellar protein, *i.e.* assuming 80% hydration (dashed lines). (b) Illustration of the studied high fat (70%, w/w) oil-in-water emulsion.

Fig. 1(a) illustrates the relative scattering length densities (SLDs) for the studied materials. Fig. 1(b) illustrates the apparent distribution of emulsion components. The exact SLD values for the employed samples are listed in Table S3 in the Supplementary Data. The scattering curves were first qualitatively discussed in terms of power law, as shown in Equation 1;

$$I(q) \propto q^{-\alpha}, \quad (1)$$

where $-\alpha$ stems from the shape of scatterers.

The scattering intensity of CAS dissolved in water (or D₂O) were subsequently analyzed in order to model the contribution of the non-adsorbed CAS in the emulsion water phases. In our notation the CAS particles were assumed to be particles composed of a number of individual protein chains. For $q < 0.45$ 1/nm the data were modelled in terms of core-shell type particles, whose interparticle interference is given by Percus-Yevick (P-Y) structure factor $S_{PY}(q, R_{HS}, \eta)$ for hard spheres [28]. The intensity was understood as shown in Equation 2;

$$I(q) = n(V_p \Delta\rho)^2 \left[\langle F^2 \rangle + \langle F \rangle^2 (S_{PY} - 1) \right], \quad (2)$$

where n is the number density of particles, V_p is the total volume of protein in the particle, $\Delta\rho$ is the scattering contrast between protein and water and $\langle F^2 \rangle$ and $\langle F \rangle$ are the particle form factor and scattering amplitude, both normalized to one. The parameters of P-Y function are the hard sphere radius R_{HS} and the particle volume fraction η . At high η , a peak develops at $q = 2\pi/D$, where $D = 2R_{HS}$ may be interpreted as particle diameter and also the distance between particles in a condensed system.

The scattering intensities of emulsions were understood as shown in Equation 3;

$$I(q) = \frac{S}{V} \left[(\rho_e - \rho_o)^2 + (\rho_e - \rho_w)^2 - 2(\rho_e - \rho_o)(\rho_e - \rho_w) \cos(qT) \right] \frac{2\pi}{q^4}, \quad (3)$$

where ρ_e, ρ_o and ρ_w are scattering length densities of emulsion (interface), oil and water where and

S/V is the specific surface area. T is the film thickness at the oil-water interface.

3. Results and discussion

3.1. Pure compounds

3.1.1. CAS in water

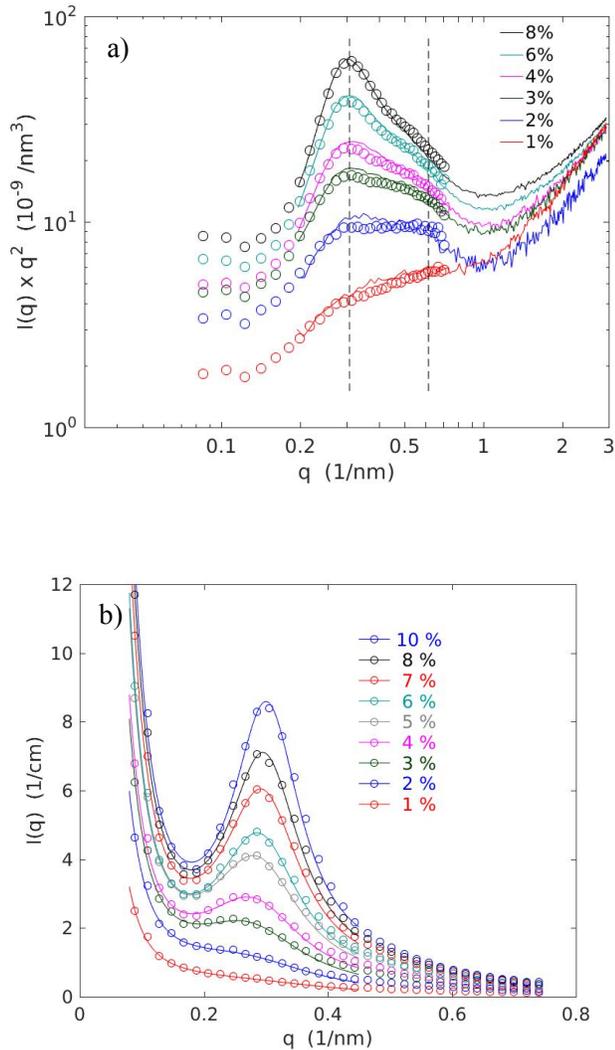


Fig 2. (a) SAXS (solid lines) and SANS (circles) patterns of CAS solutions in H_2O and D_2O , respectively, for various concentrations (Each SAXS intensity normalized to agree with respective SANS curve.) Dashed vertical lines mark positions of 1st and 2nd order maxima for periodicity $D = 20.4$ nm (b) SANS patterns (circles) with the corresponding fits to the Eq. 2 (solid lines). The CAS concentration varies from 1% to 10%.

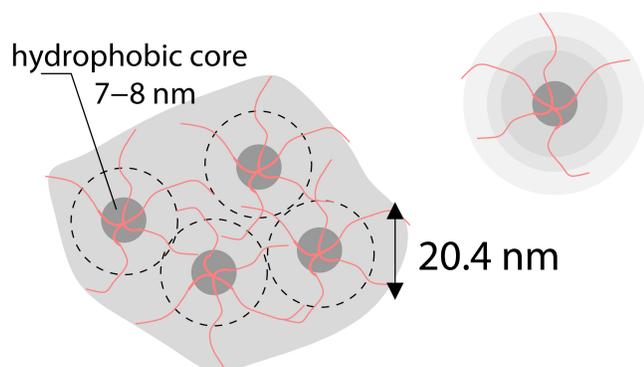


Fig 3. Schematic illustration of the structure of studied CAS aggregates in water.

We note that the properties of CAS depend strongly on manufacturer, counterions, counterion concentration etc. (See for example Smialowska *et al.* [29]). This motivates a careful structural consideration of the here employed CAS and the pure CAS-in-water systems before moving to the emulsions.

Fig.2 shows the scattering data of CAS-in-water systems and discussed system is illustrated in Fig. 3. The fitting parameters are compiled in Table S4 in the Supplementary Data. Fig. 2 shows the effect of CAS concentration on the formation of aggregates. Both SAXS and SANS agree with the formation of uniform size aggregates, whose number increases linearly with concentration. In this paper, we denote the observed units in water as particles and their clusters as aggregates, although the clusters containing several CAS molecules are sometimes called micelles and the CAS molecules within these clusters as submicelles (see e.g., [22]).

As the mass and composition (water content) of these particles were not known exactly, it was not possible to use *a priori* known values for η . Moreover, the parameter η does not grow linearly with CAS mass, which indicates that the hard sphere model does not describe realistically the ordering of soft particles in dense CAS solutions (see Fig. S1 in the Supplementary Data). Therefore, the parameter

η was taken as a fitting parameter describing order in the system rather than an exact volume fraction of the particles.

Two form factors were tried in the fitting. Initially we tried a form factor, composed of dense core $R_C = 4$ nm and shell $R_S = R_{HS}$, but results indicated that a very dense core would be sufficient to fit the results adequately, so the shell part was dropped. A second form factor that tried was a multi-star chain, which is a collection of N random walk chains linked at the center of the particle. This approximates N individual protein chains linked by hydrophobic interactions at the hard core (Fig. 3).

This model does not explain an upturn at smallest angles, which may be caused by aggregation of the particles (or elsewhere called as submicelles [22]) into larger units. For the fitting, this upturn was treated as background as shown in Equation 4;

$$I_{bg} = const. q^{-2} \quad (4)$$

The diameter of the particles is taken as twice the hard sphere radius of the P-Y fit which levels off quite well at 20.4 nm above 3 % concentration. Below this, the hard sphere radius becomes meaningless as the average distance between particles increases which causes a shift in the scattering maxima to a smaller angle. The difference in peak location was not attributed to the change in size but their packing, which indicated CAS aggregates were more closely packed at higher concentrations compared to lower concentrations. This could be explained by the loss of the water molecules in between CAS aggregates when the concentration of CAS was increased.

Guinier radius and zero-angle intensity ($I(q=0)$) to yield molar mass are shown in the Fig. 2 in the Supplementary Data. In this consideration molecule weight of CAS is estimated from a normalized

scattering curves, which are extrapolated to zero concentration for all q , thus setting $S_{p-y} \rightarrow 1$. This consideration may not be highly accurate, because our instruments do not make it possible to measure sufficiently low q values. For the latter, we may estimate from scattering data that the particles consist of a dense core of about 100 kDa protein weight and the total protein mass in the particle is about 400-500 kDa.

Our ideas are consistent with earlier neutron observations of Stothart *et al.* [20], who studied CAS submicelles obtained from whole milk micelles by acidification using SANS in concentration 16 mg/ml in 0.7 M NaCl solution in D₂O. The authors observe both the radius of gyration $R_g = 6.5$ nm and molar mass 300 kg/mol, while we observe $R_g = 16.5$ nm and molar mass = 430 kg/mol. If we use the obtained hard sphere radius and the voluminosity mg/mL (ca. 20 % dry weight) observed by Stothart, the calculated molar mass is 540 kg/mol per particle. Note that 10 % concentration would infer 9 mL/g, which corresponds to 300 kg/mol. This is then the theoretical lower limit of the molecule size for which value the submicelles would fill the whole volume, i.e. $\eta = 1$.

Our results also agree with the X-ray work of Kumosinski *et al.*, who showed a core-shell model for the particle, where 21 % of the molar mass resides in a dense core of radius 4.4 nm surrounded by less dense shell of radius 11.4 nm [21]. The total mass was 285 kg/mol. It was also reported previously that CAS forms aggregates at the size of 10-11 nm in radius, similar to submicelles formed by casein [30, 31]. On the other hand, larger sizes were also reported by Huppertz *et al.* that suspensions of CAS appear as particles with $R_g \approx 20$ nm and hydrodynamic radius ~ 10 nm [32, 33].

3.1.2. PC in water

The small-angle scattering data of employed PC in water systems divided by the concentration of the PC in H₂O or D₂O are plotted in Fig. S3 in the Supplementary Data. All samples showed a q^{-3} power law and Lorentzian shaped Bragg peaks that stem from the known layer-like structure and whose area increased with the increasing concentration [34]. Due to the higher resolution, we used X-ray data for the line shape analysis. The long period corresponding to the thickness of PC bilayers and the coherence length ξ corresponding to the thickness of the PC multilayers are compiled in Table 1 (*vide infra*). When the PC concentration was increased from 0.4% to 0.9%, the bilayer thickness increased from 6.29 to 6.33 nm, respectively. Calculated coherence lengths (size of the structures) were 97.51 nm and 87.54 nm, respectively. This corresponds to ca. 15 bilayers.

3.2.Oil-water interface of 70% oil-in-water emulsions

3.2.1. Droplet size

The prepared high fat emulsions stabilized with CAS and PC contained 70.0% of oil and 27.2% of water. The samples appear milky having a lower viscosity than mayonnaise (70-80% fat), but being more viscous than milk. Samples did not show any observable instability during the measurements. Droplet size of the emulsions produced with hexadecane or fish oil are shown in Table S4 in the Supplementary Data. The difference between their $D[3,2]$ and specific surface area was not significant.

70% fish oil-in-water emulsions with different combinations of CAS and PC were also analyzed in order to study differences between presence of both emulsifiers together, absence of one of the emulsifiers, and different concentrations of emulsifiers. Table S5 in the Supplementary Data shows the droplet size and specific surface area of these emulsions. Emulsion CAS/PC 1.05/1.75 had smaller droplets compared to emulsion with only CAS 1.05 which could be due to the contribution of PC at the oil-water interface. Emulsion CAS 2.8 had smaller droplets compared to emulsion CAS 1.05 due to the higher concentration of CAS as an emulsifier; smaller droplets also resulted in higher surface area. Large droplet sizes of emulsions PC 1.75 and PC 2.8 indicated that the emulsions were not as physically stable as emulsions produced with only CAS or combinations of CAS and PC. The estimated CAS contents left in the aqueous phase after emulsion preparation are also compiled in Table S5 in the Supplementary Data. The data show that replacement of CAS by PC increases the amount of CAS in the aqueous phase.

3.2.2. SAXS and SANS

Fig. 4 plots the SANS patterns of different oil-in-water emulsions with fish oil and deuterated hexadecane. The solid lines are expected total scattering from PC monolayer and CAS particles including constant background (*vide infra*). The dashed blue line (above) shows the calculated contribution of a single monolayer film in a nominally matched sample (deuterated hexadecane:hexadecane 0.945:0.055). The black dashed line (below) shows the contribution of CAS particles (40%) alone.

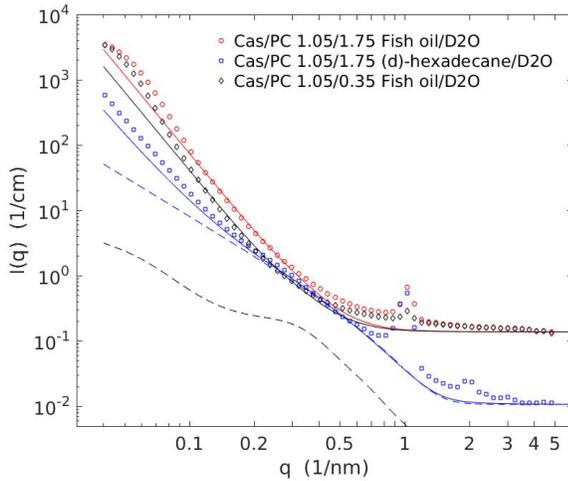


Fig 4. SANS patterns (circles) for selected emulsions. The solid lines are expected total scattering from PC monolayer and CAS particles with a constant background contribution, while dashed lines represent the individual scattering contribution from CAS particles and PC monolayer. See text for details.

The reasoning for the above-mentioned model (solid lines) is as follows. The scattering curves with fish oil are dominated by q^{-4} slope which arises from globular particles. The scattering curve with deuterated hexadecane follows q^{-2} slope. This means scattering comes from a thin film since the region only extends to $q \propto 1/T$, where T represents the interface thickness. This allows us to identify

well defined layer with $T \approx 3.2$ nm . As this number corresponds to the PC monolayer, we propose that the interface contains a well-defined PC monolayer. Following this idea, fits to the PC monolayer to oil-water interface in the case of fish oil and deuterated hexadecane are shown in in Fig. S4 in the Supplementary Data.

Calculations of scattering patterns are based on true densities where the specific surface S/V is left as a free parameter. The curves furthermore include calculated incoherent background. In case of deuterated hexadecane, there is also calculated contribution from the molecular level inhomogenities that arise from mixing large deuterated and nondeuterated molecules. As hexadecane is a linear molecule, this leads to a q^{-1} tail contribution which is calculated and detailed in Fig. S4 in the Supplementary Data. A similar tail may also be present in fish oil which is a mixture of different fatty acids in triglyceride form.

A thin film at oil-water interfacial area was estimated from Eq. 3. The value for S/V was found to be 3500 1/cm for fish oil and 11000 1/cm for deuterated hexadecane. Using $S/V = 6\varphi/D_{droplet}$, the corresponding droplet diameters determined from SAXS and SANS are $D_{droplet} = 12 \mu\text{m}$ and $D_{droplet} = 3.9 \mu\text{m}$. This agrees with the droplet size measurements (Supporting Data) but also implies that fish oil and hexadecane may have slightly different droplet sizes.

In addition, the SANS curves include a broad feature at $0.2 - 0.5 \text{ nm}^{-1}$ arising from CAS and Bragg peaks at about 1 nm^{-1} arising from PC (Fig. 4). The second order peak is visible only with deuterated oil when the incoherent background is low. These features are better resolved in SAXS data (Fig. 5).

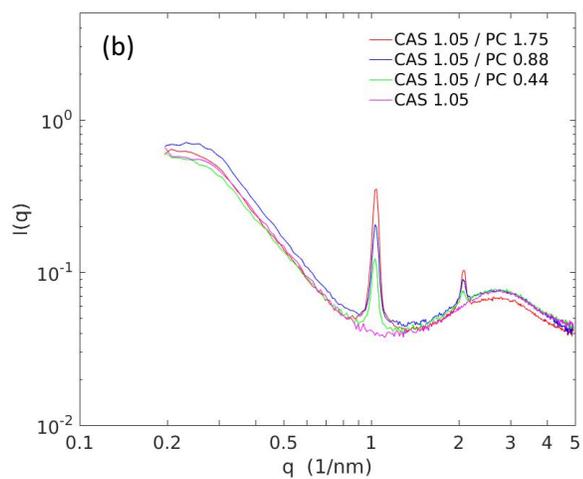
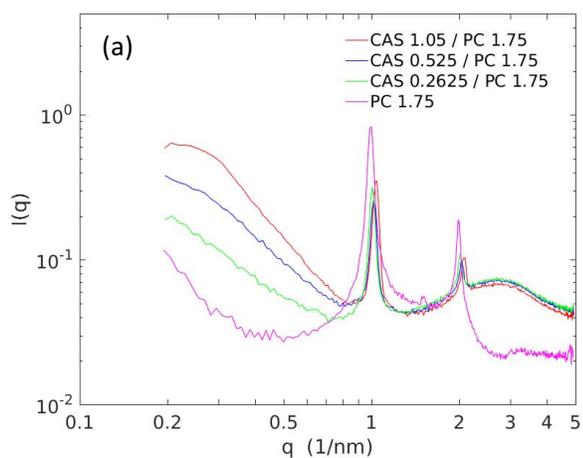


Fig 5. (a) SAXS patterns of employed fish oil-in-water emulsions with various combinations of CAS (0.2 – 1.05 %) and PC (1.75%) concentration, (b) SAXS patterns of employed fish oil-in-water emulsions with constant CAS fraction (1.05%) and various PC concentrations (0.44-1.75 %).

Table 1.

The long period L_p for PC bilayers and the coherence length ξ for PC multilayers. Above: PC water systems with increasing concentration. Below: PC bilayers in emulsions for various CAS/PC ratios

PC, <i>conc.</i>	0.4%	0.9%	0.4%	0.9%	1.8%	6%	
L_p (nm)	6.29	6.33	6.22	6.28	6.35	6.35	
ξ (nm)	97.5	87.5	47	40	40	40	
CAS/PC	1.05/1.75	-/2.8	1.05/0.88	1.05/0.44	0.525/1.75	0.263/1.75	-/1.75
L_p (nm)	6.06	6.33	6.06	6.13	6.17	6.25	6.32
ξ (nm)	110.6	88.4	108.4	107.7	110.9	120.0	116.4

Fig. 5 plots the SAXS patterns for oil-in-water emulsion with the constant PC concentration and increasing CAS concentration as well as with constant CAS concentration and increasing PC concentration. In order to magnify the scattering contribution of PC, the latter curves where the scattering contribution of CAS is subtracted are shown in Fig. S5 (the Supplementary Data). The long period for PC bilayers and the coherence length ξ for the PC multilayers in emulsions for various CAS/PC ratios are compiled in Table 1.

The location of the PC peak shifted significantly to the higher q values with the increasing concentration of CAS (0 to 1.05%) (Fig. 5a). The reason for this is that the periodic repeat distance of the PC bilayers decreased, which could be due to either decrease in the water amount in between 2 PC bilayers or increased order. This change in the overall arrangement of the bilayer stacking could be due to the interaction between CAS and PC at the oil-water interface.

The packing of PC molecules may be influenced by the presence of CAS. It was suggested by Fang and Dalgleish that a complex between DOPC and β -casein was formed involving the hydrophobic domains

of the two components, whereas it was not evident for DOPC and α_{s1} -casein, which makes the removal of casein from the interfacial region highly specific [35].

The CAS contribution increases with increasing CAS concentration (Fig. 5a). From the phase considerations (see section 3.1) we estimate that about 20% CAS remains free in the water phase. The rest remains bound to the interface but we cannot say how strong this binding is. The fact that PC peaks are proportional to PC concentration implies that there is no significant interaction between CAS and PC located in the multilayers in the bulk phase. However, the multilayers become larger in emulsions, which points to increased interactions or a confinement effect between droplets.

In the case of emulsion, we need to consider both free CAS, which presumably is in the micellar form, and additionally a thin surface of PC and CAS formed at the oil-water interface. We analyzed the micellar CAS using the same fitting procedure as for pure CAS solution and the value obtained for η were taken as measure of the amount of micellar CAS in the water phase. Thus, if the η value in emulsion is the same as, say in 3% CAS solution, then the weight fraction of the micellar CAS in the water phase is assumed to be 3% and the intensity due to micellar CAS then $I_{mc} = \varphi_w I_{3\%}$, where φ_w is the water volume fraction in emulsion and $I_{3\%}$ is the intensity in the pure CAS water system. In general, the intensity was a linear interpolation between two closest pure CAS measurements.

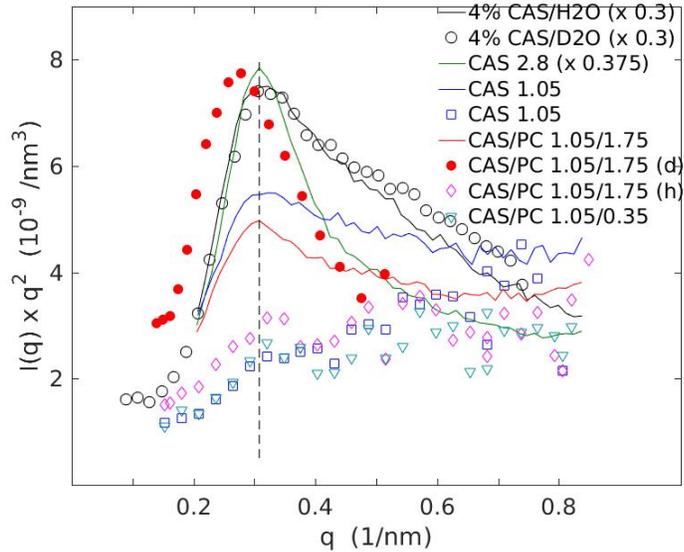


Fig 6. SAXS (solid lines) PC 1.75% emulsion subtracted from CAS 1.05% + PC 1.75%, SANS (circles) subtracted contribution from the PC monolayer as a power law background, and SANS (diamonds) CAS 4%.

Fig. 6 shows the comparison between the broad CAS peak as seen in emulsion by SANS and SAXS. The peak position for CAS in water is shown for comparison. The contribution from the proposed PC monolayer is subtracted and the intensity curves are normalized to the same water phase fractions and CAS/water scattering contrast. This comparison shows how the peak moves to the smaller scattering angles in emulsion and at the same time the peak intensity drops.

The SAXS curves show very slight decrease in peak position (increase in particle distance) and both the intensity level and peak width (related to the P-Y volume fraction, η) corresponds to 40-60 % CAS in particles. Since the amount of free CAS is 20 % at maximum (Table S5, the Supplementary Data), the SAXS data implies that either there is a time dependence to this amount or the adsorbed CAS exhibits similar inter-particle interference effects as free CAS.

The SANS measurements in fish oil (open symbols) typically showed a weak maximum at the same position as in the original CAS in water system. The width of the peak is difficult to establish, but the intensity is consistent with 20 % amount of free CAS. Thus, the adsorbed CAS seemingly does not contribute to scattering at this q -range.

The SANS measurement in deuterated hexadecane emulsions (solid symbols) showed significantly larger intensity and the characteristic distance between CAS particles is increased from 20.4 nm for the CAS in water up to 26 nm for CAS in emulsion (Fig. 6).

This means that even though CAS particles were not tightly packed on the interface forming a well-defined film (the interface is rather dominated by a well-defined PC monolayer), the CAS particles remain loosely bound and influenced by its vicinity forming supposedly a patchy array on it. This observation should be compared to the studies that have shown surfactants replaced proteins adsorbed at the oil-water interface. This phenomenon was related to the type of the surfactant, e.g., hydrophilic-lipophilic balance, number of alkyl chains attached to the molecule, as well as the ways of surfactant incorporation during emulsion production, such as addition of the surfactant before or after homogenization [17, 18]. Previous studies reported that Tween 60 replaced all the protein adsorbed at the oil-water interface. However, PC did not act in the same way, but instead interacted with protein at the interface and formed a mixed interfacial layer [15, 36].

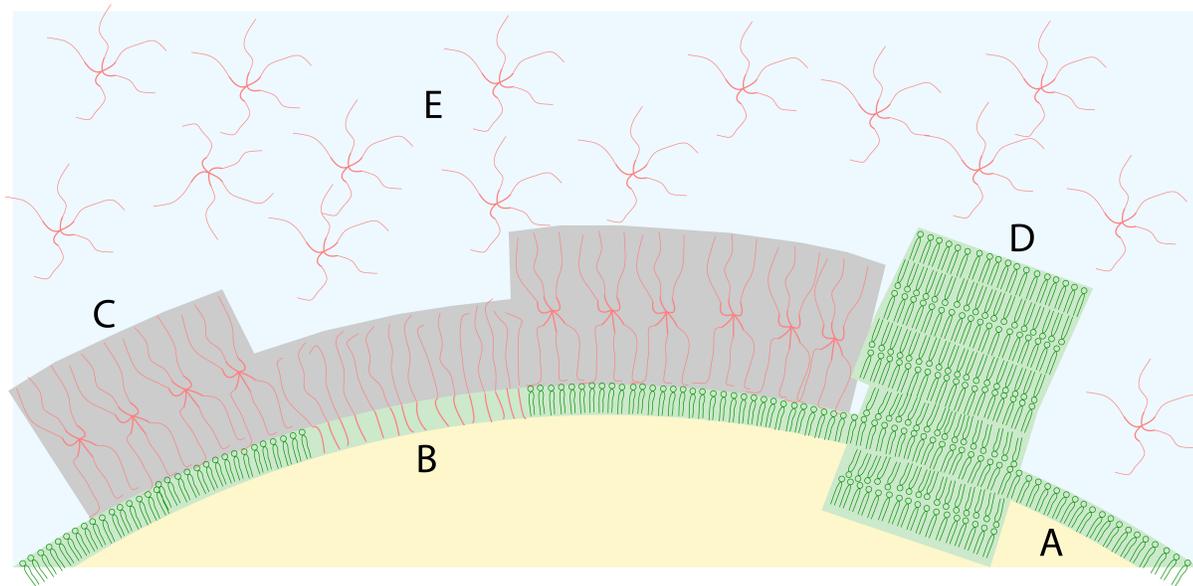


Fig 7. Proposed structure for the here studied 70% oil-in-water interface. PC monolayer (A) with CAS particles loosely bound on the PC layer without forming a continuous coverage (B-C). Excess PC forms multilayers and some may be bound to the interface without forming continuous coverage (D). The unabsorbed CAS remains in the water phase (estimated to be about 20 %) (E). Not drawn to scale.

Fig. 7 outlines qualitative interpretation of our results. A PC monolayer separates oil and water phases. 80% CAS particles are influenced or loosely bound to the interface but are not forming continuous coverage and true 2-dimensional system. The distance between aggregated CAS particles is increased compared to CAS aggregates in pure CAS in water system. PC multilayers become larger in the presence of interface compared to the pure PC in water.

4. Conclusions

Combined use of SANS and SAXS as characterization tools enlightened the interfacial structure of high fat (70%) oil-in-water emulsions. Oil-water interfacial structure was characterized by identifying thickness of the interfacial layer and interaction between emulsifiers adsorbed at the oil-water interface, as well as obtaining information on adsorption behavior of the combined use of CAS and PC in high fat delivery emulsions. A PC monolayer (3.2 nm thick) separates oil and water phases. 80% CAS particles are influenced or loosely bound to the interface but are not forming continuous coverage and true 2-dimensional system. The distance between aggregated CAS particles was increased compared to CAS aggregates in pure CAS in water system. PC multilayers become larger in the presence of the interface compared to the pure PC mixtures and bilayers become larger with increasing PC concentration.

Acknowledgements

We thank Lipoid GmbH, Arla Foods Ingredients a.m.b.a and Maritex A/S Norway for chemicals and the Danish Council for Independent Research Technology and Production Sciences for financing the project Mapping and Characterizing of Lipid Oxidation in Emulsified Systems (MAPOX, DFF-4184-0123A).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/colsurfb.XXXXXX>

References

- [1] J. Allaire, P. Couture, M. Leclerc, A. Charest, J. Marin, M.-C. Lépine, D. Talbot, A. Tchernof, B. Lamarche, *Am J Clin Nutr*, 104 (2016) 280-287.
- [2] T. Wysoczański, E. Sokoła-Wysoczańska, J. Pékala, S. Lochyński, K. Czyz, R. Bodkowski, G. Herbinger, B. Patkowska-Sokoła, T. Librowski, *Curr. Med. Chem.*, 23–8 (2016) 816–831.
- [3] P. D. Nichols, A. McManus, K. Krail, A. J. Sinclair, M. Miller, *Nutrients*, 6 (2014) 3727-3733.
- [4] C. Song, C. Shieh, Y. Wu, A. Kalueff, S. Gaikwad, K. Su. *Prog. Lipid Res-*, 62 (2016) 41-54.
- [5] B. Yesiltas, P. J. García-Moreno, A.-D. M. Sørensen, C. Jacobsen. *Eur. J. Lipid Sci. Technol.*, 119 (2017) 1600484.
- [6] B. Yesiltas, P. J. García-Moreno, A.-D. M. Sørensen, S. Anankanbil, Z. Guo, C. Jacobsen, *J. Agric. Food Chem.*, DOI: 10.1021/acs.jafc.8b04091.
- [7] A. F. Horn, N. S. Nielsen, C. Jacobsen, *Int. J. Food Sci. Technol.*, 47 (2012) 1097–1108.
- [8] C. Jacobsen, M. B. Let, N. S. Nielsen, A. S. Meyer, *Trends Food Sci. Technol*, 19 (2008) 76–93.
- [9] C. C. Berton-Carabin, M. H. Ropers, C. Genot, *Compr. Rev. Food Sci. Food Saf.*, 13 (5) (2014) 945–977.
- [10] C. Berton, M.-H. Ropers, M. Viau, C. Genot, *J. Agric. Food Chem.*, 59, (2011) 5052–5061.
- [11] E. Dickinson, *Colloids and Surfaces B: Biointerfaces*, 20 (2001) 197–210.
- [12] Y. Fang, D. G. Dalgleish, *J. Col. Interface Sci.*, 156 (1993) 329-334.
- [13] D. G. Dalgleish, M. Srinivasan, H. Singh, *J. Agric. Food Chem.*, 43 (1995) 2351-2355.

- [14] R. Waninge, P. Walstra, J. Bastiaans, H. Nieuwenhuijse, T. Nylander, M. Paulsson, N. Bergenståhl, *J. Agric. Food Chem.*, *53* (2005) 716-724.
- [15] Y. Fang, D. G. Dalgleish, *J. Am. Oil. Chem. Soc.*, *73*(4) (1996) 437–442.
- [16] D. G. Dalgleish, *Trends Food Sci. Technol.*, *8* (1997) 1-6.
- [17] D. J. McClements, E. Decker, *J. Agric. Food Chem.*, *66* (2018) 20–35.
- [18] C. C. Berton-Carabin, L. Sagis, & K., Schroën, *Annu. Rev. Food Sci. Technol.*, *9*, (2018) 24.1–24.37.
- [19] M. B. Munk, F. H. Larsen, F. W. J. van den Berg, J. C. Knudsen, J. C., M. L. Andersen, *Langmuir*, *30*, (2014) 8687–8696.
- [20] P. H. Stothart, D. J. Cebula, *J. Mol. Biol.*, *160* (1982) 391-395.
- [21] T. F. Kumosinski, H. Pessen, H. M. Farrell, H. Brumberger, *Arch. Biochem. Biophys*, *266* (1988) 548-61.
- [22] C. G. de Kruif, T. Huppertz, V. S. Urban, A. V. Petukhov, *Adv. Coll. Interface Sci.*, *171–172* (2012) 36–52.
- [23] V. Rampon, A. Riaublanc, M. Anton, C. Genot, D. J. McClements, *J. Agric. Food Chem.*, *51*, (2003) 5009-5905.
- [24] P. J. García-Moreno, A. F. Horn, C. Jacobsen, *J. Agric. Food Chem.*, *62*, (2014) 1142–1152.
- [25] C. Jacobsen, A. S. Meyer, J. Adler-Nissen, *J. Food Lipids*, *5*, (1998) 87–101.
- [26] A. Wacha, Z. Varga, A. Bóta, *J. Appl. Cryst.*, *47*, (2014) 1749–1754.
- [27] A. Wacha, *J. Appl. Cryst.*, *48*, (2015) 1843–1848.

- [28] J. K. Percus, G. J. Yevick, *J. Phys. Rev.*, 110 (1958) 1-13.
- [29] A. Smialowska, L. Matia-Merino, B. Ingham, A. J. Carr, *Colloids Surf. A.*, 522 (2017) 113–123.
- [30] P. Thomar, T. Nicolai, *Food Hydrocoll.*, 49 (2015) 224-231.
- [31] D. Farrer, A. Lips, *Int. Dairy J.*, 9 (1999) 281-286.
- [32] T. Huppertz, I. Gazi, H. Luyten, H. Nieuwenhuijse, A. Alting, E. Schokker, *Int. Dairy J.*, 74 (2017) 1-11.
- [33] T. Huppertz, P.F. Fox, A.L. Kelly, in Rickey Y. Yada (Eds.), *Proteins in Food Processing*, Vol.2, Woodhead Publishing, Cambridge, 2018, Chapter 3.
- [34] P. Balgavý, M. Dubnicková, N. Kucerka, M. A. Kiselev, S. P. Yaradaikin, D. Uhríková, *Biochim. Biophys. Acta*, 1512 (2001) 40-52.
- [35] Y. Fang, D. G. Dalgleish, in E. Dickinson, D. Bergenståhl (Eds.), *Food Colloids Protein, Lipids and Polysaccharides*, Woodhead Publishing, Cambridge, 2004.
- [36] I. Heertje, H. van Aalst, J. C. G. Blonk, A. Don, J. Nederlof, E. H. Lucassen-Reynders, *Lebensm.-Wiss. u.-Technol.*, 29 (1996) 217–226.

Supplementary data

Tables S1 and **S2** describe the employed samples used in scattering measurements.

Table S3 lists the employed contrast considerations.

Table S4 lists droplet sizes and specific surface areas of fish oil-in-water and hexadecane emulsions

Table S5 Droplet sizes, specific surface areas and relative protein content in the aqueous phases of fish oil-in-water emulsions.

Table S6 documents the fitting procedure for CAS in water systems.

Table S1.

Composition of employed samples used in SAXS measurements.

Codes	Conc. (% w / w)			
	CAS	PC	H ₂ O	Fish oil
CAS/PC 1.05/1.75	1.05	1.75	27.2	70
CAS 2.8	2.8		27.2	70
PC 2.8		2.8	27.2	70
CAS/PC 1.05/0.88	1.05	0.88	28.1	70
CAS/PC 1.05/0.44	1.05	0.44	28.5	70
CAS/PC 0.53/1.75	0.53	1.75	27.7	70
CAS/PC 0.26/1.75	0.26	1.75	28.0	70
CAS 1.05	1.05		29.0	70
PC 1.75		1.75	28.3	70
CAS 1%	1		99.0	
CAS 2%	2		98.0	
CAS 3%	3		97.0	
CAS 4%	4		96.0	
CAS 6%	6		94.0	
CAS 8%	8		92.0	
PC 1.8%		1.8	98.2	
PC 0.9%		0.9	99.1	
PC 0.4%		0.4	99.6	
Fish oil				100

Table S2.

Composition of employed samples used in SANS measurements.

		<i>Conc. (% w / w)</i>						
	Codes	CAS	PC	D ₂ O	Fish oil	Hexadecane	d-Hexadecane	NaCl
SANS/Yellow Submarine	CAS/PC 1.05/1.75 Fish oil-in-D ₂ O	1.05	1.75	27.2	70			
	CAS/PC 1.05/1.75 Hexadecane-in-D ₂ O	1.05	1.75	27.2		16.8	53.2	
	CAS/PC 1.4/0.4 Fish oil-in-D ₂ O	1.4	0.4	28.2	70			
	CAS/PC 1.4/0.8 Fish oil-in-D ₂ O	1.4	0.8	27.8	70			
	PC 6%		6	94				
	CAS 10%	10		90				
	CAS 8%	8		92				
	CAS 7%	7		93				
	CAS 6%	6		94				
	CAS 5%	5		95				
	CAS 4%	4		96				
	CAS 3%	3		97				
	CAS 2%	2		98				
	CAS 1%	1		99				
	CAS 0.4%	0.4		99.6				
Aqueous p.	3.5	5.8	90.7					
SANS/Larmor	CAS/PC 1.05/1.75 Fish oil-in-D ₂ O	1.05	1.75	27.2	70			
	CAS/PC 1.05/1.75 Hexadecane-in-D ₂ O	1.05	1.75	27.2		16.8	53.2	
	CAS/PC 1.05/0.88 Fish oil-in-D ₂ O	1.05	0.88	28.1	70			
	CAS/PC 1.05/0.35 Fish oil-in-D ₂ O	1.05	0.35	28.6	70			
	PC 6%		6	94				
	PC 1.8%		1.8	98.3				
	PC 0.9%		0.9	99.1				
	PC 0.4%		0.4	99.6				
	CAS 5%	5		95				
	CAS 5% + NaCl 5 %	5		90				5

Table S3.

Scattering contrast considerations

Compound	Chemical formula	Molecular weight (g/mol)	Volume (\AA^3)	Density (g/cm^3)	SLD (nm ³)	s_{ic} (nm ³)	e (nm ³)
H ₂ O		18.0006	29.89	1.000	-56.17	5371	334.6
D ₂ O		20.0272	29.89	1.113	640.5	137	334.6
hex	C ₁₆ H ₃₄	226.2029	485.93	0.773	-42.92	5616	267.5
d-hex	C ₁₆ D ₃₄	260.6554	486.88	0.889	684.25	143	267.0
PC	C ₄₂ H ₈₀ NO ₈ P	757.4982	1221.2	1.03	33.38	5258	342.3
CAS (*)	(*)	22951	29271	1.302	179.6	4364	418.8
CAS(**)	(**)	23297	29271	1.322	301.3	3451	418.8
Om-3	C ₆₀ H ₉₂ O ₆	908.77	1622.6	0.93	55.1	4551	318.0

Coherent SLD are in units $\text{fm} / \text{nm}^3 = 10^{-6} \text{nm}^{-2}$

Incoherent s in units $\text{barn} / \text{nm}^3 = 10^{-3} \text{1/cm} \rightarrow$ divide further by 4π

(*) CAS was calculated based on alpha caseinate with formula C₁₀₃₅ H₁₅₉₀ N₂₆₅ O₃₁₆ S₅

(**) CAS was calculated based on deuterated caseinate with formula C₁₀₃₅ D₃₄₂H₁₂₄₈ N₂₆₅ O₃₁₆ S₅, where it is assumed that 342 hydrogens are exchanged in D₂O. This gives contrast match point at 41 % in deuterated water.

Table S4

Droplet sizes (diameters) and specific surface areas of fish oil-in-water and hexadecane emulsions

Emulsions	Volume Weighted Mean D[4,3] (μm)	Surface Weighted Mean D[3,2] (μm)	Specific Surface Area (m^2/g)
CAS/PC 1.05/1.75 Fish oil-in-D ₂ O	12.87 \pm 0.23	11.61 \pm 0.20	0.56 \pm 0.01
CAS/PC 1.05/1.75 Hexadecane-in-D ₂ O	14.42 \pm 0.63	11.62 \pm 0.07	0.60 \pm 0.00

Table S5

Droplet sizes, specific surface areas and relative protein content in the aqueous phase of fish oil-in-H₂O emulsions produced with different combinations of CAS and PC.

Emulsion code	Surface Weighted Mean D[3,2] (μm)	Volume Weighted Mean D[4,3] (μm)	Specific Surface Area (m ² /g)	Non-adsorbed CAS in the aqueous phase after
CAS/PC	13.13 ± 0.39	18.02 ± 0.70	0.65 ± 0.02	22
CAS 1.05	20.04 ± 0.21	23.02 ± 0.23	0.43 ± 0.00	23
PC 1.75	9.71 ± 12.84	55.59 ± 54.78	7.03 ± 9.29	0
CAS 2.8	0.82 ± 0.01	8.54 ± 0.17	10.40 ± 0.14	10
PC 2.8	12.07 ± 2.05	46.53 ± 11.34	0.72 ± 0.12	0

Table S6

Fitting parameters using two different form factor for particle form with increasing CAS concentration. The first two parameters are for spherical (shell) model and the second two for multi-arm model. The actual volume fraction of the particles is calculated using particle size $R_{HS} = 10.2$ nm and molar mass 540 kDa.

Conc.	1 %	2 %	3 %	4 %	5 %	6%	7 %	8 %	10%
R_{HS}	11.6	11.5	11.2	11.0	10.9	10.8	10.7	10.6	10.5
η^*	0.10	0.15	0.20	0.22	0.25	0.27	0.28	0.29	0.31
R_{HS}	8.9	9.8	10.1	10.2	10.2	10.3	10.2	10.2	10.2
η^*	0.13	0.17	0.22	0.24	0.27	0.29	0.30	0.31	0.33
η	0.04	0.08	0.12	0.17	0.21	0.25	0.29	0.33	0.42

Fig. S1 plots the Percus-Yevick (P-Y) volume fractions for CAS in water systems. With increasing polydispersity, the fitting approaches expected linear correspondence between volume fraction and CAS concentration. The limiting slope at small concentrations agrees with ca. 540 kDa mass of protein per particle, assuming 80 % hydration. This also agrees with the absolute intensity (see Fig. S2 below)

Fig. S2 plots the CAS mass considerations. The blue curve describes the form factor that was used for fitting of the peak region corresponding to the particle core. The black curve represents particle whose size is according to Guinier fit, which may overestimate the physical shell size, but gives reasonable extrapolation of the total mass. Therefore the particles may be estimated to have dense core of approximately 100 kDa and less dense shell approximately 400 kDa.

Fig. S3 plots combined small-angle scattering data of employed PC in water systems divided by the concentration of the PC in H₂O or D₂O.

Fig. S4 shows calculation of scattering pattern for CAS/PC emulsion in D₂O/fish oil and D₂O/deuterated hexadecane. Both calculation are based on true densities having (S/V) as a free parameter. The curves furthermore include calculated incoherent background, which had to be reduced by 25 %. Also shown are the calculated contribution from random mixture of supposedly rigid D16 and H16 chains based on composition and approximate molecule dimensions and molecular volume of hexadecane as q^{-1} . The fit to the D₂O/deuterated hexadecane scattering without this contribution is shown for comparison.

Fig. S5 plots SAXS patterns from CAS/PC emulsions after subtraction of CAS contribution.

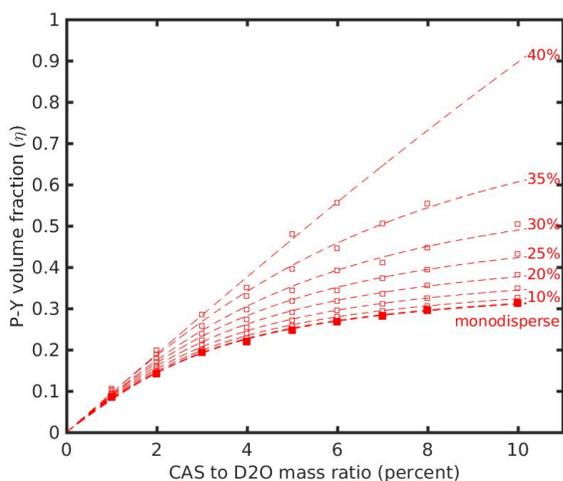


Fig S1. The determined P-Y volume fraction for CAS against the CAS to D₂O mass ratio using either monodisperse or polydisperse distribution on RHS.

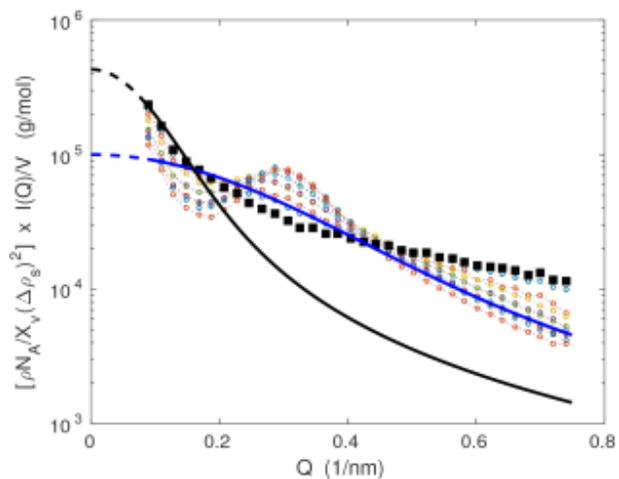


Fig. S2. Measured SANS scattering curves of CAS in D₂O normalized by concentration and multiplied by constant such that y-axis intersection gives directly the protein mass. The black markers show a curve that has been extrapolated to zero concentration for each Q-value.

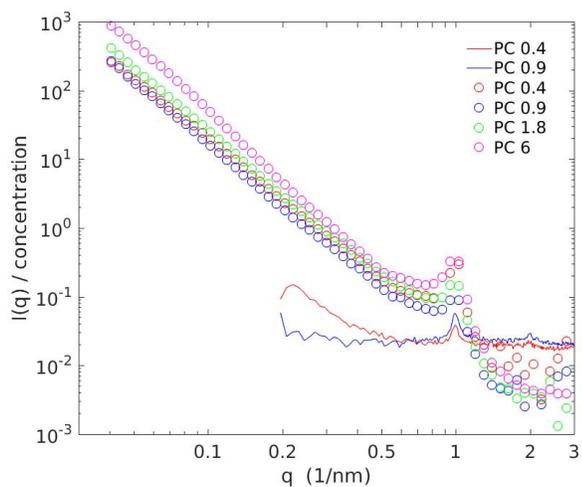


Fig S3. SAXS (solid lines) and SANS (circles) patterns of employed PC in $\text{D}_2\text{O}/\text{H}_2\text{O}$ for various concentrations. Each SAXS intensity is normalized to agree with respective SANS curve.

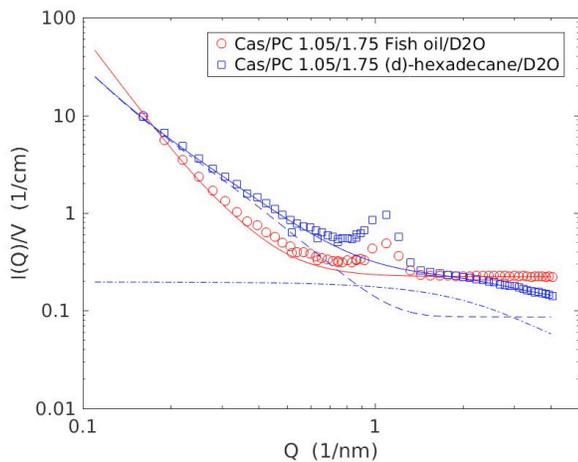


Fig. S4. Calculation of scattering pattern for CAS/PC emulsion in D_2O /fish oil (red) and D_2O /deuterated hexadecane. The contribution of individual rigid hexadecane solvent molecules (dashed line) and the fit to the scattering pattern of D_2O /deuterated hexadecane without this contribution (dash-dot line).

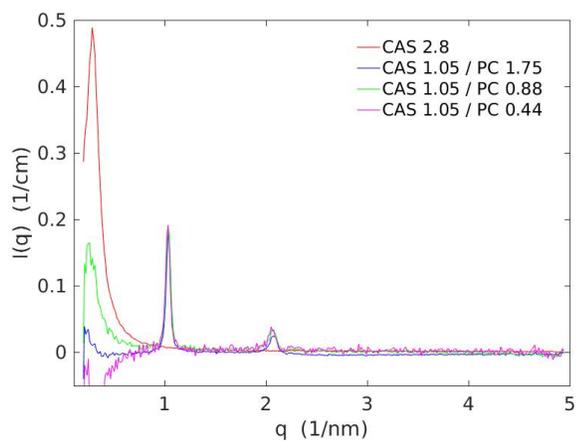


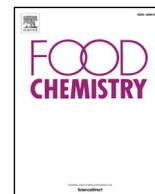
Fig S5. SAXS patterns from CAS/PC emulsions with varying PC concentration. From all curves, the contribution of pure CAS 1.05 emulsion was subtracted to bring out the contribution added PC. Apart from the uppermost curve, the curves are divided by PC concentration.

PAPER IV

Yesiltas, B., Sørensen, A. M., García-Moreno, P. J., Anankanbil, S., Guo, Z., Jacobsen, C., 2018.

**Combination of sodium caseinate and succinylated alginate improved stability of
high fat fish oil-in-water emulsions.**

Food Chemistry, 255, 290–299.



Combination of sodium caseinate and succinylated alginate improved stability of high fat fish oil-in-water emulsions



Betül Yesiltas^a, Ann-Dorit Moltke Sørensen^a, Pedro J. García-Moreno^a, Sampson Anankanbil^b, Zheng Guo^b, Charlotte Jacobsen^{a,*}

^a Division of Food Technology, National Food Institute, Technical University of Denmark, Denmark

^b Division of Lipid Biotechnology and Engineering, Department of Engineering, Aarhus University, Denmark

ARTICLE INFO

Keywords:

Lipid oxidation
Omega-3
50–70% o/w emulsion
Emulsifier
Modified alginate
Physical stability
Oxidative stability
Cod liver oil

ABSTRACT

Sodium caseinate (CAS) and commercial sodium alginate (CA), long chain modified alginate (LCMA) or short chain modified alginate (SCMA) were used in combination for emulsifying and stabilizing high fat (50–70%) fish oil-in-water emulsions. Physical (creaming, droplet size, viscosity and protein determination) and oxidative (primary and secondary oxidation products) stabilities of the emulsions were studied during 12 days of storage. Creaming stability was higher for emulsions produced with alginates and CAS compared to emulsions prepared with only CAS. Combined use of CAS + LCMA performed better in terms of physical stability compared to emulsions produced with only CAS. However, the oxidative stability of this emulsion was inferior probably due to the presence of an unsaturated carbon chain in LCMA structure. CAS + SCMA emulsions not only showed better physical stability such as smaller droplet size, lower creaming and higher viscosity, but also had an improved oxidative stability than emulsions produced with only CAS.

1. Introduction

Long chain (LC) omega-3 polyunsaturated fatty acids (PUFA) have been found to decrease cardiovascular diseases and improve immune system and mental health (Song et al., 2016; Wysoczański et al., 2016; Nichols, McManus, Krail, Sinclair, & Miller, 2014). LC omega-3 PUFAs such as eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic (DHA, 22:6) can only be synthesized from alpha linolenic acid in the human body with a limited conversion rate. As fish oil is a good source for LC omega-3 PUFAs, efforts have been made to enrich food products with fish oil to reach the recommended daily intake levels of LC omega-3 PUFAs (EFSA Panel on dietetic Products, 2010).

Oil-in-water emulsions are often used as a base for delivering bioactive compounds such as omega-3 PUFAs by enhancing their solubility and oxidative stability in food systems (McClements, Decker, & Weiss, 2007). Many studies have been carried out evaluating low fat (up to 30%) fish oil-in-water emulsions for enriching food systems with omega-3 PUFAs (Let, Jacobsen, & Meyer, 2007; Berton-Carabin, Ropers, & Genot, 2014; García-Moreno, Guadix, Guadix, & Jacobsen, 2016). However, to the best of the authors' knowledge, only a few studies focusing on the stabilization of high fat (> 50%) fish oil-in-water emulsions have been reported (Horn et al., 2011; Horn, Nielsen, Jensen,

Horsewell, & Jacobsen, 2012; Yesiltas, García-Moreno, Sørensen, & Jacobsen, 2017). High fat fish oil-in-water delivery emulsions provide advantages when enriching foods with omega-3 PUFAs due to their higher fish oil content compared to low fat emulsions, which means that lower amounts of emulsions would be required for enrichment. This allows minimum modification of the original recipe and makes the enrichment process easier, especially for foods containing high amounts of fat such as mayonnaise, cream cheese and dressings.

Nevertheless, both physical and oxidative stability challenges arise in high fat fish oil-in-water emulsions, which might differ from low fat emulsions. Hadnadev, Dokic, Krstonosic, & Hadnadev (2013) reported that increasing oil content might result in larger droplet sizes at lower emulsifier concentration and less intense homogenization. Thus, it might make the high fat emulsions more prone to creaming, although they normally have higher viscosity compared to low fat emulsions (e.g. due to the higher ratio oil:water). Moreover, high concentration of dispersed phase leads to an emulsion packed with droplets and thereby larger total interfacial area. This might favor oxidation of omega-3 PUFA as oxidation is claimed to be initiated at the interface and then propagated to the lipid phase (Jacobsen, Adler-Nissen, & Meyer, 1999; McClements & Decker, 2000; Sørensen et al., 2007). Lipid oxidation occurs due to the high content of fish oil rich in omega-3 PUFAs which

* Corresponding author at: National Food Institute, Technical University of Denmark, Kemitorvet, Building 204, 2800 Kgs. Lyngby, Denmark.

E-mail addresses: betye@food.dtu.dk (B. Yesiltas), adms@food.dtu.dk (A.-D.M. Sørensen), pejeg@food.dtu.dk (P.J. Garcia-Moreno), sampsonanankanbil@eng.au.dk (S. Anankanbil), guo@eng.au.dk (Z. Guo), chja@food.dtu.dk (C. Jacobsen).

<https://doi.org/10.1016/j.foodchem.2018.02.074>

Received 4 August 2017; Received in revised form 27 January 2018; Accepted 13 February 2018

Available online 14 February 2018

0308-8146/ © 2018 Elsevier Ltd. All rights reserved.

makes the emulsion susceptible to oxidation.

Physical and oxidative stability are commonly achieved by using emulsifiers and stabilizers, which have good emulsifying and stabilizing properties as well as antioxidant activity. Hence, selection of appropriate emulsifiers is crucial for the final physical and oxidative stability of the emulsion. Sodium caseinate (CAS) and sodium alginate (CA) are commercial emulsifier and stabilizer, respectively; which have previously been reported to be used in combination in oil-in-water emulsions (Pallandre, Decker, & McClements, 2007; Sosa-Herrera, Lozano-Esquivel, Ponce de León-Ramírez, & Martínez-Padilla, 2012). A previous study in our lab showed that high fat emulsions prepared at pH 7 and stabilized with CAS were more oxidatively stable compared to other emulsions stabilized with phospholipid based emulsifiers and to neat fish oil (Horn et al., 2011). This is mainly explained by the metal chelating activity of CAS and to its flexible structure, which allowed a better coverage of the droplets (Pallandre et al., 2007; Sosa-Herrera et al., 2012; Berton-Carabin et al., 2014). Furthermore, our recent study indicated that combinations of CAS and CA (used as thickening agent) successfully stabilized high fat (50–70%, w/w) fish oil-in-water emulsions both in terms of physical and oxidative stability, but it would still be an advantage if oxidative stability could be further improved (Yesiltas et al., 2017).

Alginates have recently been modified with succinic anhydride in order to increase their antioxidant properties and make them surface active (Falkeborg et al., 2014; Falkeborg and Guo, 2015). For instance, Falkeborg and Guo (2015) reported that 30% oil-in-water emulsions stabilized with 3% of alginate modified with dodecenyl succinic anhydride (SAC12) had lower creaming and higher oxidative stability compared to emulsions stabilized with β -lactoglobulin or CA. This was attributed to: i) a reduction in droplet size as a result of the improved interfacial properties of the modified alginate, ii) an improved physical barrier at the oil-water interface due to the presence of the modified alginate, which protect the lipid from pro-oxidants, and iii) an enhanced radical scavenging and metal chelating properties of the modified alginates in emulsions due to the additional carboxyl groups originating from modification as well as bringing the antioxidant active sites of the molecule close to the interface. Therefore, considering these properties, it was hypothesized that the combined use of CAS and CA/modified alginates would provide better physical stability compared to emulsions produced only with CAS by decreasing the droplet size and creaming. Moreover, modified alginates, which had improved emulsifying abilities and antioxidant activities, were expected to locate at the water-oil interface, thereby providing better oxidative stability compared to CA in high fat fish oil-in-water emulsions. In addition, the chain length of the modified alginates is expected to influence their location in emulsions and, thereby their physical and oxidative stabilities. Scientific significance of employing modified stabilizers/emulsifiers in improving the stability of oil-in-water emulsions is to understand the behaviors of these modified compounds which might provide interfacial engineering solutions for satisfying the needs of food industry for producing health promoting food products.

In light of the above, this study aimed to improve physical and oxidative stability of high fat oil-in-water omega-3 delivery emulsions by evaluating the combined use of CAS and modified alginates. Three types of alginates with hypothetically different interfacial and antioxidant properties were assayed in combination with CAS: a) commercially available sodium alginate (commercial alginate – CA), b) alginate modified with succinic anhydride (SACO) (short chain modified alginate – SCMA), and c) alginate modified with dodecenyl succinic anhydride (SAC12) (long chain modified alginate – LCMA). Particularly, the effect of oil content (50, 60 and 70%, w/w) as well as the combination of emulsifiers/stabilizers (CAS, CAS + CA, CAS + LCMA, CAS + SCMA) were evaluated with respect to the physical (creaming, droplet size, viscosity and protein determination) and oxidative stability (primary and volatile secondary oxidation products) of high-fat fish oil-in-water emulsions.

2. Materials and methods

2.1. Materials

Cod liver oil was provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway), and stored at -40°C until use. Peroxide value was 0.12 ± 0.08 meq peroxide/kg oil. Alpha-, gamma- and delta-tocopherol results were reported as 250 ± 1.9 , 118 ± 1.2 and 48 ± 0.9 $\mu\text{g/g}$ cod liver oil, respectively. Sodium caseinate (Miprodan 30) was kindly donated by Arla Foods Ingredients Amba (Viby J, Denmark). Arla reported a protein content of 92% in sodium caseinate for Miprodan 30. Commercial sodium alginate (Grindsted® Alginate FD 170) was provided by DuPont (Brabrand, Denmark). Modified alginates SAC0 (SCMA) and SAC12 (LCMA) were produced according to the method described by Falkeborg, Paitaïda, Shu, Pérez, & Guo (2015). SCMA was produced by modifying commercial sodium alginate with SAC0 and LCMA was produced by modifying commercial sodium alginate with SAC12 which includes an unsaturated double bond (see the Supplementary material for chemical structures of both modified alginates). The degree of succinylation of SCMA and LCMA were $28.63 \pm 0.02\%$ and $35.30 \pm 0.01\%$, respectively.

2.2. Emulsion preparation and sampling

Aqueous phase of emulsions were prepared by dissolving both emulsifiers in distilled water and left overnight on a stirrer at 4°C and the day after pH was adjusted to 7.0 with 2M HCl or 2M NaOH. Emulsions were produced in 500 g batches in a Stephan Universal mixer (Stephan, UMC5, Hameln, Germany) equipped with an emulsification blade as described by Horn et al. (2011). Fish oil concentration with 3 different levels (50, 60 and 70%, w/w) and combination of emulsifiers with 4 different types (CAS + LCMA, CAS + SCMA, CAS + CA and only CAS) were the two factors set for the experimental design. According to our previous study, 1.4% (w/w) total emulsifier and 1.2:1 ratio CAS:CA were found to be the optimum values for stabilizing high fat emulsions using combinations of CAS and CA (Yesiltas et al., 2017). Thus, these were the values used in this study for total amount of CAS + alginate and ratio CAS:alginate. Twelve emulsions were produced (Table 1). After emulsions were produced, Fe^{2+} (0.03% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solubilized in water, corresponding to approximately $100 \mu\text{m}$ in the final emulsion) and 0.05% sodium azide were added into the emulsions in order to accelerate oxidation and prevent microbial growth, respectively. Each emulsion was divided in portions of 85 g, which were stored in 100 mL glass bottles at room temperature in darkness for 12 days and samples were taken on days 0, 2, 5, 8 and 12. Creaming index was measured on days 1, 5, 9 and 12. Droplet size and viscosity of emulsions were measured on days 1 and 12. Protein content in the aqueous phase was measured using frozen samples from day 5. Peroxide value, tocopherols and volatile compounds were analyzed on days 0, 2, 5, 8 and 12. Samples for lipid oxidation analysis were kept at -40°C until analysis.

2.3. Characterization of emulsions

2.3.1. Creaming index

Creaming of emulsion samples were followed in 100 mL storage bottles. Creaming index was calculated by measuring the height of total emulsion (a) and height of water phase separated in the bottom of the bottle (b); following that (b) divided by (a) and multiplied by 100. This gave the percentage of the creaming of the emulsion sample on a specific sampling point and this was calculated for all sampling points without replicates.

2.3.2. Droplet size

Droplet size of the emulsions was measured by laser diffraction (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK) using the method described by Let et al. (2007) and Horn et al. (2011).

Table 1
Experimental design showing sample name, emulsifier combination and oil content (%).

Emulsion code	Emulsifiers ^a	Fish oil%, w/w of total emulsion
CAS + LCMA-50%	CAS + LCMA (sodium caseinate + long chain modified alginate)	50
CAS + LCMA-60%	CAS + LCMA (sodium caseinate + long chain modified alginate)	60
CAS + LCMA-70%	CAS + LCMA (sodium caseinate + long chain modified alginate)	70
CAS + SCMA-50%	CAS + SCMA (sodium caseinate + short chain modified alginate)	50
CAS + SCMA-60%	CAS + SCMA (sodium caseinate + short chain modified alginate)	60
CAS + SCMA-70%	CAS + SCMA (sodium caseinate + short chain modified alginate)	70
CAS + CA-50%	CAS + CA (sodium caseinate + commercial alginate)	50
CAS + CA-60%	CAS + CA (sodium caseinate + commercial alginate)	60
CAS + CA-70%	CAS + CA (sodium caseinate + commercial alginate)	70
CAS-50%	CAS (sodium caseinate)	50
CAS-60%	CAS (sodium caseinate)	60
CAS-70%	CAS (sodium caseinate)	70

^a Total emulsifier content was 1.4% (w/w, of total emulsion) for all emulsions and the ratio of CAS to alginates was 1.2.

Results were given as the volume weighted mean diameter $D[4,3] = \sum n_i d_i^4 / \sum n_i d_i^3$. Measurements were carried out in duplicates.

2.3.3. Apparent viscosity

Viscosity was measured using a stress-controlled rheometer (Stresstech, Reologica Instruments AB, Lund, Sweden) equipped with a CC25 standard bob cup system in a temperature vessel. Measurements were done at 25 °C over a shear stress range from 0.0125 to 400 Pa. Apparent viscosity results were obtained at a shear rate of 20 s⁻¹ and expressed in Pa·s. Viscosities were measured twice on each emulsion.

2.3.4. Relative protein content in the aqueous phase

Protein determination in the aqueous phase of emulsions was done mainly based on the method described by Jacobsen, Meyer, & Adler-Nissen (1998). Emulsions (~20 g) were centrifuged for 10 min at 25,400g and 10 °C (Sorvall RC-6 PLUS, Thermo Fisher Scientific, Osterode, Germany; rotor SS-34) and the oil phase was removed by the use of a pipette. The rest was mixed with distilled water (1:2) and then subjected to ultracentrifugation (Beckman Ultracentrifuge L8-60M, Fullerton, CA; rotor 21102) for 16 h at 106,979g and 15 °C, and once again the aqueous phase was extracted by the use of a syringe. The aqueous phase was diluted 1:6 in distilled water and protein concentration was determined using a BCA protein assay reagent kit (Pierce, Thermo Scientific, Rockford, IL, USA) by measuring in a spectrophotometer (Shimadzu, UV mini 1240, Kyoto, Japan) at 562 nm. Results were presented as relative protein content in the aqueous phase which was obtained by calculating the percentage of protein content in the aqueous phase compared to total protein amount added into the emulsion.

2.4. Lipid oxidation in emulsions

2.4.1. Primary oxidation products – peroxide value (PV)

For determination of primary oxidation products, a lipid extract was prepared according to the method described by Bligh and Dyer (1959) using 5 g of emulsion for each extraction and a reduced amount of solvent (60 mL of methanol and chloroform, 1:1). PV was subsequently measured on the lipid extracts by colorimetric determination of iron thiocyanate on a spectrophotometer (Shimadzu, UV mini 1240, Kyoto, Japan) at 500 nm, as described by Shantha and Decker (1994). Measurements were carried out in duplicate.

2.4.2. Tocopherol content – HPLC

Tocopherol contents of emulsions were determined by HPLC (Agilent 1100 Series; Column: Waters Spherisorb 3 μm Silica; 4.6 × 150 mm) using lipid extracts (see Section 2.4.1) which were further evaporated and re-dissolved in heptane. Tocopherol analyses were carried out according to the official AOCS method (AOCS, 1998) in duplicates.

2.4.3. Secondary volatile oxidation products – dynamic headspace (DHS) GC–MS

Secondary volatile oxidation products were analyzed according to the method described by Jacobsen et al. (1999). Approximately 4 g of emulsion was mixed with 2 mL antifoam and 10 mL distilled water in a 100 mL purge bottle. The bottle was heated in a water bath at 60 °C while purging with nitrogen (flow 150 mL/min, 30 min). Volatile compounds were trapped on Tenax GR tubes and separated in a gas chromatograph (Agilent Technologies, 6890N Network GC System) on a 30 m DB 1701 fused silica capillary column (0.25 mm i.d., 1 μm film thickness; Agilent Technologies, J&W GC Columns, USA). The oven program had an initial temperature of 45 °C for 5 min, increasing with 1.5 °C/min until 55 °C, with 2.5 °C/min until 90 °C, and with 12.0 °C/min until 220 °C, where the temperature was held for 4 min. The individual volatile compounds were analyzed by mass spectrometry (Agilent 5973 Network Mass Selective Detector, Agilent Technologies, electron ionization mode, 70 eV; mass to charge ratio scan between 30 and 250) and identified by MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard). Quantification was done through calibration curves where the external standards employed were 2-ethylfuran, 1-penten-3-one, 1-penten-3-ol, (*E*)-2-pentenal, hexanal, (*E*)-2-hexenal, (*Z*)-4-heptenal, 2-pentyl-furan, (*E*)-2-heptenal, benzaldehyde, (*E,E*)-2,4-heptadienal, nonanal, (*E,Z*)-2,6-nonadienal. Measurements were made in triplicate for each sample.

2.5. Statistical analyses

Statgraphics XVII (Statpoint Technologies, Inc., Virginia, USA) was used to carry out the statistical analysis. Multifactor analysis of variance (ANOVA) was performed followed by Fisher's least significant difference. The significance of all terms in the models was judged statistically by computing the p-value at a confidence level 1-α = 95%. Principal component analysis (PCA) was done by Latentix 2.12 (Latentix, Copenhagen, Denmark). The PCA was carried out with the emulsions as objects and creaming, viscosity, droplet size, oil content, protein content in the aqueous phase, peroxide value and volatile compounds as variables. Data set were autoscaled to make the variables contribute equally to the model.

3. Results and discussion

3.1. Characterization of emulsions

3.1.1. Relative protein content in the aqueous phase

Relative protein content in the aqueous phase decreased significantly ($p < 0.05$) with increasing fish oil content from 50% to 70% for the emulsions produced with CAS + CA (added CAS was 0.76% of the total emulsion) (Fig. 1a). This indicated that more CAS was adsorbed at the oil-water interface when the fish oil content increased;

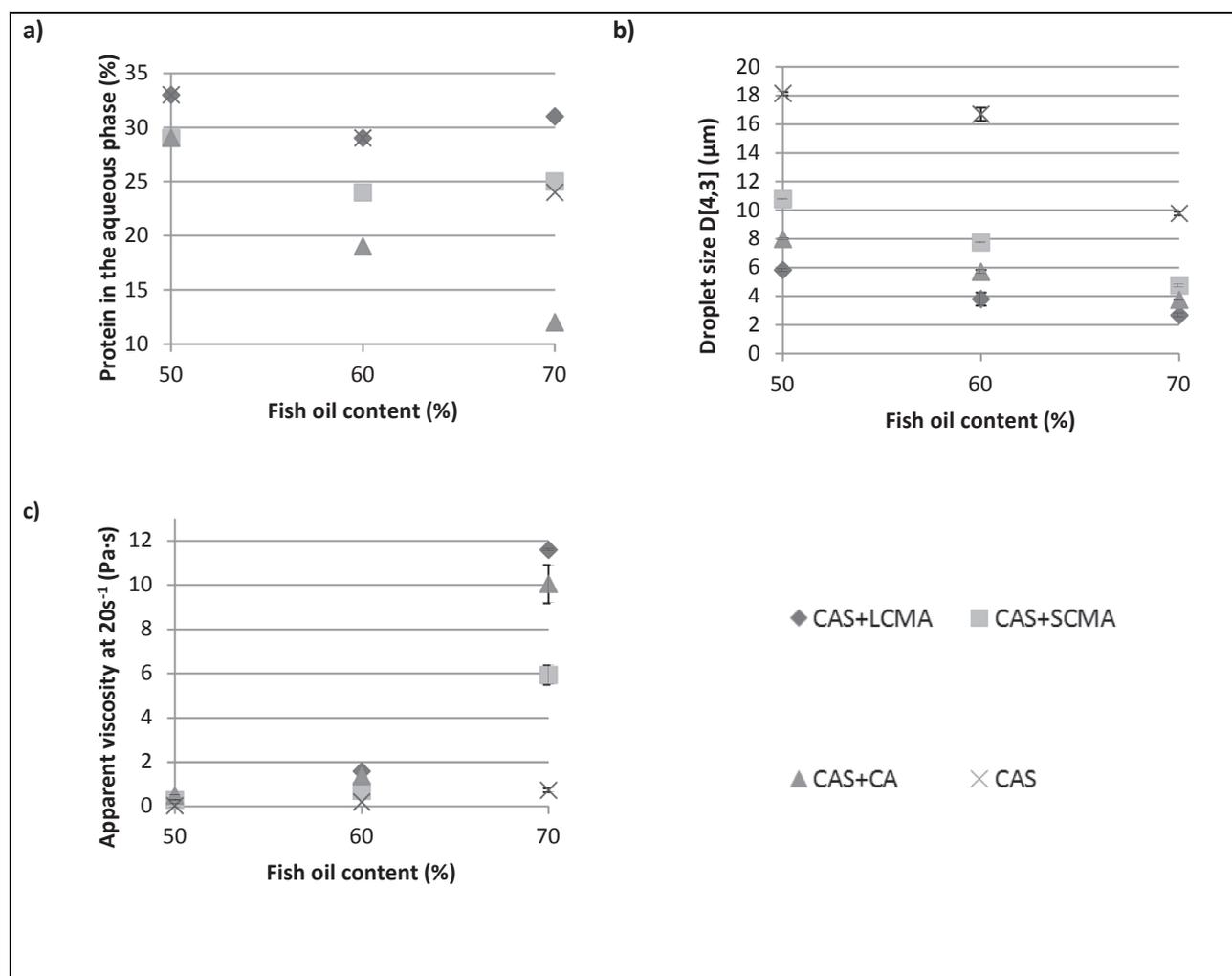


Fig. 1. Effect of fish oil content and emulsifiers on: a) relative protein content in the aqueous phase, b) droplet size (day 1), and c) apparent viscosity at 20 s^{-1} (day 1).

which correlated well with the larger interfacial area obtained ($D[3,2]$ of 5.25 ± 0.12 and $2.07 \pm 0.20\ \mu\text{m}$ for CAS + CA 50% and CAS + CA 70%, respectively) as a consequence of the higher oil content and smaller droplet sizes. Moreover, the relative protein content in the aqueous phase of CAS + LCMA-70% (31%) or CAS + SCMA-70% (25%) was higher compared to CAS + CA-70% (12%); thereby it led us to conclude that LCMA and SCMA were better incorporated at the interface (i.e. replacing CAS) compared to CA. This finding was in line with the study carried out by Falkeborg and Guo (2015), who pointed out that CA tends to locate at the water phase instead of being adsorbed at the oil-water interface.

3.1.2. Droplet size

Droplet size of the emulsions did not show any significant increase during storage; therefore only data from day 1 was shown (Fig. 1b). Droplet size decreased significantly ($p < 0.05$) with the increasing fish oil content from 50% to 70% for all emulsions independently of the combination of emulsifiers used. This could be due to the relative increase in the emulsifier content in the aqueous phase when the oil content in the emulsion increased and more concentrated water phase was obtained, which in the end directly affects the viscosity of the final emulsion (Tesch & Schubert, 2002; Aken, 2006). The mechanism might work as follows: high viscosity of the water phase might allow better disruption of oil droplets by Stephan mixer. Moreover, having higher oil fraction in an emulsion also increases the viscosity of the mixture inside the Stephan mixer, which may also enhance the disruption of oil droplets.

Droplet size increased significantly depending on the emulsifiers employed in the following order CAS + LCMA < CAS + CA < CAS + SCMA < CAS. Falkeborg and Guo (2015) also found that 30% oil-in-water emulsions produced by stirring using only LCMA as emulsifier had significantly smaller droplets ($7.64\ \mu\text{m}$) compared to emulsions stabilized only with CA ($22.02\ \mu\text{m}$), which indicated the superior emulsifying properties of LCMA. Emulsions prepared with only CAS had significantly bigger droplet sizes compared to the rest of the emulsions, which indicates that combining commercial or modified alginates with CAS contributed to obtaining smaller droplet sizes. As commented above, this might be due to the increase in viscosity of the water phase in the presence of CAS and alginates together, which allowed more efficient disruption of the oil in small droplets in the Stephan mixer. Moreover, emulsions prepared with CAS + LCMA had significantly smaller droplets than CAS + SCMA, which indicated that modifying CA with a long fatty acid chain led to a faster adsorption at the water-oil interface during homogenization and to the stabilization of more oil droplets before they coalesced. These results were in agreement with the significantly higher protein content in the water phase of emulsions prepared with CAS + LCMA compared to CAS + SCMA (Fig. 1a), which indicated that more LCMA adsorbed at the water-oil interface compared to SCMA. LCMA had a slightly higher modification degree (35.3%) than the SCMA (28.6%). The modification degree may also impact the differences observed in droplet size between LCMA and SCMA due to the increase in surface activity with increased modification. However, the impact of the modification degree has to be further evaluated.

On the contrary, even though SCMA showed better emulsifying capacity compared to CA (according to results from protein content in the aqueous phase); the emulsions stabilized with CAS + CA had smaller droplets than the emulsion stabilized with CAS + SCMA. The latter correlated well with the higher viscosity of the emulsion stabilized with CAS + CA (Fig. 1c); which permitted a more severe disruption of the oil in droplets in the homogenizer employed. In any case, these findings suggest that CA and SCMA had different mechanisms for physically stabilizing the emulsions; CA worked as a stabilizer whereas SCMA worked as an emulsifier in the emulsion system.

3.1.3. Apparent viscosity

All emulsions showed shear thinning behavior. This is in line with another study which reported that the addition of xanthan gum to 20% v/v menhaden oil-in-water emulsions emulsified with whey protein isolate also led to shear thinning behavior (Sun, Gunasekaran, & Richards, 2007). Apparent viscosity of the emulsions at 20 s^{-1} shear rate did not change significantly during storage except for CAS + LCMA-70% (from 11.59 ± 0.04 to 10.83 ± 0.11 Pa·s), CAS + LCMA-50% (from 0.26 ± 0.01 to 0.20 ± 0.00 Pa·s) and CAS + SCMA-50% (from 0.28 ± 0.01 to 0.17 ± 0.03 Pa·s), which significantly decreased after 12 days of storage. Therefore, data was shown only for day 1 in Fig. 1c. CAS + LCMA-50% and CAS + SCMA-50%, which had significant decrease in their viscosity, contained 50% fish oil and had creaming during storage (Fig. 2). However, even though CAS + LCMA-70% had a significant decrease in its viscosity, it still had the highest viscosity value within all emulsion samples with no creaming during 12 days of storage (Fig. 2).

Results indicated that both fish oil content and emulsifier types had a significant effect on viscosity ($p < 0.05$). It was observed that the viscosity of the emulsions became higher with increasing fish oil content; whereas the droplet sizes became smaller (Section 3.1.2). This could be due to the high concentration of dispersed phase which leads to an emulsion packed with droplets i.e. close packing of emulsion droplets and thereby larger total interfacial area. This negative correlation between droplet size and viscosity might also be explained by the increased friction forces between smaller droplets caused by expanded surface-to-volume ratio of the dispersed phase, which also results in less mobility in the emulsion and therefore higher viscosity compared to having bigger droplets (Pal, 1996). Moreover, relative increase of the emulsifier content in the water phase, due to increasing oil phase at a fixed emulsifier concentration, promotes increased viscosity of the water phase. This increase in the viscosity of the water phase leads to high shear forces and results in higher viscosity of final emulsion as explained in detail under the Section 3.1.2.

Alginates are known for their thickening properties, which increase the viscosity of the products they are incorporated into (Antonov, Van Puyvelde, & Moldenaers, 2004; Chen, McClements, & Decker, 2010). Fig. 1c also shows that substitution of some of the CAS with different

alginates increased the viscosity of the emulsions significantly for all fish oil concentrations (50, 60 and 70%). For 60% and 70% fish oil contents, viscosity of the emulsions was higher when LCMA was used followed by CA and SCMA.

3.1.4. Creaming index

Creaming decreased with increasing oil content for all emulsions, which had the same total emulsifier content, but different emulsifier combinations (Fig. 2). Emulsions produced with 50% fish oil showed 10–33% creaming at the last day of the storage, whereas emulsions produced with 70% fish oil did not have any creaming except for emulsion produced with only CAS (5% creaming). Increased creaming for 50% oil-in-water emulsions compared to 70% emulsions was supported by the fact that gravitational separation increases with decreasing droplet concentration. It has a direct link to concentration of dispersed phase, since the movement of a droplet is prevented by the surrounding droplets (McClements et al., 2007). Mayonnaise was shown as a good example for retarding gravitational separation where the droplet concentration is high (e.g. 80% fat content) (McClements et al., 2007). Sun and Gunasekaran (2009) also reported that increasing oil phase volume fraction played an important role on decreasing creaming and increasing viscosity in menhaden oil-in-water emulsions emulsified with combined use of 1 or 2 wt% whey protein isolate and 0.2 wt% xanthan gum when the oil fraction increased from 20 to 40% v/v. Likewise, an increase in oil concentration (5–40%, w/w oil) led to a decrease in creaming independently of examined different triethanolamine oleate concentrations (3–20%) and homogenization time ranges (5–60 min), which was due to an increase in packing density and inter droplet interactions (Hahnadev et al., 2013).

Since emulsions with 70% fish oil stabilized with combinations of CAS and LCMA/SCMA/CA did not show creaming, the finding confirmed the contribution of alginates on retarding creaming and improving physical stability of emulsions. Additionally, emulsions prepared with only CAS had lower viscosity compared to the rest using combinations of CAS and LCMA/CA/SCMA (Section 3.1.3). The most stable emulsions against creaming were produced with CAS + CA which was due to CA's tendency to locate in the water phase instead of being located at the interface, which hindered droplets movement (Falkeborg and Guo, 2015). All emulsions with 50% fish oil content had creaming; however, results were different depending on the emulsifier types.

3.2. Lipid oxidation measurements of emulsions

3.2.1. Primary oxidation products – peroxide value (PV)

Primary oxidation products increased significantly between days 0, 2 and 5; however, there was no significant increase between days 5, 8 and 12 (Fig. 3). Emulsions prepared with 70% of fish oil oxidized significantly more than emulsions prepared with 60% and 50% fish oil.

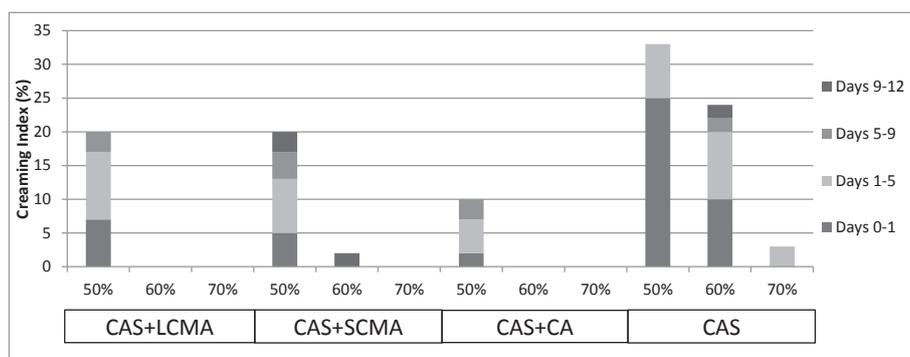


Fig. 2. Creaming index of emulsions during storage. The bars indicate the total creaming over 12 days of storage; whereas, the different grey tones indicate the creaming formed between sampling points.

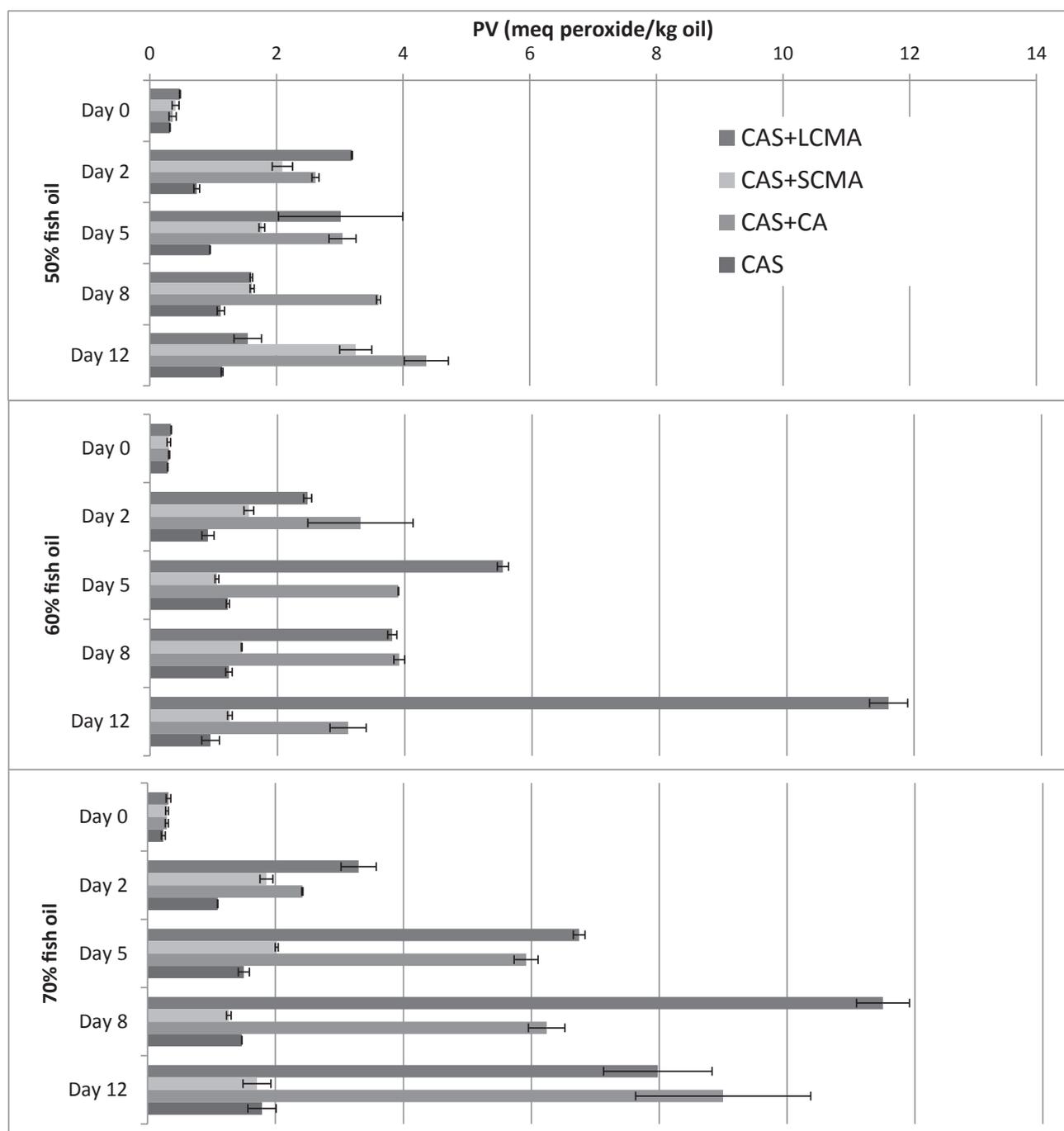


Fig. 3. Peroxide values of emulsions during storage.

High oil concentration led to lower oxidative stability, which contradicts the results found in some other studies (Osborn & Akoh, 2004; Sun & Gunasekaran, 2009; Berton-Carabin et al., 2014). However, it is important to point out that all the 50% emulsions and some of the 60% emulsions had creaming during their storage. First of all, creaming led to oil droplets being in contact with a lower amount of water phase as the droplets accumulated on the top part of the bottle and most of the water phase stayed at the bottom. Secondly, creamed droplets were closely packed, therefore some parts of the interface were in contact with other droplets' interface instead of water phase. Thereby, creaming limited the interaction between prooxidants (e.g. traces of iron) present in the aqueous phase and lipids in these emulsions.

Emulsifier type also had a significant effect on formation of primary oxidation products, as also reported by Fomuso, Corredig, & Akoh

(2002) where lecithin, Tween 20, whey protein isolate, mono-/diacylglycerols, and sucrose fatty acid ester were investigated in two different concentrations (0.25 and 1%) in 10% structured lipid oil-in-water emulsions. CAS + LCMA-60%, CAS + CA-70% and CAS + LCMA-70% were oxidized significantly more than the rest of the emulsions (< 4.4 meq peroxide/kg oil) having peroxide values of 11.6, 9.0 and 7.9 meq peroxide/kg oil, respectively (Fig. 3). Emulsions produced with CAS + LCMA had significantly higher PVs compared to the rest of the emulsions; this could be due to the unsaturated nature of LCMA (Falkeborg et al., 2015). Emulsions produced with CAS + CA had significantly higher PVs than emulsions produced with CAS + SCMA and only CAS. This might be due to the lower content of CAS in the aqueous phase of CAS + CA emulsions compared to CAS + SCMA or CAS emulsions (Fig. 1a); since CAS has metal chelating activity and

traps metal ions present in the aqueous phase (Gallaher, Hollender, Peterson, Roberts, & Coupland, 2005; Horn et al., 2012). CAS + SCMA emulsions had similar PVs as CAS emulsions and showed improved physical stability such as less creaming and higher viscosity. Moreover, better antioxidative properties of SCMA than CA and LCMA might be due to the presence of a terminal carboxyl group in its structure (Falkeborg et al., 2015), which may be involved in chelation of metal ions both at the water-oil interface and aqueous phase (Hudson, 1990). Additionally, protein determination results showed that the amount of CAS dissolved in the water phase was higher for the CAS + LCMA emulsions compared to CAS + SCMA emulsions (Fig. 1a), which indicates that SCMA was present in higher concentration in the water phase than LCMA. Hence, SCMA was expected to behave as a better metal chelator in emulsions due to its preferable location in the aqueous phase.

3.2.2. Tocopherol content

Alpha-, gamma- and delta-tocopherols were quantified on day 0 to be in the range of 220.9 ± 1.8 – 232.1 ± 2.5 , 41.6 ± 0.7 – 43.7 ± 1.5 and 100.9 ± 2.0 – 106.6 ± 4.0 mg/kg emulsion, respectively. There

were no significant decrease in delta-tocopherol and gamma-tocopherol during storage for all emulsion samples. Emulsions produced with CAS + LCMA-50, 60 and 70% as well as CAS + SCMA-60 and 70% showed a significant decrease in alpha-tocopherol content during 12 days storage (see Supplementary material). This indicated that the formation of primary and secondary oxidation products during storage was reduced by the antioxidant activity of alpha-tocopherol. Even though the consumption of alpha-tocopherol showed that it acted as an antioxidant in these emulsions and prevented some of the oxidation, CAS + LCMA emulsions still had higher oxidation compared to CAS + SCMA-60 and 70% which did not show high PV values (Section 3.2.1). Emulsions produced with only CAS or CAS + CA did not show any significant decrease in their alpha-tocopherol content during their storage.

3.2.3. Secondary oxidation products – dynamic head space (DHS) GC–MS

Fig. 4 shows the content of selected volatiles, namely 1-penten-3-ol, (Z)-4-heptenal, (E,E)-2,4-heptadienal and (E,Z)-2,6-nonadienal during storage. These compounds were chosen due to their higher concentration compared to others, as well as being representative for the rest of

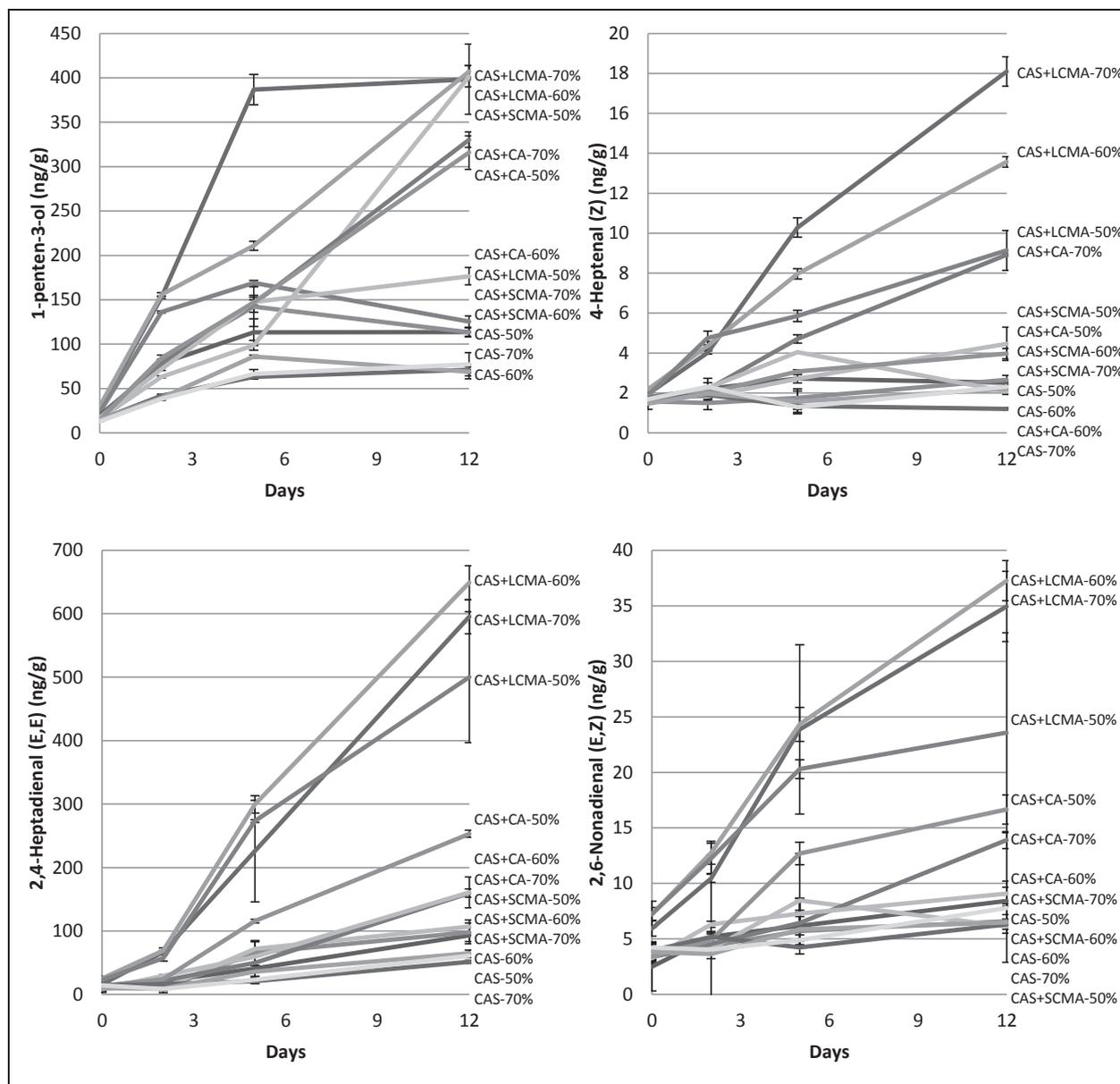


Fig. 4. Development of 1-penten-3-ol (ng/g), (Z)-4-Heptenal (ng/g), (E,E)-2,4-Heptadienal (ng/g), (E,Z)-2,6-nonadienal (ng/g) in emulsions during storage.

the volatile compounds except for nonanal which was not identified for CAS + LCMA samples. Moreover, these 4 volatile compounds are secondary oxidation products formed from oxidation of omega-3 PUFAs such as EPA and DHA (Hernandez, 2011). It was observed that the emulsions produced with CAS + LCMA were separated from the other emulsions on the 2nd day of storage by having higher concentrations of volatile compounds (Fig. 4). Emulsions CAS + LCMA-70% and CAS + LCMA-60% formed significantly higher concentrations of 1-penten-3-ol, (Z)-4-heptenal, (E,E)-2,4-heptadienal and (E,Z)-2,6-nonadienal compared to CAS + SCMA and only CAS emulsions in all levels of fish oil content on day 12. As emulsions produced with alginates and CAS have a negative surface charge due to sodium caseinate is negatively charged above its isoelectric point (pH 4.7–5.2) (Swaisgood, 1992) and both modified and commercial alginates are negatively charged due to carboxylate groups in their structure (Aken, 2006; Falkeborg et al., 2015); metal ions are expected to move towards water-oil interface due to attractive electrostatic forces. Although some of them could be chelated by carboxyl groups of LCMA or amino acid residues of CAS (Hudson, 1990; Tong, Sasaki, McClements, & Decker, 2000; Faraji, McClements, & Decker, 2004), the non-chelated metal ions could interact with the double bond which is located on the long carbon chain part of LCMA (Falkeborg et al., 2015). This is not the case for emulsions stabilized with CA and SCMA, since these alginates do not present any double bond in their structure. Additionally, CAS + LCMA emulsions had the smallest droplet sizes compared to other emulsions (Fig. 1b) and, therefore, highest interfacial area which might have favored initiation of lipid oxidation chain reactions (Jacobsen et al., 1999).

CAS + CA-70% had significantly higher formation of 1-penten-3-ol and (Z)-4-heptenal compared to CAS + SCMA-70%; whereas they had similar formation of (E,E)-2,4-heptadienal and (E,Z)-2,6-nonadienal at the last day of the storage. Least formation of primary and secondary oxidation products was observed for emulsions produced with only CAS; however, these emulsions had creaming (5–33%), which limited the interaction between prooxidants in the water phase and lipids. Therefore, it is worth noting that emulsions showed no creaming (Fig. 2) were oxidatively less stable due to more contact of oil droplet surface with prooxidants existing in the water phase such as metal ions. Another reason could be that the formed cream layer had an increased viscosity, which may hinder the diffusion of reactants (Genot, Meynier, & Riaublanc, 2003). Moreover, the higher viscosity of the emulsions

produced with CAS and alginates was expected to lead to a superior oxidative stability, as the movement of prooxidant metal ions in the water phase was expected to be slowed down (Sun et al., 2007).

On the other hand, physically stable emulsions such as CAS + SCMA-70% and CAS + SCMA-60% did not differ significantly from emulsions produced with only CAS for the formation of 1-penten-3-ol, (Z)-4-heptenal, (E,E)-2,4-heptadienal and (E,Z)-2,6-nonadienal (Fig. 4). Presumably, this is due to the fact that SCMA has one more carboxyl group in its structure compared to LCMA, which is a metal chelator. Moreover, SCMA is expected to be located more in the water phase than LCMA (see section 3.1.1), which might lead to a superior metal chelating activity in the water phase, whereas LCMA attracted metal ions at the oil-water interface.

3.3. Principal component analysis (PCA)

PCA model was used for combining all results in order to visualize the overall picture by using different data obtained from physical and oxidative stability measurements (Fig. 5). PCA showed that PV and volatile compounds were explained by PC1 (62%). Groups were formed according to emulsifier type along the PC1 axis of the PCA bi-plot which was ranked as CAS, CAS + SCMA, CAS + CA and CAS + LCMA when moving from left to right. This information suggested that oxidation was highly affected by different emulsifier combinations used in emulsions. Oil content was well explained by PC2; groups were formed according to fish oil content which was decreasing from 70% to 50% with increasing PC2 scores. When moving from upper-left to the lower-right side of the PCA plot, viscosity was increasing and droplet size and creaming were decreasing. These findings suggested that these parameters were affected both from oil content and emulsifier type (as discussed in Section 3.1), and were explained by PC1 and PC2, respectively. PC3, PC4 and PC5 were also investigated; however, they did not reveal additional information about the data.

Droplet size and creaming were located close to each other; suggesting that the emulsions with larger droplet sizes were more likely to cream. Also, oil content and viscosity were placed close to each other, confirming that higher oil content increases viscosity of the final emulsion which was in line with the discussion under the Section 3.1.3.

Emulsions CAS + LCMA-50, 60, 70% and CAS + CA-70% were located to the right side of the PCA bi-plot and so were the primary and secondary oxidation products. This shows that these emulsions had

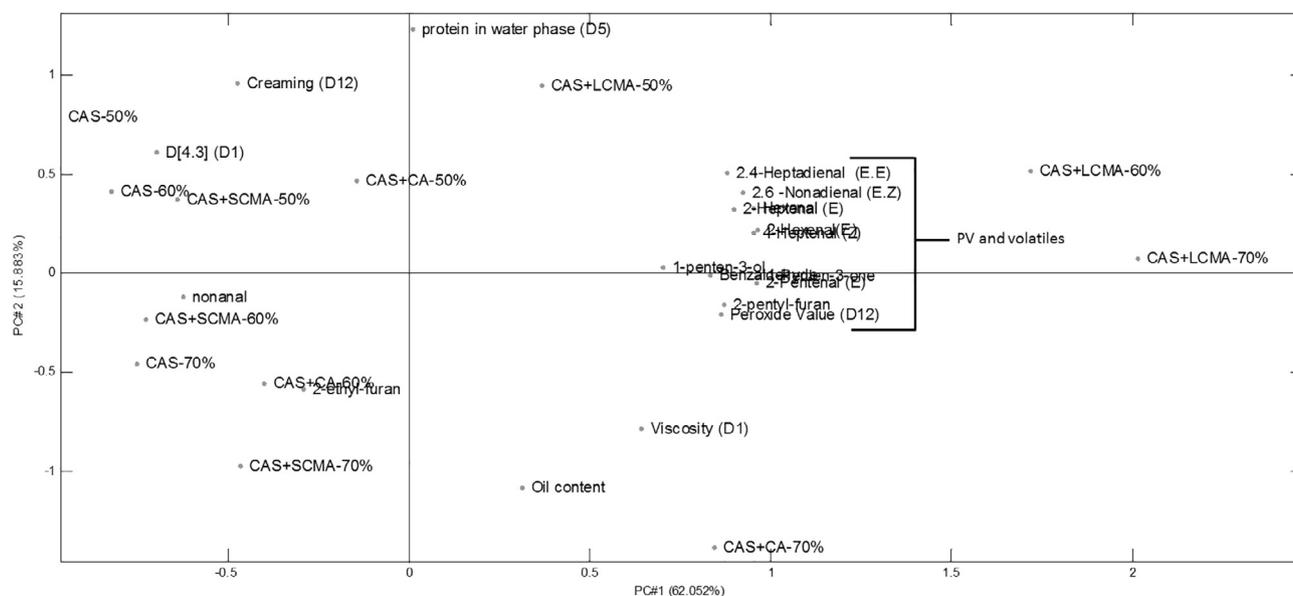


Fig. 5. Principal component analysis (PCA) plot.

lower oxidative stability compared to others. On the other hand, emulsions with only CAS, CAS + SCMA, CAS + CA-50% and CAS + CA-60% were located far from primary and secondary oxidation products, which indicated that they were more oxidatively stable. Among these samples, emulsions produced with only CAS were not physically stable in terms of creaming, had larger droplet sizes and lower viscosity (see creaming, droplet size and viscosity loadings in Fig. 5). In contrast, CAS + SCMA-70%, CAS + SCMA-60% and CAS + CA-60% were physically more stable in terms of creaming and there were no significant increase in droplet size during their storage (Sections 3.1.2). Within these three emulsions, CAS + SCMA-70% and CAS + CA-60% were located closer to oxidation products compared to CAS + SCMA-60%. Therefore, CAS + SCMA-60% was the most oxidatively stable emulsion having the smallest surface area which limits the interaction of oil and prooxidants.

4. Conclusion

Emulsions produced with alginates and CAS improved creaming stability compared to emulsions prepared with only CAS. High fish oil concentration also enhanced creaming stability regardless of emulsifier type. Both fish oil content and emulsifier type had significant effect on droplet size, viscosity, protein content in the water phase and peroxide value. As expected, emulsions produced with CAS + LCMA had better physical stability such as having smaller droplets and higher viscosity than the rest of the emulsions. This was due to LCMA's improved emulsifying capacity by being more surface active due to its long chain. However, oxidative stability of the CAS + LCMA emulsions was low compared to emulsions produced with CAS + SCMA and only CAS, due to the unsaturated carbon chain in LCMA structure, which triggered lipid oxidation. As SCMA had the advantage of having terminal hydroxyl group as an extra metal chelator either in the water phase or at the water-oil interface, emulsions produced with CAS + SCMA showed almost as good oxidative stability as emulsions produced with only CAS. Moreover, CAS + SCMA had improved physical stability as evaluated by its smaller droplet size and lower degree of creaming and higher viscosity compared to CAS emulsions. Therefore, these results show the potential of combining CAS and SCMA for the physical and oxidative stabilization of high fat (60–70%) fish oil-in-water emulsions.

Acknowledgments

We would like to thank Arla Foods Ingredients amba and Dupont for donating sodium caseinate and sodium alginate, respectively. We would also like to thank Maritex A/S, Norway for providing cod liver fish oil. Furthermore, we are grateful to Inge Holmberg and Thi Thu Trang Vu for their skillful work in the laboratory. Finally, we would like to thank the Danish Council for Independent Research Technology and Production Sciences for financing the project Mapping and Characterizing of Lipid Oxidation in Emulsified Systems (MAPOX).

The authors have declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2018.02.074>.

References

Aken, G. A. (2006). Polysaccharides in food emulsions. In A. M. Stephen, G. O. Phillips, & P. A. Williams (Eds.), *Food polysaccharides and their applications* (pp. 521–539). Taylor & Francis Group LLC.

Antonov, Y. A., Van Puyvelde, P., & Moldenaers, P. (2004). Interfacial tension of aqueous biopolymer mixtures close to the critical point. *International Journal of Biological Macromolecules*, *34*, 29–35.

AOCS Official Method Ce 8-89 (1998). *Determination of tocopherols and tocotrienols in vegetable oils and fats by HPLC*. Champaign, IL, USA: AOCS.

Berton-Carabin, C. C., Ropers, M., & Genot, C. (2014). Lipid oxidation in oil-in-water emulsions: Involvement of the interfacial layer. *Comprehensive Reviews in Food Science and Food Safety*, *13*, 945–977.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry Physiol.* *37*, 911–917.

Chen, B., McClements, D. J., & Decker, A. (2010). Role of continuous phase anionic polysaccharides on the oxidative stability of menhaden oil-in-water emulsions. *Journal of Agriculture and Food Chemistry*, *58*, 3779–3784.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) (2010). Scientific opinion on dietary reference values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. *EFSA Journal*, *8*, 1461–1568.

Falkeberg, M., Cheong, L., Gianfco, C., Sztukiel, K. M., Kristensen, K., Glasius, M., ... Guo, Z. (2014). Alginate oligosaccharides: Enzymatic preparation and antioxidant property evaluation. *Food Chemistry*, *164*, 185–194.

Falkeberg, M., & Guo, Z. (2015). Dodecanyl succinylated alginate (DSA) as a novel dual-function emulsifier for improved fish oil-in-water emulsions. *Food Hydrocolloids*, *46*, 10–18.

Falkeberg, M., Paitaida, P., Shu, A. N., Pérez, B., & Guo, Z. (2015). Dodecanyl succinylated alginate as a novel material for encapsulation and hyperactivation of lipases. *Carbohydrate Polymers*, *133*, 194–202.

Faraji, H., McClements, D. J., & Decker, E. A. (2004). Role of continuous phase protein on the oxidative stability of fish oil-in-water emulsions. *Journal of Agriculture and Food Chemistry*, *52*, 4558–4564.

Fomoso, L. B., Corredig, M., & Akoh, C. C. (2002). Effect of emulsifier on oxidation properties of fish oil-based structured lipid emulsions. *Journal of Agriculture and Food Chemistry*, *50*, 2957–2961.

Gallaher, J. J., Hollender, R., Peterson, D. G., Roberts, R. F., & Coupland, J. (2005). Effect of composition and antioxidants on the oxidative stability of fluid milk supplemented with an algae oil emulsion. *International Dairy Journal*, *15*, 333–341.

Genot, C., Meynier, A., & Riaublanc, A. (2003). Lipid oxidation in emulsions. In A. Kamal-Eldin (Ed.), *Lipid oxidation pathways* (pp. 190–244). AOCS Publishing.

García-Moreno, P. J., Guadix, A., Guadix, E. M., & Jacobsen, C. (2016). Physical and oxidative stability of fish oil-in-water emulsions stabilized with fish protein hydrolysates. *Food Chemistry*, *203*, 124–135.

Hadnadev, T. D., Dokic, P., Krstonosic, V., & Hadnadev, M. (2013). Influence of oil phase concentration on droplet size distribution and stability of oil-in water emulsions. *European Journal of Lipid Science and Technology*, *115*, 313–321.

Hernandez, E. M. (2011). Processing of omega-3 oils. In E. M. Hernandez, & M. Hosokawa (Eds.), *Omega-3 oils applications in functional foods* (pp. 107–128). Illinois: AOCS Press (Chapter 5).

Horn, A. F., Nielsen, N. S., Andersen, U., Søgaard, L. H., Horwell, A., & Jacobsen, C. (2011). Oxidative stability of 70% fish oil-in-water emulsions: Impact of emulsifiers and pH. *European Journal of Lipid Science and Technology*, *113*, 1243–1257.

Horn, A. F., Nielsen, N. S., Jensen, L. S., Horwell, A., & Jacobsen, C. (2012). The choice of homogenisation equipment affects lipid oxidation in emulsions. *Food Chemistry*, *134*, 803–810.

Hudson, B. J. F. (1990). *Food antioxidants*. Elsevier Applied Food Science Series.

Jacobsen, C., Meyer, A. S., & Adler-Nissen, J. (1998). Oxidation mechanisms in real food emulsions: Method for separation of mayonnaise by ultracentrifugation. *Journal of Food Lipids*, *5*, 87–101.

Jacobsen, C., Adler-Nissen, J., & Meyer, A. S. (1999). Effect of ascorbic acid on iron release from the emulsifier interface and on the oxidative flavor deterioration in fish oil enriched mayonnaise. *Journal of Agriculture and Food Chemistry*, *47*, 4917–4926.

Let, M. B., Jacobsen, C., & Meyer, A. S. (2007). Lipid oxidation in milk, yoghurt, and salad dressing enriched with neat fish oil or pre-emulsified fish oil. *Journal of Agricultural and Food Chemistry*, *55*, 7802–7809.

McClements, D. J., & Decker, E. A. (2000). Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of Food Science*, *65*, 1270–1282.

McClements, E. A., Decker, E. A., & Weiss, J. (2007). Emulsion-based delivery systems for lipophilic bioactive components. *Journal of Food Science*, *72*, 109–124.

Nichols, P. D., McManus, A., Krail, K., Sinclair, A. J., & Miller, M. (2014). Recent advances in omega-3: Health benefits, sources, products and bioavailability. *Nutrients*, *6*, 3727–3733.

Osborn, H. T., & Akoh, C. C. (2004). Effect of emulsifier type, droplet size, and oil concentration on lipid oxidation in structured lipid-based oil-in-water emulsions. *Food Chemistry*, *84*, 451–456.

Pal, R. (1996). Effect of droplet size on the rheology of emulsions. *AIChE J.* *42*, 3181–3190.

Pallandre, S., Decker, E. A., & McClements, D. J. (2007). Improvement of stability of oil-in-water emulsions containing caseinate-coated droplets by addition of sodium alginate. *Journal of Food Science*, *72*(9).

Shantha, N. C., & Decker, E. A. (1994). Rapid, sensitive, iron based spectrophotometric methods for determination of peroxide values of food lipids. *Journal of AOAC International*, *77*, 421–424.

Song, C., Shieh, C., Wu, Y., Kalueff, A., Gaikwad, S., & Su, K. (2016). The role of omega-3 polyunsaturated fatty acids eicosapentaenoic and docosahexaenoic acids in the treatment of major depression and Alzheimer's disease: Acting separately or synergistically? *Progress in Lipid Research*, *62*, 41–54.

Sosa-Herrera, M. G., Lozano-Esquivel, I. E., Ponce de León-Ramírez, Y. R., & Martínez-Padilla, L. P. (2012). Effect of added calcium chloride on the physicochemical and rheological properties of aqueous mixtures of sodium caseinate/sodium alginate and respective oil-in-water emulsions. *Food Hydrocolloids*, *29*, 175–184.

Sørensen, A.-D. M., Baron, C. P., Let, M. B., Brüggemann, D. A., Pedersen, L. R. L., & Jacobsen, C. (2007). Homogenization conditions affect the oxidative stability of fish

- oil enriched milk emulsions: Oxidation linked to changes in protein composition at the oil-water interface. *Journal of Agriculture and Food Chemistry*, 55, 1781–1789.
- Sun, C., Gunasekaran, S., & Richards, M. P. (2007). Effect of xanthan gum on physico-chemical properties of whey protein isolate stabilized oil-in-water emulsions. *Food Hydrocolloids*, 21, 555–564.
- Sun, C., & Gunasekaran, S. (2009). Effects of protein concentration and oil-phase volume fraction on the stability and rheology of menhaden oil-in-water emulsions stabilized by whey protein isolate with xanthan gum. *Food Hydrocolloids*, 23, 165–174.
- Swaisgood, H. E. (1992). Proteins. In P. F. Fox (Ed.). *Advanced dairy chemistry*. London: Elsevier Applied Science.
- Tesch, S., & Schubert, H. (2002). Influence of increasing viscosity of the aqueous phase on the short-term stability of protein stabilized emulsions. *Journal of Food Engineering*, 52, 305–312.
- Tong, L. M., Sasaki, S., McClements, D. J., & Decker, E. A. (2000). Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. *Journal of Agriculture and Food Chemistry*, 45, 1473–1478.
- Wysoczański, T., Sokoła-Wysoczańska, E., Pékala, J., Lochyński, S., Czyz, K., Bodkowski, R., ... Librowski, T. (2016). Omega-3 fatty acids and their role in central nervous system – A review. *Current Medicinal Chemistry*, 23–8, 816–831.
- Yesiltas, B., García-Moreno, P. J., Sørensen, A.-D. M., & Jacobsen, C. (2017). Physical and oxidative stability of high fat fish oil-in-water emulsions stabilized with combinations of sodium caseinate and sodium alginate. *European Journal of Lipid Science and Technology*, 119, 1600484.

PAPER V

Yesiltas, B., García-Moreno, P. J., Sørensen, A. M., Anankanbil, S., Guo, Z., Jacobsen, C., **2018.**

Effects of modified DATEMs with different alkyl chain lengths on improving oxidative and physical stability of 70% fish oil-in-water emulsions.

Journal of Agricultural and Food Chemistry, 66, 12512-12520.

Effects of Modified DATEMs with Different Alkyl Chain Lengths on Improving Oxidative and Physical Stability of 70% Fish Oil-in-Water Emulsions

Betül Yesiltas,[†] Pedro J. García-Moreno,[†] Ann-Dorit M. Sørensen,[†] Sampson Anankanbil,[‡] Zheng Guo,[‡] and Charlotte Jacobsen^{*,†}

[†]National Food Institute, Technical University of Denmark, DK-2800 Lyngby, Denmark

[‡]Department of Engineering, Aarhus University, 8000 Aarhus, Denmark

Supporting Information

ABSTRACT: The objective of this study was to produce oxidatively and physically stable 70% fish oil-in-water emulsions by combined use of sodium caseinate (CAS), commercial diacetyl tartaric acid esters of mono- and diglycerides (DATEM), and modified DATEM. First, the optimal formula was determined using DATEM and CAS. Subsequently, modified DATEMs (DATEM C12 and DATEM C14) were designed for investigating both the effects of different alkyl chain lengths and caffeic acid conjugation to the emulsifier on physical and oxidative stability of the emulsions. Emulsions produced with modified DATEMs showed better oxidative stability compared with emulsion using commercial DATEM plus an equivalent amount of free caffeic acid, confirming the advantage of having antioxidant covalently attached to the emulsifier. Results indicated that DATEM_C14 replaced more CAS compared with DATEM_C12 from the interface in 70% fish oil-in-water emulsion. Emulsions produced with DATEM_C14 had significantly decreased amounts of primary and secondary oxidation products compared with emulsions using DATEM_C12.

KEYWORDS: *modified emulsifiers, DATEM and sodium caseinate, lipid oxidation, oil–water interface, caffeic acid, high fat delivery emulsions*

1. INTRODUCTION

There has been increasing evidence of the health benefits of long-chain (LC) omega-3 (n-3) polyunsaturated fatty acids (PUFAs), such as eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA). These health benefits are mainly reducing the risk of cardiovascular diseases,^{1,2} maintaining normal blood pressure and triglyceride levels in the blood,³ improving brain development in the infant,⁴ and supporting mental health⁵ as well as the immune system.⁶ However, consumption of these bioactive compounds has been reported to be below daily recommended levels.³ Therefore, there is high interest from food researchers and industry to enrich food products with LC n-3 PUFAs. Nevertheless, LC n-3 PUFAs are highly prone to oxidation, and there are many factors affecting oxidation in complex food systems such as mayonnaise, milk, cream cheese, and dressings, which are basically oil-in-water emulsions. This has led researchers to study lipid oxidation in fish oil-in water emulsions, which have been used as a delivery system for LC n-3 PUFAs.^{7–9} The most important factors affecting lipid oxidation are type and concentration of the ingredients (e.g., oil, emulsifiers, etc.), antioxidants existing or added in the system, oil–water interface structure and distribution of emulsifiers and antioxidants in the emulsion, surface charge of the oil droplets, and other physical parameters of oil-in-water emulsions such as droplet size and viscosity.^{7–9}

Different approaches have been suggested for protecting emulsified lipids from oxidation, and improving the properties of the oil–water interfacial layer is one of them. Previous studies claimed that oxidation is initiated at the oil–water interface.

Therefore, attention has been focused on enhancing interfacial layer capabilities such as increasing antioxidant activities at the interface as well as using a combination of emulsifiers, which are expected to provide better interfacial characteristics.^{8,10} In order to enhance the physical structure of interface layer, proteins and low-molecular-weight emulsifiers (LMWEs) are suggested to be combined together for the emulsification of oils.^{11,12} Sodium caseinate (CAS), as a milk protein and a common emulsifier in the food industry, has been combined with LMWEs (e.g., phospholipids, mono- and diglycerides, etc.). This approach resulted in displacement of adsorbed proteins at the oil–water interface by the LMWEs, which also resulted in the ability of the LMWEs to help cover the interface through the small gaps between proteins.^{11,13,14} This might lead to a well-covered oil–water interface by the formation of protein-phospholipid complexes, which then prevents migration of pro-oxidants through the interfacial layer from the water phase to the oil phase, where LC n-3 PUFAs are located.

In order to improve the oxidative stability of emulsions, phenolic acids have been used because of their antioxidant properties. Sørensen et al. studied the effects of adding phenolic compounds into low fat (10%) fish oil-in-water emulsions and investigated their interaction with emulsifiers as well as effects of pH and iron addition on oxidative stability of LC n-3 PUFAs.¹⁵

Received: July 31, 2018

Revised: November 6, 2018

Accepted: November 6, 2018

Published: November 6, 2018

Table 1. Experimental Design for Determining the Effects of Having Modified DATEMs

sample codes	sample descriptions	commercial DATEM (%)	modified DATEM (%)	caffeic acid (%)
DATEM	only DATEM (commercial)	0.93	-	-
DATEM_C12_10%	10% of DATEM was replaced by DATEM C12	0.84	0.09	0.03 ^a
DATEM_C12_30%	30% of DATEM was replaced by DATEM C12	0.65	0.28	0.09 ^a
DATEM_C12_60%	60% of DATEM was replaced by DATEM C12	0.37	0.56	0.18 ^a
DATEM_C14_10%	10% of DATEM was replaced by DATEM C14	0.84	0.09	0.03 ^a
DATEM_C14_60%	60% of DATEM was replaced by DATEM C14	0.37	0.56	0.17 ^a
DATEM_caf_low	DATEM with added caffeic acid at low concn	0.93	-	0.03
DATEM_caf_high	DATEM with added caffeic acid at high concn	0.93	-	0.17

^aCommercial caffeic acid was not added for this sample. Percentages was calculated according to the added modified DATEM, which had caffeic acid attached to the compound itself.

Moreover, researchers also focused on adding phenolipids in the emulsion systems to obtain better oxidative stability by having antioxidant effects at the oil–water interface. Phenolipids are expected to be surface-active because of their amphiphilic character and adsorb at the oil–water interface where the oxidation is claimed to be initiated by prooxidants (e.g., metal ions). The effects of having different chain lengths of phenolipids on oxidative stability have been studied in different emulsion systems.^{16–22} The different efficacies of phenolipids with different chain lengths were related to their location/distribution in the emulsion system, oil phase, oil–water interface, and aqueous phase.

Caffeic acid acts as an antioxidant by scavenging free radicals and chelating metals especially iron.²³ Caffeic acid efficacy in O/W emulsions was shown to be dependent on pH, iron addition, and emulsifier type.¹⁵ Antioxidant properties and efficacy of alkyl caffeates, ferulates, and coumarates have been investigated, and only caffeic acid and caffeates were able to form a complex with iron via their catechol group in the phenolic ring.¹⁹ The same study also reported that caffeic acid and alkyl caffeates showed the highest radical scavenging activity and reducing power, which was followed by ferulic acid and alkyl ferulates, whereas coumaric acid and alkyl coumarates showed the lowest efficiency. It was also shown that the medium alkyl chain length (octyl caffeate) was found to have higher antioxidant activity than shorter (caffeic acid, methyl-, propyl caffeate) or longer (hexadecyl caffeate) alkyl chains, which was related to their presence at the interfacial region.²² Alemán et al. also showed that caffeates with short- to medium-chain length (C4, C8, and C12) were found to be more effective antioxidants in fish-oil-enriched mayonnaise.¹⁸

Commercial DATEM is a commonly used surfactant in the food industry, mainly in baking. In order to combine advantageous effects of both caffeates and DATEM (antioxidative and surface active effects, respectively), we aimed to use modified DATEM as a LMWE, which was modified with caffeic acid, and C12 or C14 alkyl chains, into high-fat fish oil-in-water emulsions stabilized in combination with CAS. Chain lengths were selected according to the results from previous studies conducted with phenolipids which showed that the short- to medium-chain length (C4–C12) performed the best.¹⁸ Considering the larger head part of the DATEM molecules compared with a phenolipid, we decided to study the longest short- to medium-chain length, which is C12 and one even longer (C14), in order to adjust the hydrophilic–lipophilic balance (HLB) of the molecules. A recent study reported that DATEM C14 led to lower lipid oxidation (TBARS) when compared with DATEM C12 or C16 in 30% fish oil-in-water emulsions.²⁴

The first part of this study focused on finding an optimal recipe for emulsifying high fat fish oil-in-water emulsions using CAS and commercial DATEM. For that purpose, we used Box–Behnken design combined with Response Surface Methodologies (RSM). The effects of fish oil content, emulsifier content, and ratio of CAS to DATEM on physical and oxidative parameters were evaluated. The second part of the study aimed at investigating the use of CAS in combination with different concentrations of modified DATEMs with caffeic acid and different fatty acid chain lengths (C12 and C14). The use of modified DATEM was compared to commercial DATEM with and without free caffeic acid to evaluate the effect of having: (i) caffeic acid attached to the emulsifier on oxidative stability of high-fat fish oil-in-water emulsions and (ii) different chain lengths on the distribution of emulsifiers in high-fat emulsion systems as well as its effect on physical and oxidative stability.

2. MATERIALS AND METHODS

2.1. Materials. Cod liver oil used for the optimization experiment was provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway), and stored at $-40\text{ }^{\circ}\text{C}$ until use. The fatty acid (% w/w) content of the fish oil was as follows: C14:0 (3.0), C16:0 (8.9), C16:1 n-7 (8.2), C18:0 (1.9), C18:1 n-9 (16.0), C18:1 n-7 (5.2), C18:2 n-6 (1.8), C18:3 n-3 (0.8), C20:1 n-9 (11.6), C20:5 n-3 (9.3), C22:1 n-11 (6.1), and C22:6 n-3 (11.6). Cod liver oil used for modified DATEM experiment was provided by Vesteraalens A/S (Sortland, Norway) and stored at $-40\text{ }^{\circ}\text{C}$ until use. Peroxide value was determined as 0.12 ± 0.08 mequiv peroxides/kg oil. The fatty acid (% w/w) content of the fish oil was as follows: C14:0 (0.2), C16:0 (9.4), C16:1 n-7 (8.6), C18:0 (2.0), C18:1 n-9 (16.2), C18:1 n-7 (4.6), C18:2 n-6 (1.8), C18:3 n-3 (0.1), C20:1 n-9 (12.6), C20:5 n-3 (9.1), C22:1 n-11 (5.9), and C22:6 n-3 (11.1). Alpha-, beta-, gamma-, and delta tocopherol contents were 250 ± 2 , 0 ± 0 , 118 ± 1 , $48 \pm 1\text{ }\mu\text{g toc/g oil}$, respectively. Sodium caseinate, CAS (Miprodan 30) was kindly donated by Arla Foods Ingredients a/s (Viby J, Denmark). Arla reported a protein content of 92% in sodium caseinate for Miprodan 30. DATEM (PANODAN AB 100 VEG-FS MB, PD 244-18.3 EN) was provided by Danisco (Brabrand, Denmark). Caffeic acid was purchased from Sigma-Aldrich. Modified DATEMs with caffeic acid and different alkyl chains C12 or C14 were synthesized as described in a previous study.²⁵ All other chemicals and solvents used were of analytical grade.

2.2. Experimental Designs. **2.2.1. Experimental Design for Optimal Formula Determination.** The optimal recipe was determined by Box–Behnken design using three different levels for three factors which were fish oil content (50, 60, and 70%), total emulsifier content (1.4, 2, and 2.8%), and the ratio between CAS and DATEM (0.4, 1.2, and 2). Experimental design and details are shown in the [Supporting Information](#).

2.2.2. Experimental Design for Emulsions Produced with Modified DATEMs. Sample codes and sample descriptions amounts of added ingredients are listed in [Table 1](#). All samples include 70 wt % fish oil and 2.8 wt % total emulsifier with a ratio of CAS to total DATEM of 2. Commercial DATEM was replaced by modified DATEM in a range of 0

to 60%. Emulsions presenting commercial DATEM and free caffeic acid were also added as controls.

2.3. Emulsion Preparation and Sampling. Prior to emulsification, emulsifiers (CAS, DATEM, and modified DATEMs) were dissolved in distilled water and stirred overnight at 4 °C. Aqueous phases were adjusted to pH 7 using 1 M HCl and 2 M NaOH. Emulsions were produced in 500 g batches in a Stephan Universal mixer (Stephan, UMC5, 1995, Hameln, Germany) as explained by Horn et al.²⁷ Sodium azide (0.05% w/v) and 100 μM Fe^{2+} were added into emulsions in order to prevent microbial activity and accelerate lipid oxidation, respectively. All emulsions were divided into 100 mL bottles in approximately 90 g and stored at room temperature for up to 12 days in the dark. Samples were collected at days 0, 2, 5, 8, and 12 for oxidative stability analysis.

2.4. Methods for Characterization of Emulsions. **2.4.1. Creaming Index.** Creaming was measured on days 1, 5, 8, and 12 in the stored bottles without replicates. Creaming index was calculated using eq 1:

$$\text{CI} (\%) = b/a * 100 \quad (1)$$

where a is the height of total emulsion, and b is the height of water phase separated at the bottom of the bottle. Creaming index was calculated as in percentage for each emulsion sample and sampling point.

2.4.2. Droplet Size. Droplet size of the emulsions was measured by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, U.K.) on days 0 and 12 using the method described by Let, Jacobsen, Meyer and Horn et al.^{26,27} Results were presented as the surface weighted ($D[3,2]$) and volume weighted ($D[4,3]$) mean diameter, which were calculated according to the eq 2 and 3, respectively:

$$D[3,2] = \sum n_i d_i^3 / \sum n_i d_i^2 \quad (2)$$

$$D[4,3] = \sum n_i d_i^4 / \sum n_i d_i^3 \quad (3)$$

where n is the number of droplet with a specific diameter, and d is the diameter. Measurements were carried out as duplicates.

2.4.3. Apparent Viscosity. Apparent viscosity was measured using a stress-controlled rheometer (Stresstech, Reologica Instruments AB, Lund, Sweden) on days 1 and 12. The rheometer was equipped with a CC25 standard bob cup system in a temperature vessel. Fifteen milliliters of emulsion was measured over a shear stress range from 0.0125 to 200 Pa at 25 °C. Results were calculated on a specific shear rate (20 s^{-1}) for each emulsion in Pascal second (Pa·s). Samples were measured in duplicates.

2.4.4. Zeta Potential. Zeta potential measurements were done using Zetasizer Nano 2S (Malvern Instruments, Ltd.) in order to determine the surface charge of the emulsion droplets. Each sample (0.32 g sample) was diluted in distilled water (40 g of distilled water) before measuring and placed in DTS-1070 disposable folded capillary cell (Malvern Instruments, Ltd., United Kingdom). The zeta potential range was set to -100 to $+50$ mV, and measurements were carried out in duplicate at 25 °C on day 2.

2.4.5. Relative Protein Content in the Aqueous Phase. Protein content of the aqueous phase was measured based on the method described by Jacobsen, Meyer, and Adler-Nissen.²⁸ In order to separate the aqueous phase, the emulsion sample (~ 20 g) was centrifuged for 10 min at 25 400g and 10 °C (Sorvall RC-6 PLUS, Thermo Fisher Scientific, Osterode, Germany; rotor SS-34). Supernatant (oil phase) was removed by the use of a pipet. The rest was mixed with distilled water (1:2) and then subjected to ultracentrifugation (Beckman Ultracentrifuge L8-60M, Fullerton, CA; rotor 21102) for 16 h at 106 979g and 15 °C. The Dumas method (Elementar, Mt. Laurel, NJ, U.S.A.) was used for the determination of protein concentration. Approximately 1 g of aqueous phase was placed in the sample tray, and further steps were automated including sample combustion in a chamber at a high temperature (900 °C) in the presence of oxygen. The content of crude protein was estimated by using a conversion factor (6.25). Protein concentration is reported as percent of aqueous phase. Measurements were carried out in duplicates.

2.5. Methods for Lipid Oxidation Measurements of Emulsions. **2.5.1. Primary Oxidation Products—Peroxide Value**

(PV). Determination of primary oxidation products was done according to the Bligh and Dyer method²⁹ with slight changes. Lipid extract was prepared using 5 g of emulsion for each extraction and a reduced amount of solvent (30.0 mL of methanol and chloroform, 1:1). PV was subsequently measured on the lipid extracts by colorimetric determination of iron thiocyanate on a spectrophotometer (Shimadzu, UV mini 1240, Kyoto, Japan) at 500 nm, as described by Shantha and Decker.³⁰ Measurements were carried out in duplicate.

2.5.2. Tocopherol Content: HPLC. Tocopherol content of emulsions was determined by HPLC (Agilent 1100 Series; Column: Waters Spherisorb 3 μm Silica; 4.6×150 mm). Tocopherol analysis was carried out according to the official AOCS method³¹ using lipid extracts (see section 2.5.1) that were further evaporated and redissolved in heptane. Measurements were carried out in duplicates.

2.5.3. Secondary Oxidation Products: Dynamic Head Space GC-MS. Volatile secondary oxidation products were analyzed according to the method described by Yesiltas et al.³² Emulsion samples (approximately 4 g) were mixed with 2 mL of antifoam and 10 mL of distilled water in a 100 mL purge bottle. The bottle was heated in a water bath at 60 °C while purging with nitrogen (flow 150 mL/min, 30 min). Volatile compounds were trapped on Tenax GR tubes. The volatiles were separated in a gas chromatograph (Agilent Technologies, 6890N Network GC System, DE, U.S.A.) on a 30 m DB 1701 fused silica capillary column (0.25 mm i.d., 1 μm film thickness; Agilent Technologies, J&W GC Columns, CA, U.S.A.). The oven program had an initial temperature of 45 °C for 5 min, increasing with 1.5 °C/min until 55 °C, with 2.5 °C/min until 90 °C, and with 12.0 °C/min until 220 °C, where the temperature was held for 4 min. The individual compounds were analyzed by mass-spectrometry (Agilent 5973 Network Mass Selective Detector, Agilent Technologies, 70 eV; mass to charge ratio scan between 30 and 250) and identified by MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard). The volatile compounds 2-ethyl-furan, 1-penten-3-one, 1-penten-3-ol, (*E*)-2-pentenal, hexanal, (*E*)-2-hexenal, (*Z*)-4-heptenal, 2-pentyl-furan, (*E*)-2-heptenal, benzaldehyde, (*E,E*)-2,4-heptadienal, nonanal and (*E,Z*)-2,6-nonadienal were selected for quantification and analyzed in all emulsions. In the optimization study, a calibration curve was prepared by injecting standards directly on the TENAX GR tubes. Another calibration curve was prepared for the samples in the second part of this study by injecting standards into an emulsion produced according to the optimal formula (CAS and commercial DATEM were used as emulsifier). Then volatiles were collected the same way as the emulsion samples. This was carried out in order to maintain similar release conditions for standard volatile compounds and volatiles in the emulsion samples.

2.6. Response Surface Methodology and Statistical Analysis. Statgraphics XVII (Statpoint Technologies, Inc., Virginia, U.S.A.) was used to generate the statistical analysis and the regression models for the parameters, which were used for determining physical and oxidative stability of the emulsions produced for the recipe optimization study. First, the output variables (Y : viscosity at day 1, droplet size at day 1, creaming at day 12, peroxide values at day 12 and volatile compounds at day 12) were related to the input variables (X : fish oil content, total emulsifier content and ratio between CAS and DATEM) by second-order polynomial equation as follows, eq 4:

$$Y = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i < j}^3 b_{ij} X_i X_j \quad (4)$$

where the coefficients b_i and b_{ii} are related to the linear and quadratic effects, respectively, of each input factor on the output variable, and the cross-product coefficients b_{ij} represent the interactions between two input variables. Second, the analysis of variance (ANOVA) was carried out. The significance of all terms in the models was evaluated statistically at the confidence level $1 - \alpha = 95\%$. Contour maps were drawn using the regression coefficients. Optimal values for each input variables, which maximized the quality of the emulsions in terms of physical and oxidative stability, were determined for each output variable by using RSM.³³

Table 2. Droplet Size, Viscosity, Zeta Potential, and Protein Content in the Aqueous Phase Results of Emulsions

	D[3,2] (μm) (day 1)	D[4,3] (μm) (day 1)	apparent viscosity (Pa·s) at 20 s ⁻¹ (day 1)	zeta potential (mV)	protein in the aqueous phase (%)
DATEM	2.35 ± 0.02 ^b	2.73 ± 0.01 ^b	7.27 ± 0.10 ^d	(-) 63.4 ± 1.3 ^{cd}	4.85 ± 0.01 ^c
DATEM_C12_10%	2.32 ± 0.02 ^b	2.73 ± 0.01 ^b	7.40 ± 0.24 ^{de}	(-) 63.6 ± 1.1 ^{cd}	4.49 ± 0.02 ^a
DATEM_C12_30%	2.43 ± 0.02 ^d	2.81 ± 0.02 ^{e,1}	7.74 ± 0.11 ^{e,1}	(-) 64.4 ± 1.4 ^c	4.70 ± 0.08 ^b
DATEM_C12_60%	2.71 ± 0.00 ^f	3.20 ± 0.00 ^f	5.84 ± 0.06 ^{b,1}	(-) 75.3 ± 2.3 ^a	4.65 ± 0.04 ^b
DATEM_C14_10%	2.20 ± 0.03 ^a	2.57 ± 0.02 ^a	6.49 ± 0.10 ^{c,1}	(-) 60.6 ± 1.1 ^d	5.05 ± . ^s
DATEM_C14_60%	2.46 ± 0.01 ^{d,1}	2.87 ± 0.01 ^{d,1}	5.89 ± 0.20 ^b	(-) 66.3 ± 2.2 ^{bc}	4.86 ± 0.07 ^c
DATEM_caf_low	2.39 ± 0.02 ^c	2.78 ± 0.01 ^c	6.84 ± 0.46 ^{c,1}	(-) 64.8 ± 1.7 ^c	4.63 ± 0.04 ^b
DATEM_caf_high	2.63 ± 0.02 ^e	3.14 ± 0.03 ^e	5.37 ± 0.33 ^{a,1}	(-) 68.6 ± 2.4 ^b	4.62 ± 0.11 ^b

¹Samples changed significantly during 12 days of storage time. ^{a–f}Letters indicate the significant differences between samples for the same physical parameter. ^sThis sample has only one replicate, due to a measurement error.

2.7. Principle Component Analysis (PCA). Principal component analysis (PCA) was done by Latentix 2.12 (LatentiX, Copenhagen, Denmark) for the emulsions produced for the recipe optimization study. The PCA was carried out with the emulsions as objects and creaming, viscosity, droplet size, zeta potential, peroxide value, alpha-tocopherol, and volatile compounds as variables. Data were autoscaled to make the variables contribute equally to the model.

3. RESULTS AND DISCUSSION

3.1. Selection of Optimal Formula. An optimal formula was selected according to the results of physical and oxidative

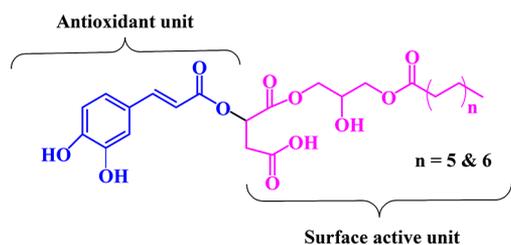


Figure 1. Molecular structures of modified DATEMs (DATEM modified with caffeic acid and C12 or C14 alkyl chain lengths)

parameters of the emulsions, which were produced based on the Box–Behnken experimental design. Results were analyzed using RSM as described in detail by Yesiltas et al.,³⁴ and the optimal formula was determined as follows: 70% fish oil, 2.8% total emulsifier, and 2 as the ratio of CAS to commercial DATEM. As this study was performed as a preliminary study for finding the optimal recipe, detailed results are shown in the Supporting Information. Overall, emulsion viscosity was significantly increased when increasing the amount of fish oil. Creaming

and zeta potential of the droplets significantly decreased with increased fish oil content and total emulsifier content. Droplet size significantly decreased with increasing fish oil content, total emulsifier content, and the ratio between CAS and DATEM. Additionally, the ratio between CAS and DATEM played a significant role for oxidative stability; having a higher amount of CAS resulted in less formation of primary and secondary oxidation products (see the Supporting Information).

3.2. Physical Characterization of Emulsions Produced with Modified DATEMs. Physical characteristics of the emulsions were identified according to the droplet size, apparent viscosity, zeta potential, and relative protein content in the aqueous phase (Table 2). There was no creaming for these emulsions during 12 days of storage.

3.2.1. Droplet Size. During 12 days of storage, surface weighed (D[3,2]) mean diameters did not change significantly except for DATEM_C14_60% (from 2.46 ± 0.01 to 2.49 ± 0.01 μm) as well as volume weighed (D[4,3]) mean diameters for DATEM_C14_60% (from 2.87 ± 0.01 to 2.89 ± 0.00 μm) and DATEM_C12_30% (from 2.81 ± 0.02 to 2.75 ± 0.00 μm) (Table 2). Increased amounts of both modified DATEMs (DATEM_C12 and DATEM_C14) led to significantly larger droplet sizes (both in D[3,2] and D[4,3] mean diameters) compared with commercial DATEM emulsion at day 1. This was also the case for the emulsions produced with commercial DATEM and caffeic acid. Compared to commercial DATEM emulsion, only DATEM_C12_10% and DATEM_C14_10% had similar and significantly smaller droplets, respectively. These differences could be due to the different chain lengths of DATEM, which can affect the adsorption behavior of the emulsifiers by increasing the lipophilic characteristic of the emulsifiers. On the other hand, when caffeic acid is attached to

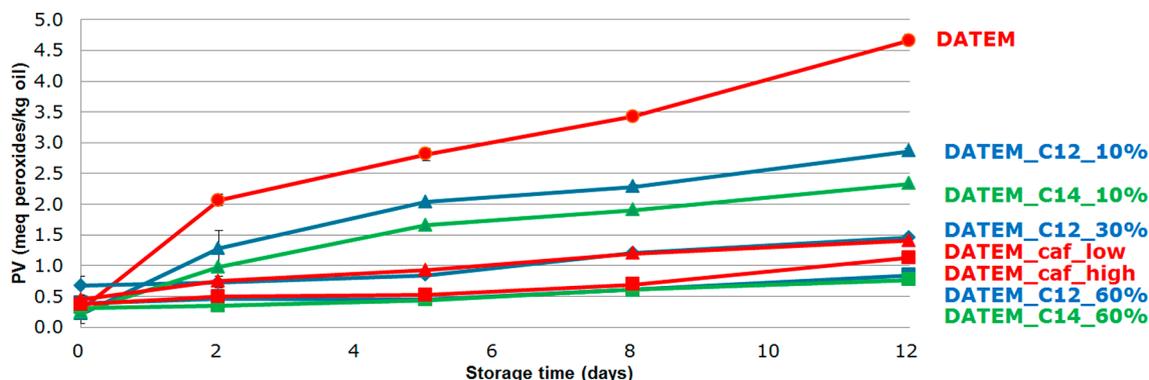


Figure 2. Primary oxidation products formed during the storage of the emulsion samples. Sample codes refer to Table 1.

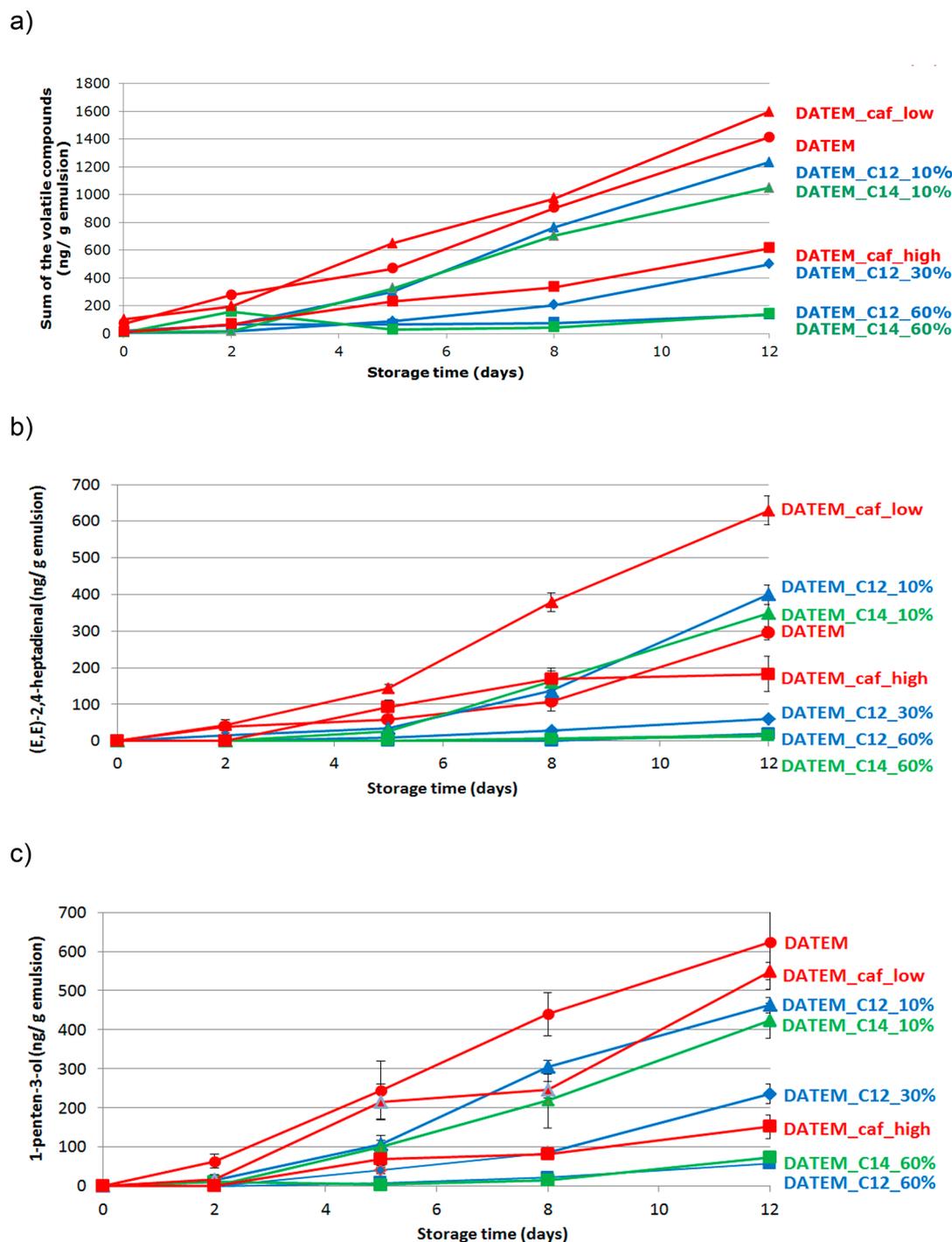


Figure 3. Volatile secondary oxidation products formed during the storage of the emulsion samples (a) sum of the volatile compounds, (b) (*E,E*)-2,4-heptadienal, and (c) 1-penten-3-ol. Sample codes refer to Table 1.

DATEM, it increases the size of the headgroup of the DATEM emulsifier, which is then expected to affect the HLB of the emulsifier.³⁵ This could lead to higher hydrophilic characteristic; thereby, increase the emulsifier's affinity toward water phase, which slows down the adsorption of modified DATEMs at the oil–water interface compared with DATEM. Other studies also reported that phenolic compounds interacted with emulsifiers (e.g., proteins) in emulsions, and this might have affected the functionality as well as emulsifying properties of emulsifiers at the oil–water interface.^{15,36,37} Mattia et al. observed that catechin addition into emulsions emulsified with Tween20/ β -

lactoglobulin resulted in larger droplet sizes compared with control, which indicated a possible negative effect of phenols on emulsifying activities of emulsifiers.³⁸ This phenomenon was attributed to having protein–protein interactions mediated by catechin in 20% olive oil-in-water emulsions. In our study, increase in droplet size could be also explained by protein-DATEM/modified DATEM interactions, which might have been favored in the presence of caffeic acid and carboxylic acid units in the structures of DATEM.³⁹ It was shown that DATEM has a strong ability toward the formation of hydrogen bonds with amidic groups of the gluten-proteins.³⁵

Thus, the smaller droplet size for the emulsions with lower amounts of modified DATEMs (DATEM_C12_10% and DATEM_C14_10%) compared with higher amounts of modified DATEMs (DATEM_C12_60% and DATEM_C14_60%) could be explained by the higher amount of commercial DATEM which exhibited higher surface activity than DATEM C12 and C14. However, this cannot explain the differences observed in droplet size for DATEM_C14_10% emulsion and the DATEM emulsion.

3.2.2. Apparent Viscosity. All the emulsions were non-Newtonian and showed shear thinning behavior. Same characteristics were also reported by Yesiltas et al. for high fat oil-in-water emulsions stabilized with CAS and alginates.³² Apparent viscosity of the samples showed that 60% replacement of the DATEM with modified DATEMs provided significantly less viscous emulsions. This was the case also for the emulsions which had 10% of its DATEM replaced by DATEM C14. Emulsions produced with commercial DATEM and caffeic acid also had significantly lower viscosities compared with DATEM emulsion without caffeic acid. This shows that the decrease in viscosity was affected by the presence of caffeic acid in the emulsions, which could be related to the changes in droplet size discussed under the section 3.2.1. Changes in the viscosity correlated well with the droplet size results, where viscosity decreased with the larger droplet size (except for emulsion DATEM_C14_10%). This could be explained by the fact that smaller droplet sizes led to a close packing of emulsion droplets and thereby larger interfacial area, which can be related to increased friction forces between droplets at an expanded surface-to-volume ratio of the dispersed phase. This could result in less mobility of the oil droplets in the emulsion and therefore higher viscosity.^{32,40}

3.2.3. Zeta Potential. Zeta potential results are a measure of the surface charge of the droplets in the emulsions. Surface charge of the emulsions was negative due to molecular charges of both CAS⁴¹ and DATEM⁴² at pH 7. Zeta potential values for emulsions ranged between -60.6 ± 1.1 and -75.3 ± 2.3 mV (Table 2). Only DATEM_C12_60% and DATEM_caf_high were found to be significantly different compared with DATEM emulsion. Moreover, an increased amount of modified DATEM resulted in more negatively charged oil droplets. This could be due to having higher concentration of caffeic acid at the interface which interacted with iron as well and became more negatively charged. This was also observed in another study where addition of iron caused more negatively charged droplets in citrem-stabilized emulsions (pH 6) where phenolic compounds were also present. It was reported that emulsions with added caffeic acid had much higher negative charge compared with emulsions with added rutin or naringenin, after iron was added.¹⁵ In addition, the anionic nature of modified DATEM could have contributed to the increased negative charge of oil droplets.

3.2.4. Relative Protein Content in the Aqueous Phase. Results of relative protein content in the water phases of the emulsions are presented in Table 2. The protein content in the water phase is a measure of the amount of protein (CAS), which has been replaced by modified DATEM at the interface or nonadsorbed proteins in the aqueous phase. It was observed that emulsions produced with DATEM C14 replaced more CAS compared with DATEM C12. This could be due to the effect of chain length on surface activity of modified DATEMs; meaning DATEM C14 had a higher affinity to oil–water interface compared with DATEM C12 in 70% fish oil-in-water emulsions. This was the case for both 10% and 60% replacement of

DATEM with modified DATEMs. Protein content in the aqueous phase was similar for DATEM_C14_60% and DATEM, which confirmed that replacing 60% of the DATEM with DATEM C14 did not affect the protein replacement at the oil–water interface. On the other hand, DATEM_C12_10% had less protein in the aqueous phase, thereby more proteins at the oil–water interface compared with DATEM_C14_10%. This could be attributed to lower surface activity of DATEM C12 compared with DATEM C14 or DATEM.

On the other hand, results from emulsions with commercial DATEM and added free caffeic acid showed that the amount of CAS in the water phase was lower compared with the control (emulsion with commercial DATEM and no caffeic acid), which suggested that the caffeic acid presence decreased the adsorption of commercial DATEM at the oil–water interface. Even though DATEM_caf_low and DATEM_caf_high had larger droplet size and thereby lower surface area compared with emulsion with only DATEM, these emulsions had more CAS adsorbed at the interface. This decreased amount of CAS replacement by commercial DATEM could, presumably, be explained by the interactions between caffeic acid and commercial DATEM, which resulted in lower amounts of CAS replacement at the oil–water interface compared with control. Sorensen et al. reported that partitioning results showed that caffeic acid and ester of caffeic acid (caffeates) had a different distribution in the aqueous phase of Citrem- and Tween 80-stabilized emulsions.⁴³ Less caffeic acid and caffeates were present in the aqueous phase of Tween 80-stabilized emulsions, which indicated that Tween 80 had a stronger interaction with caffeic acid and caffeates compared with Citrem. Thus, it is possible that free caffeic acid had interactions with DATEM, which affected its surface activity.

3.2.5. Color. Iron-caffeic acid complex was formed after iron was added into emulsions produced with modified DATEM which had caffeic acid in their structure (Figure 1) as well as emulsions produced with commercial DATEM and free caffeic acid. It could easily be observed as a color change to light gray. It is known that phenols interact with iron and form phenol-iron complexes because of their metal-chelating properties.^{15,44}

3.3. Lipid Oxidation in Emulsions Produced with Modified DATEMs. Oxidative stability of emulsions was evaluated by measuring the formation of primary and secondary oxidation products during 12 days of storage as well as the consumption of tocopherols.

3.3.1. Primary Oxidation Products. Peroxide value of the emulsions had a significant increase during 12 days of storage (Figure 2). The most oxidized emulsion was DATEM without caffeic acid with 4.7 ± 0.1 peroxides/kg oil at day 12. High-fat (70%) fish oil-in-water emulsions produced with CAS and succinylated alginate or only CAS showed better oxidative stability (1.7 ± 0.2 and 1.8 ± 0.2 peroxides/kg oil, respectively) compared with emulsions produced with CAS and DATEM, whereas emulsions produced with CAS and dodecyl succinylated alginate or CAS and commercial alginate (8.0 ± 0.8 and 9.0 ± 1.4 peroxides/kg oil, respectively) showed less oxidative stability.³² Emulsions with the same oil content and produced with CAS and phosphatidylcholine had values ranging between 3.5–5.0 which was similar to emulsions with CAS and DATEM.^{32,34} These differences could be due to antioxidative activity of different emulsifiers as well as different amounts of total emulsifier and the ratios between emulsifiers.

Emulsions were oxidatively more stable when the concentration of caffeic acid was increased for all emulsifier

combinations no matter whether DATEM was modified or not. Emulsions with the highest concentration of modified DATEMs (DATEM_C12_60% and DATEM_C14_60%) were more oxidatively stable (0.8 ± 0.0 and 0.8 ± 0.0 peroxides/kg oil) compared with the DATEM_caf_high (1.1 ± 0.0 peroxides/kg oil) which had the same concentration of caffeic acid added in the free form at day 12. Conversely, this effect was observed vice versa at the lower concentration of caffeic acid. DATEM_C12_10% and DATEM_C14_10%, which had the caffeic acid attached to the emulsifier itself, oxidized significantly more than DATEM_caf_low, which had the same concentration of caffeic acid added in the free form. This led to the conclusion that having caffeic acid attached to the emulsifier, and when using a high concentration of the modified emulsifier, restrained the formation of peroxides compared with the emulsions produced with the commercial DATEM and equivalent concentrations of caffeic acid in free form.

3.3.2. Tocopherol Content. Alpha-, gamma-, and delta-tocopherols were quantified on day 0 to be in the range of 145.1 ± 8.0 – 161.0 ± 3.5 , 67.8 ± 2.1 – 73.0 ± 0.2 , and 27.5 ± 0.6 – 29.7 ± 0.2 $\mu\text{g/g}$ emulsion, respectively (data not shown). Beta-tocopherol content was very low (1 – 2 $\mu\text{g/g}$ emulsion); therefore, it was not considered. Alpha-tocopherol content of the emulsion samples significantly decreased at day 12 compared with day 0 except for DATEM_C12_10%. Likewise, delta-tocopherol content of the emulsions was also decreased from day 0 to day 12 except for DATEM_C12_10% and DATEM_C12_60%. However, gamma-tocopherol content was not significantly different when days 0 and 12 were compared. Tocopherols are natural chain-breaking antioxidants, which react with free radicals by donating hydrogen atoms. Therefore, consumption of tocopherols indicates that some of the free radicals were deactivated by tocopherols, which presumably helped to decrease the formation of oxidation products. Emulsions which had decreased in the tocopherol content would have had higher levels of oxidation products formed during the storage. Moreover, in the presence of caffeic acid, tocopherols can be regenerated through hydrogen donation mechanism. Thus, it was reported that the consumption rate of tocopherols was faster in the control sample compared with caffeic acid supplemented fish muscle, which confirmed that caffeic acid efficiently protected tocopherols for retarding the depletion of alpha-tocopherol by hydrogen donation.^{45,46}

3.3.3. Volatile Secondary Oxidation Products. Emulsions showed a similar trend in the content of most of the volatile compounds during storage. Therefore, the sum of the volatile compounds formed during storage was shown in Figure 3a, together with most abundant two volatile compounds, (*E,E*)-2,4-heptadienal and 1-penten-3-ol in Figure 3b,c (see Supporting Information for some of the other individual volatile compounds). Although there were some differences, in general terms, similar trends were observed for 1-penten-3-ol, (*E,E*)-2,4-heptadienal and the sum of volatiles. Emulsions produced with modified DATEMs showed better oxidative stability compared with the control (commercial DATEM without added caffeic acid). Moreover, added caffeic acid in low concentration into the emulsion showed a pro-oxidative effect (see DATEM_caf_low). The prooxidant effect of caffeic acid was also reported in 10% fish oil-in-water emulsions produced with Tween 80 with added iron at pH 6.¹⁵ It was attributed to caffeic acid's ability to reduce endogenous Fe^{3+} in the fish oil or emulsifier to Fe^{2+} .^{15,23} Emulsions produced with the modified DATEMs showed better oxidative stability compared with emulsions produced with

commercial DATEM with added equivalent concentration of caffeic acid both at low and high concentrations of caffeic acid. This suggests that having caffeic acid attached to the emulsifier improved oxidative stability compared with the emulsions containing free caffeic acid in the same concentration, which presumably support the claim that oxidation is initiated at the interface. Having caffeic acid located at the interface improved oxidative stability of the emulsions due to their radical trapping mechanisms. As shown in Figure 3, DATEM_C14 showed slightly better oxidative stability compared with DATEM_C12, which could be due to the effect of the chain length on their affinity to the oil–water interface. Protein content in the aqueous phase (see section 3.2.4) supported that DATEM_C14 replaced more CAS at the interface when compared with DATEM_C12. This could be the reason for the higher oxidative stability of DATEM_C14 emulsion.

As a conclusion, the tandem use of CAS, commercial and modified DATEMs allowed production of physically and oxidatively stable 70% fish oil-in-water emulsions. DATEM modified with caffeic acid and C14-alkyl chain replaced more CAS from the oil–water interface compared with DATEM modified with caffeic acid and C12-alkyl chain in 70% fish oil-in-water emulsions. Covalent attachment of caffeic acid to DATEM significantly improved oxidative stability compared with the emulsions produced with physical combinations of commercial DATEM and caffeic acid. These results highlight the importance of bringing antioxidants to the oil–water interface by the new approach of attaching antioxidants to an emulsifier molecule. When 60% of the commercial DATEM was substituted with either of the modified DATEMs (C12 or C14), viscosity was significantly decreased compared with emulsions with commercial DATEM, which might make high fat emulsions incorporation into low viscosity food systems easier.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.jafc.8b04091](https://doi.org/10.1021/acs.jafc.8b04091).

Table 1. Box–Behnken design for emulsions; Figure 1. Principle component analysis of the emulsions for the optimization study; Figure 2. Response surface methodology results for the optimization study: (a) Pareto charts, (b) contour plots; and Figure 3. Volatile compounds formed during storage of emulsions produced with modified DATEMs: (a) 1-penten-3-one, (b) 2-pentenal, and (c) hexanal (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: chja@food.dtu.dk. Tel.: +45 23 27 90 75.

ORCID

Betül Yesiltas: 0000-0002-4587-9990

Funding

We would like to thank the Danish Council for Independent Research Technology and Production Sciences for financing the project Mapping and Characterizing of Lipid Oxidation in Emulsified Systems (MAPOX).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank Arla Foods Ingredients and Dupont for donating sodium caseinate and DATEM, respectively. We would also like to thank Maritex A/S, Norway for providing cod liver fish oil. Furthermore, we are grateful to Inge Holmberg, Lis Berner, Yuka Omura Lund and Thi Thu Trang Vu for their skillful work in the laboratory.

■ ABBREVIATIONS USED

CAS, sodium caseinate; DATEM, diacetyl tartaric acid esters of mono- and diglycerides; DHA, docosahexaenoic acid; DHS, dynamic head space; EPA, eicosapentaenoic acid; HPLC, high pressure liquid chromatography; LC PUFAs, long chain polyunsaturated fatty acids; LMWEs, low molecular weight emulsifiers; PV, peroxide value; PCA, principle component analysis; RSM, response surface methodology

■ REFERENCES

(1) Calder, P. C.; Yaqoob, P. Marine omega-3 fatty acids and coronary heart disease. *Curr. Opin. Cardiol.* **2012**, *27*, 412–419.

(2) Song, C.; Shieh, C.; Wu, Y.; Kalueff, A.; Gaikwad, S.; Su, K. The role of omega-3 polyunsaturated fatty acids eicosapentaenoic and docosahexaenoic acids in the treatment of major depression and Alzheimer's disease: Acting separately or synergistically? *Prog. Lipid Res.* **2016**, *62*, 41–54.

(3) EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Scientific opinion on dietary reference values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. *EFSA J.* **2010**, *8*, 1461–1568.

(4) Cheatham, C. L.; Colombo, J.; Carlson, S. E. n-3 Fatty acids and cognitive and visual acuity development: methodologic and conceptual considerations. *Am. J. Clin. Nutr.* **2006**, *83*, 1458S–1466S.

(5) Wysoczański, T.; Sokola-Wysoczańska, E.; Pékala, J.; Lochyński, S.; Czyz, K.; Bodkowski, R.; Herbinger, G.; Patkowska-Sokola, B.; Librowski, T. Omega-3 fatty acids and their role in central nervous system – A review. *Curr. Med. Chem.* **2016**, *23*, 816–831.

(6) Nichols, P. D.; McManus, A.; Krail, K.; Sinclair, A. J.; Miller, M. Recent advances in omega-3: Health benefits, sources, products and bioavailability. *Nutrients* **2014**, *6*, 3727–3733.

(7) Jacobsen, C.; Let, M. B.; Nielsen, N. S.; Meyer, A. S. Antioxidant strategies for preventing oxidative flavour deterioration of foods enriched with n-3 polyunsaturated lipids: a comparative evaluation. *Trends Food Sci. Technol.* **2008**, *19*, 76–93.

(8) Berton-Carabin, C. C.; Ropers, M. H.; Genot, C. Lipid Oxidation in Oil-in-Water Emulsions: Involvement of the Interfacial Layer. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13* (5), 945–977.

(9) Jacobsen, C. Some strategies for the stabilization of long chain n-3 PUFA-enriched foods: A review. *Eur. J. Lipid Sci. Technol.* **2015**, *117*, 1853–1866.

(10) Genot, C.; Meynier, A.; Riaublanc, A. Lipid oxidation in emulsions. In *Lipid oxidation pathways*; Kamal-Eldin, A., Ed.; AOCS Press: Champaign, IL, 2003; pp 190–244.

(11) García-Moreno, P. J.; Horn, A. F.; Jacobsen, C. Influence of casein-phospholipid combinations as emulsifier on the physical and oxidative stability of fish oil-in-water emulsions. *J. Agric. Food Chem.* **2014**, *62*, 1142–1152.

(12) McClements, D. J.; Bai, L.; Chung, C. Recent Advances in the Utilization of Natural Emulsifiers to Form and Stabilize Emulsions. *Annu. Rev. Food Sci. Technol.* **2017**, *8*, 205–236.

(13) Fang, Y.; Dalgleish, D. G. Casein adsorption on the surfaces of oil-in-water emulsions modified by lecithin. *Colloids Surf., B* **1993**, *1*, 357–364.

(14) Munk, M. B.; Larsen, F. H.; van den Berg, F. W. J.; Knudsen, J. C.; Andersen, M. L. Competitive Displacement of Sodium Caseinate by Low-Molecular-Weight Emulsifiers and the Effects on Emulsion Texture and Rheology. *Langmuir* **2014**, *30*, 8687–8696.

(15) Sørensen, A.-D. M.; Haahr, A.-M.; Becker, E. M.; Skibsted, L. H.; Bergenstahl, B.; Nilsson, L.; Jacobsen, C. Interactions between Iron, Phenolic Compounds, Emulsifiers, and pH in Omega-3-Enriched Oil-in-Water Emulsions. *J. Agric. Food Chem.* **2008**, *56*, 1740–1750.

(16) Laguerre, M.; Lopez Giraldo, L. J.; Lecomte, J.; Figueroa-Espinoza, M. C.; Barea, B.; Weiss, J.; Decker, E. A.; Villeneuve, P. Chain length affects antioxidant properties of chlorogenate esters in emulsion: The cutoff theory behind the polar paradox. *J. Agric. Food Chem.* **2009**, *57*, 11335–11342.

(17) Laguerre, M.; Lopez Giraldo, L. J.; Lecomte, J.; Figueroa-Espinoza, M. C.; Baréa, B.; Weiss, J.; Decker, E. A.; Villeneuve, P. Relationship between hydrophobicity and antioxidant ability of “phenolipids” in emulsion: A parabolic effect of the chain length of rosmarinic esters. *J. Agric. Food Chem.* **2010**, *58*, 2869–2876.

(18) Alemán, M.; Bou, R.; Guardiola, F.; Durand, E.; Villeneuve, P.; Jacobsen, C.; Sørensen, A.-D. M. Antioxidative effect of lipophilized caffeic acid in fish oil enriched mayonnaise and milk. *Food Chem.* **2015**, *167*, 236–244.

(19) Sørensen, A.-D. M.; Durand, E.; Laguerre, M.; Bayrasy, C.; Lecomte, J.; Villeneuve, P.; Jacobsen, C. Antioxidant properties and efficacies of synthesized alkyl caffeates, ferulates and coumarates. *J. Agric. Food Chem.* **2014**, *62*, 12553–12562.

(20) Sørensen, A.-D. M.; Lyneborg, K. S.; Villeneuve, P.; Jacobsen, C. Alkyl chain length impacts the antioxidative effect of lipophilized ferulic acid in fish oil enriched milk. *J. Funct. Foods* **2015**, *18*, 959–967.

(21) Costa, M.; Losada-Barreiro, S.; Paiva-Martins, F.; Bravo-Díaz, C.; Romsted, L. S. A direct correlation between the antioxidant efficiencies of caffeic acid and its alkyl esters and their concentrations in the interfacial region of olive oil emulsions. The pseudophase model interpretation of the ‘cut-off’ effect. *Food Chem.* **2015**, *175*, 233–242.

(22) Costa, M.; Losada-Barreiro, S.; Paiva-Martins, F.; Bravo-Díaz, C. Physical evidence that the variations in the efficiency of homologous series of antioxidants in emulsions are a result of differences in their distribution. *J. Sci. Food Agric.* **2017**, *97*, 564–571.

(23) Gülçin, I. Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). *Toxicology* **2006**, *217*, 213–220.

(24) Anankanbil, S.; Pérez, B.; Fernandes, I.; Widzisz, K. M.; Wang, Z.; Mateu, N.; Guo, Z. A new group of synthetic phenolic-containing amphiphilic molecules for multipurpose applications: Physico-chemical characterization and cell-toxicity study. *Sci. Rep.* **2018**, *8*, 832.

(25) Anankanbil, S.; Pérez, B.; Yang, J.; Banerjee, C.; Guo, Z. A novel array of interface confined molecules: Assembling natural segments for delivery of multi-functionalities. *J. Colloid Interface Sci.* **2017**, *508*, 230–236.

(26) Let, M. B.; Jacobsen, C.; Meyer, A. S. Lipid oxidation in milk, yoghurt, and salad dressing enriched with neat fish oil or pre emulsified fish oil. *J. Agric. Food Chem.* **2007**, *55*, 7802–7809.

(27) Horn, A. F.; Nielsen, N. S.; Andersen, U.; Søgaard, L. H.; Horwell, A.; Jacobsen, C. Oxidative stability of 70% fish oil-in-water emulsions: Impact of emulsifiers and pH. *Eur. J. Lipid Sci. Technol.* **2011**, *113*, 1243–1257.

(28) Jacobsen, C.; Meyer, A. S.; Adler-Nissen, J. Oxidation mechanisms in real food emulsions: Method for separation of mayonnaise by ultracentrifugation. *J. Food Lipids* **1998**, *5*, 87–101.

(29) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917.

(30) Shantha, N. C.; Decker, E. A. Rapid, sensitive, iron based spectrophotometric methods for determination of peroxide values of food lipids. *J. AOAC Int.* **1994**, *77*, 421–424.

(31) *Determination of tocopherols and tocotrienols in vegetable oils and fats by HPLC*; AOCS: Champaign, IL, 1998.

(32) Yesiltas, B.; Sørensen, A.-D. M.; García-Moreno, P. J.; Anankanbil, S.; Guo, Z.; Jacobsen, C. Combination of sodium caseinate and succinylated alginate improved stability of high fat fish oil-in-water emulsions. *Food Chem.* **2018**, *255*, 290–299.

(33) Myers, R. H.; Montgomery, D. C.; Anderson-Cook, C. M. *Response Surface Methodology, Process and Product Optimization Using Designed Experiments*, 3rd ed.; John Wiley & Sons, Inc.: Hoboken, NJ, 2002.

(34) Yesiltas, B.; Sørensen, A.-D. M.; García-Moreno, P. J.; Akoh, C. C.; Jacobsen, C. Physical and oxidative stability of high fat fish oil-in-water emulsions stabilized with sodium caseinate and phosphatidylcholine as emulsifiers. *Food Chem.* **2018**, DOI: [10.1016/j.foodchem.2018.09.172](https://doi.org/10.1016/j.foodchem.2018.09.172)

(35) Gaupp, R.; Adams, W. Di-acetyltartaric esters of monoglycerides (DATEM) and associated emulsifiers in bread making. In *Emulsifiers in Food Technology*; Whitehurst, R. J., Ed.; Blackwell Publishing Ltd.: Northampton, United Kingdom, 2004; pp 86–109.

(36) Pripp, A. H.; Vreeker, R.; van Duynhoven, J. Binding of olive oil phenolics to food proteins. *J. Sci. Food Agric.* **2005**, *85*, 354–362.

(37) Di Mattia, C.; Paradiso, V. M.; Andrich, L.; Giarnetti, M.; Caponio, F.; Pittia, P. Effect of Olive Oil Phenolic Compounds and Maltodextrins on the Physical Properties and Oxidative Stability of Olive Oil O/W Emulsions. *Food Biophys* **2014**, *9*, 396–405.

(38) Di Mattia, C. D.; Sacchetti, G.; Mastrocola, D.; Sarker, D. K.; Pittia, P. Surface properties of phenolic compounds and their influence on the dispersion degree and oxidative stability of olive oil O/W emulsions. *Food Hydrocolloids* **2010**, *24*, 652–658.

(39) Köhler, P. Study of the Effect of DATEM. 3: Synthesis and Characterization of DATEM Components. *LWT* **2001**, *34*, 359–366.

(40) Pal, R. Effect of Droplet Size on the Rheology of Emulsions. *AIChE J.* **1996**, *42* (11), 3181–3190.

(41) Capron, I.; Costeux, S.; Djabourov, M. Water in water emulsions: Phase separation and rheology of biopolymer solutions. *Rheol. Acta* **2001**, *40*, 441–456.

(42) McClements, D. J. Emulsion Ingredients. In *Food Emulsions: Principles, Practices, and Techniques*, 3rd ed.; CRC Press: Boca Raton, FL, 2015.

(43) Sørensen, A.-D. M.; Villeneuve, P.; Jacobsen, C. Alkyl caffeates as antioxidants in O/W emulsions: Impact of emulsifier type and endogenous tocopherols. *Eur. J. Lipid Sci. Technol.* **2017**, *119*, 1600276.

(44) Genaro-Mattos, T. C.; Maurício, A. Q.; Rettori, D.; Alonso, A.; Hermes-Lima, M. Antioxidant Activity of Caffeic Acid against Iron-Induced Free Radical Generation—A Chemical Approach. *PLoS One* **2015**, *10* (6), No. e0129963.

(45) Iglesias, J.; Pazos, M.; Andersen, L.; Skibsted, L. H.; Medina, I. Caffeic acid as antioxidant in fish muscle: Mechanism of synergism with endogenous ascorbic acid and α -tocopherol. *J. Agric. Food Chem.* **2009**, *57* (2), 675–681.

(46) Medina, I.; Undeland, I.; Larsson, K.; Storror, I.; Rustad, T.; Jacobsen, C.; Kristinová, V.; Gallardo, J. M. Activity of caffeic acid in different fish lipid matrices: A review. *Food Chem.* **2012**, *131*, 730–740.

PAPER VI

Yesiltas, B., Sørensen, A. M., García-Moreno, P. J., Anankanbil, S., Guo, Z., Jacobsen, C., 2018.

**Modified phosphatidylcholine with different alkyl chain length
and covalently attached caffeic acid affects the physical and oxidative stability of
omega-3 delivery 70% oil-in-water emulsions.**

Submitted to *Food Chemistry*.

1 **Modified phosphatidylcholine with different alkyl chain length and covalently attached**
2 **caffeic acid affects the physical and oxidative stability of omega-3 delivery 70% oil-in-**
3 **water emulsions**

4 Betül Yesiltas¹, Ann-Dorit M. Sørensen¹, Pedro J. García-Moreno¹, Sampson Anankanbil²,
5 Zheng Guo², Charlotte Jacobsen¹

6 ¹National Food Institute, Technical University of Denmark, Lyngby, Denmark

7 ²Department of Engineering, Aarhus University, Aarhus, Denmark

8 *Correspondence: Charlotte Jacobsen, National Food Institute, Technical University of
9 Denmark, Kemitorvet, Building 204, 2800 Kgs. Lyngby, Tel: +45 23 27 90 75; e-mail:
10 chja@food.dtu.dk

11

12

13

14

15

16

17

18

19

20 **Abstract**

21 This study investigated the effects of modified phosphatidylcholine (PC) with different alkyl
22 chain lengths (PC_C14 and PC_C16) and covalently attached caffeic acid on the physical
23 and oxidative stability of 70% fish oil-in-water emulsions. High fat emulsions were produced
24 using different amounts of modified PCs in combination with sodium caseinate and soy-PC.
25 Results showed that the physical stability of the emulsions was improved with increasing
26 concentrations of modified PCs, due to their high surface activity. Emulsion stabilized with
27 PC_C14 led to smaller droplets and higher viscosity, whereas PC_C16 had higher protein
28 surface load, which may result in a thicker interfacial layer. Modified PCs enhanced the
29 oxidative stability of the emulsions due to the attachment of caffeic acid to the glycerol
30 backbone of PC, which brings the antioxidant in the vicinity of oil-water interface. PC_C16 led
31 to less formation of primary and secondary oxidation products compared to PC_14 at their
32 equivalent concentrations.

33 **Key words:** modified phospholipids; phosphatidylcholine; sodium caseinate; lipid oxidation;
34 oil-water interface; caffeic acid; high fat delivery emulsions

35

36

37

38

39

1. Introduction

Evidences for health benefits of long chain (LC) omega-3 (n-3) polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) have been increasing in the last decades. Some of the effects include decreasing the risk of cardiovascular diseases, improving brain development in infants, maintaining normal blood pressure and triglyceride levels in the blood, supporting mental health as well as immune system (Calder & Yaqoob, 2012; Song et al., 2016; Cheatham, Colombo, & Carlson, 2006).

LC n-3 PUFAs are mainly available in marine fish and fish products. However, the consumption of these LC n-3 PUFAs is inadequate in most Western countries (EFSA, 2010). Therefore, increasing the consumption of these bioactive compounds has attracted interest from food researchers and industry. Due to easier hydrogen abstraction from the bis-allylic positions, LC n-3 PUFAs are highly prone to oxidation (Frankel, 2012b). Therefore, delivery systems have been developed in order to protect LC n-3 PUFAs against lipid oxidation. One of the delivery systems for LC n-3 PUFAs is oil-in-water emulsions. High fat (70%) oil-in-water emulsions were found to be advantageous for enrichment of high fat content food systems with LC n-3 PUFAs (Horn et al., 2011; Yesiltas et al., 2018a,b).

Previous studies focused on different ways of improving physical properties of the interface as well as enhancing the oxidative stability of the oil-in-water emulsion system. The strategies studied include to: i) use phenolipids with various alkyl chain lengths and phenolic compounds (Laguerre et al., 2009; Sørensen et al., 2014; Alemán et al., 2015; Sørensen et al., 2017), ii) incorporate emulsifiers with antioxidant activities (Yesiltas et al., 2019), iii) add free

61 antioxidants in the emulsion along with emulsifiers (Sørensen et al., 2008), or iv) to have
62 emulsifiers modified with various alkyl chains and covalently attached phenolic acids
63 (Anankanbil et al., 2018; Yesiltas et al., 2018b).

64 One of the approaches to limit lipid oxidation whilst ensuring an acceptable physical stability
65 is engineering an interfacial layer with optimal properties to resist oxidation, as oxidation has
66 been claimed to be initiated at the interface. In order to have a bioinspired interface structure,
67 proteins and phospholipids could be used in combination. Such a combination of emulsifiers
68 has been suggested to form an interface structure providing a better coverage of the oil
69 droplets compared to a single emulsifier (Fang and Dalgleish, 2016; García-Moreno et al.,
70 2014; Berton-Carabin et al., 2018; Yesiltas et al., 2019), which could provide a good physical
71 barrier for prooxidants` diffusion into the oil phase.

72 Previous studies have focused on the antioxidant activity at the oil-water interface of the oil-in-
73 water emulsions (Laguerre et al., 2013; Berton-Carabin et al., 2014, 2018). However,
74 lipophilicity of the surface active antioxidative compound has an important effect on its
75 antioxidative activity in heterogeneous systems. Based on polar paradox and cut-off effect
76 theories, researchers have focused on finding the most efficient lipophilicity for particular
77 surface active compounds with antioxidant activity in emulsion systems. In the studies
78 conducted using phenolipids with various lipophilicity, it was observed that the most efficient
79 alkyl chain lengths could be system and particular compound dependent (Laguerre et al.,
80 2013; Alemán et al., 2015; Sørensen et al., 2017).

81 Caffeic acid is a commonly used antioxidant with radical scavenging and metal chelating
82 (especially iron) activities and its efficacy has been shown to be dependent on pH, addition of
83 iron and emulsifier type (Gülcin, 2006; Sørensen et al., 2008). A previous study reported that
84 caffeic acid and alkyl caffeates had the highest radical scavenging activity and reducing
85 power compared to ferulic acid and alkyl ferulates, and coumaric acid and alkyl coumarates
86 (Sørensen et al., 2014). The effect of the alkyl chain length on antioxidant efficacy has also
87 been studied for caffeic acid esters. It was found that the medium alkyl chain length caffeic
88 acid (octyl caffeate) had higher antioxidant activity compared to shorter and longer alkyl chain
89 lengths in 40% soybean oil-in-water emulsions (Costa, Losada-Barreiro, Paica-Martinsa, and
90 Bravo-Díaz, 2017). Similar results were obtained when alkyl caffeates were applied in fish oil
91 enriched mayonnaise; short to medium alkyl chain (C4, C8, and C12) caffeic acid were found
92 to be more effective antioxidants (Alemán et al., 2015). Another study carried out with
93 modified diacetyl tartaric acid esters of mono- and diglycerides (DATEM) with covalently
94 attached caffeic acid and C12 or C14 alkyl chain lengths showed that the modified DATEM
95 with C14 chain length provided slightly better oxidative stability compared to the modified
96 DATEM with C12 chain length in 70% fish oil-in-water emulsions produced with CAS, DATEM
97 and modified DATEMs (Yesiltas et al., 2018b).

98 The aim of this study was to investigate the effect of modified PC with covalently attached
99 caffeic acid and different alkyl chain lengths on physical and oxidative stability of 70% fish oil-
100 in-water emulsions stabilized by sodium caseinate, soy PC and modified PCs. Soy PC and
101 modified PC in combination with CAS was used in order to decrease the viscosity of the high
102 fat omega-3 delivery emulsions when compared to emulsions stabilized only with CAS. This

103 will make it easier to apply the delivery emulsions in food systems. It was hypothesized that
104 incorporation of modified PCs in the 70% fish oil-in-water emulsions will lead to a more
105 pronounced reduction in interfacial tension compared to conventional PC with 2 alkyl chains.
106 Thus, this will result in emulsions with enhanced physical stability. Moreover, different chain
107 length (C14 and C16) of modified PCs is expected to have an impact on adsorption of the
108 molecule at the oil-water interface of the emulsions due to the different hydrophilic-lipophilic
109 balance (HLB) values. In addition, modified PCs with covalently attached caffeic acid are
110 expected to enhance oxidative stability of the emulsions compared to physical mixtures of soy
111 PC and free caffeic acids with the strategy of bringing the phenolic compounds with
112 antioxidant activity into the vicinity of the interface taking advantage of the amphiphilicity of
113 the PCs.

114 **2. Materials and Methods**

115 **2.1. Materials**

116 Cod liver oil was provided by Vesteraalens A/S (Sortland, Norway) and stored at -40 °C until
117 use. Peroxide value was determined as 0.09 ± 0.00 meq peroxides/kg oil. The fatty acid (%
118 w/w) content of the fish oil was as follows: C14:0 (4.0), C16:0 (9.2), C16:1 n-7 (8.3), C18:0
119 (2.2), C18:1 n-9 (15.8), C18:1 n-7 (4.1), C18:2 n-6 (2.5), C18:3 n-3 (0.2), C20:1 n-9 (11.4),
120 C20:5 n-3 (8.8), C22:1 n-11 (5.4), and C22:6 n-3 (11.4). Alpha-, beta-, gamma-, and delta
121 tocopherol contents were 146 ± 7 , 0 ± 0 , 97 ± 2 , 43 ± 0.3 μg toc/g oil, respectively. Sodium
122 caseinate, CAS (Miprodan 30) was donated by Arla Foods Ingredients amba (Viby J,
123 Denmark). Arla reported a protein content of 92% in sodium caseinate for Miprodan 30.

124 Phosphatidylcholine extracted from soybean (LIPOID S100, soy PC) was provided by Lipoid
125 GmbH, Germany. Peroxide value was 1.91 meq peroxides/kg sample. Analysis of certificate
126 of the LIPOID S100 reported that 97.1% of the product was phosphatidylcholine (based on
127 dry weight) and that it contained 0.20% DL- α -Tocopherol, which is an antioxidant. Other lipid
128 molecules were also reported as follows: 1.2% lysophosphatidylcholine, 0.5% N-acyl-
129 phosphatidylethanolamine, lower than 0.1% phosphatidylethanolamine, lower than 0.1%
130 phosphatidylinositol, 0.8% non-polar lipids, and 0.3% triglycerides. The fatty acid (% w/w)
131 content of the soy PC (LIPOID S100) was analyzed and found as follows: C14:0 (0.09), C16:0
132 (12.55), C18:0 (3.77), C18:1 n-9 (8.42), C18:2 n-6 (65.82), C18:3 n-6 (7.11). Caffeic acid was
133 purchased from Sigma Aldrich. Modified PCs (PC_C14 and PC_C16) with caffeic acid and
134 different alkyl chains C14 or C16 were synthesized as described in a previous study
135 (Anankanbil et al., 2018). The fatty acid (% w/w) content of the PC_C14 and PC_C16 was as
136 follows: C14:0 (99.38%) and C16:0 (98.74%), respectively. All other chemicals and solvents
137 used were of analytical grade.

138 **2.2. Experimental design**

139 Sample codes, descriptions and amounts of ingredients are listed in Table 1. All samples
140 include 70 wt% fish oil and 2.8 wt% total emulsifier with a ratio of CAS to total PC of 1.2.
141 These values were selected based on a previous study (Yesiltas et al., 2019). Soy PC was
142 replaced by modified PCs in order to obtain various final caffeic acid concentrations, namely
143 360, 1080 and 2160 ppm in the final emulsion. Emulsions produced with soy PC and free
144 caffeic acid were also included in the experimental plan as controls.

145 **2.3. Emulsion preparation, storage and sampling**

146 Emulsifiers (CAS, PC, and modified PCs) were dissolved in distilled water and stirred
147 overnight at 4°C. Aqueous phases were adjusted to pH 7 using 2M NaOH. Emulsions were
148 produced in 500 g batches in a Stephan Universal mixer (Stephan, UMC5, 1995, Hameln,
149 Germany) as described by Horn et al. (2011). Sodium azide (0.05% w/v) and 100 µM Fe²⁺
150 were added into emulsions in order to prevent microbial growth and accelerate lipid oxidation,
151 respectively. All emulsions were divided into 100 mL bottles in approximately 90 g and stored
152 at room temperature for up to 12 days in darkness. Samples were collected at days 0, 2, 5, 8
153 and 12 for physical and oxidative stability analyses.

154 **2.4. Methods for characterization of emulsions**

155 **2.4.1. Creaming index**

156 Creaming rate was determined by the measurement of creaming index on days 1, 5, 8 and 12
157 in the stored bottles without replicates. Creaming index was calculated using equation 1:

$$158 \quad CI (\%) = b/a*100 \quad \text{(Equation 1)}$$

159 where (a) is the height of total emulsion and (b) is the height of aqueous phase separated at
160 the bottom of the bottle. Creaming index was calculated as in percentage for each emulsion
161 sample and sampling point.

162 **2.4.2. Interfacial tension of the emulsifier combinations – pendant drop method**

163 The dynamic interfacial tension of the emulsifiers at the oil-water interface was determined
164 using an automated drop tensiometer OCA20 (DataPhysics Instruments GmbH, Filderstadt,
165 Germany) at 25°C. Aqueous phases of emulsions 1 CAS, 2 PC_com, 5 PC_C14_2160 and 8

166 PC_C16_2160 (see what the codes stand for in Table 1) were prepared as described before
167 and measured in duplicates. The surfactant solution (aqueous phase) was filled into a syringe
168 with a screwed needle. For each measurement, a small drop of the aqueous phase sample
169 solution was generated using the automated syringe into a quartz glass cuvette filled with
170 MCT oil (WITARIX® MCT 60/40, IOI Oleo GmbH, Hamburg, Germany). The image of the
171 drop was recorded with a camera every 10 s for 15 min. The images were transferred to the
172 drop shape analysis software. The calculation of the interfacial tension was based on the
173 shape analysis of a pendant drop according to the Young-Laplace equation, equation 2:

$$174 \quad \Delta P = \gamma \left(\frac{1}{R_1} + \frac{1}{R_2} \right) \quad (\text{Equation 2})$$

175 where ΔP (mN/m²) is the pressure difference across the interface, γ (mN/m) is the interfacial
176 tension and R_1 and R_2 (m) are the principal radii of curvature of the pendant drop.
177 Measurements were carried out in duplicate. Changes in the interfacial tension (mN/m) were
178 plotted as a function of time (min) for each aqueous phase solution.
179

180 **2.4.3. Zeta potential**

181 Zeta potential measurements were performed using Zetasizer Nano 2S (Malvern Instruments,
182 Ltd.) in order to determine the surface charge of the emulsion droplets. Each sample (0.32 g
183 sample) was diluted in distilled water (40 g of distilled water) before measuring and placed in
184 DTS-1070 disposable folded capillary cell (Malvern Instruments, Ltd., United Kingdom). The
185 zeta potential range was set to -100 to +50 mV and measurements were done at 25°C on the
186 samples collected on day 2. Measurements were carried out in duplicate.

187 **2.4.4. Droplet size**

188 Particle size of the emulsions was determined using laser diffraction in a Mastersizer 2000
189 (Malvern Instruments, Ltd., Worcestershire, UK) on days 0 and 12 using the method
190 described by Horn et al. (2011). Results were presented as the surface weighted (D[3,2]) and
191 volume weighted (D[4,3]) mean diameter, which were calculated according to equations 3 and
192 4, respectively:

$$193 \quad D[3,2] = \frac{\sum nidi^3}{\sum nidi^2} \quad (\text{Equation 3})$$

$$194 \quad D[4,3] = \frac{\sum nidi^4}{\sum nidi^3} \quad (\text{Equation 4})$$

195 where n is the number of droplet with a specific diameter and d is the diameter.
196 Measurements were carried out as duplicates.

197 **2.4.5. Apparent viscosity**

198 Apparent viscosity was measured using a stress-controlled rheometer (Stresstech, Reologica
199 Instruments AB, Lund, Sweden) on days 1 and 12. Rheometer was equipped with a CC25
200 standard bob cup system in a temperature vessel. 15 ml of emulsion were measured over a
201 shear stress range from 0.0125 to 50 Pa at 25°C. Results were calculated on a specific shear
202 rate (20 s⁻¹) for each emulsion in Pascal second (Pa·s). Samples were measured in duplicate.

203 **2.4.6. Relative protein content in the aqueous phase and the surface load of** 204 **proteins**

205 Protein content of the aqueous phase was measured based on the method described by
206 Jacobsen, Meyer, & Adler-Nissen (1998). In order to separate the aqueous phase, emulsion
207 sample (~20 g) was centrifuged for 10 min at 25,400g and 10 °C (Sorvall RC-6 PLUS,

208 Thermo Fisher Scientific, Osterode, Germany; rotor SS-34). Supernatant (oil phase) was
209 removed by the use of a pipette. The rest was mixed with distilled water (1:1) and then
210 subjected to ultracentrifugation (Beckman Ultracentrifuge L8-60M, Fullerton, CA; rotor 21102)
211 for 1 h at 106,979g and 15 °C. Dumas method (Elementar, Mt. Laurel, NJ, USA) was used for
212 the determination of protein concentration. Approximately 1 g of aqueous phase was placed
213 in the sample tray and further steps were automated including sample combustion in a
214 chamber at a high temperature (900°C) in the presence of oxygen. Content of crude protein
215 was estimated by using a conversion factor (6.25). Protein concentration is reported as
216 percent of aqueous phase. Measurements were carried out in duplicate.

217 The surface load of proteins (Γ , mg/m²) was calculated according to the equation described
218 by Zhu et al. (2018), equation 5:

$$219 \quad \Gamma = \frac{V_C(C_{INI} - C_{SER})}{S V_{OIL}} = \frac{(1-\Phi)d_{32}}{6\Phi} (C_{INI} - C_{SER}) \quad (\text{Equation 5})$$

220 where V_C and V_{OIL} are the volume of the aqueous and oil phase (mL), S is the surface area of
221 the emulsion droplets (m²), Φ is the oil phase volume fraction, C_{INI} is the initial concentration
222 of the protein in the aqueous phase (mg/L), and C_{SER} is the non-adsorbed protein
223 concentration in the aqueous phase after emulsification (mg/L).

224 **2.5. Methods for lipid oxidation measurements of emulsions**

225 **2.5.1. Primary oxidation products—peroxide value (PV)**

226 Primary oxidation products were determined according to the Bligh and Dyer method with
227 slight changes (Bligh & Dyer, 1959). Lipid extract was prepared using 5 g emulsion for each

228 extraction and a reduced amount of solvent (30.0 mL of methanol and chloroform, 1:1). PV
229 was subsequently measured on the lipid extracts by colorimetric determination of iron
230 thiocyanate on a spectrophotometer (Shimadzu, UV mini 1240, Kyoto, Japan) at 500 nm, as
231 described by Shantha and Decker (1994). Measurements were carried out in duplicate.

232 **2.5.2. Tocopherol content - HPLC**

233 Tocopherol content of emulsions was determined by HPLC (Agilent 1100 Series; Column:
234 Waters Spherisorb 3 μm Silica; 4.6 \times 150 mm). Tocopherol analysis was carried out according
235 to the official AOCS method (1998) using lipid extracts (see Section 2.5.1) that were further
236 evaporated and re-dissolved in heptane. Measurements were carried out in duplicate.

237 **2.5.3. Secondary oxidation products—Dynamic Head Space GC-MS**

238 Volatile secondary oxidation products were analyzed according to the method described by
239 Yesiltas et al (2018a). Volatile compounds were trapped on Tenax GR tubes. The volatiles
240 were separated in a gas chromatograph (Agilent Technologies, 6890N Network GC System,
241 DE, USA) on a 30 m DB 1701 fused silica capillary column (0.25 mm i.d., 1 μm film thickness;
242 Agilent Technologies, J&W GC Columns, CA, USA). The individual compounds were
243 analyzed by mass-spectrometry (Agilent 5973 Network Mass Selective Detector, Agilent
244 Technologies, 70 eV; mass to charge ratio scan between 30 and 250) and identified by MS-
245 library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard). The volatile
246 compounds 2-ethyl-furan, 1-penten-3-one, 1-penten-3-ol, (*E*)-2-pentenal, hexanal, (*E*)-2-
247 hexenal, (*Z*)-4-heptenal, 2-pentyl-furan, (*E*)-2-heptenal, benzaldehyde, (*E,E*)-2,4-heptadienal,
248 nonanal and (*E,Z*)-2,6-nonadienal were selected for quantification and analyzed in all

249 emulsions. Calibration curve was prepared by injecting standards into an emulsion produced
250 with CAS and soy PC. Then volatiles were collected the same way as the emulsion samples.
251 This was carried out in order to maintain similar release conditions for standard volatile
252 compounds and volatiles in the emulsion samples.

253 **2.6. Statistical analysis and principle component analysis (PCA)**

254 Statgraphics XVII (Statpoint Technologies, Inc., Virginia, USA) was used to carry out the
255 analysis of variance (ANOVA) using Fisher's least significant difference test. The significance
256 was evaluated statistically at the confidence level $1-\alpha = 95\%$.

257 PCA was done using Latentix 2.12 (LatentiX, Copenhagen, Denmark). The PCA was carried
258 out with the emulsions as objects and creaming, viscosity, droplet size, zeta potential,
259 peroxide value, alpha-tocopherol, and volatile compounds as variables. The data was
260 transformed using the autoscale function in Latentix and the PCA models were calculated.

261 **3. Results and Discussion**

262 **3.1. PCA of all emulsions**

263 Two different PCA models were calculated; one where all time points for each of the variables
264 were included and one where only the last time point (day 12) of the variables was included.
265 The conclusion that could be made from these 2 models was more or less the same. For the
266 sake of simplicity, only the model calculated with the last time point for the variables is shown
267 in Fig. 1. The first principle component (PC1) and second principle component (PC2)
268 explained 43 and 29% of the variation in the data, respectively. The scores plot showed that
269 the 1_CAS emulsion located differently compared to the rest of the emulsions, indicating that

270 it behaved differently with respect to the physical and oxidative parameters measured. The
271 loadings plot showed that the location of CAS was mostly explained by the high viscosity as
272 well as higher protein content in the aqueous phase (non-adsorbed CAS). Oxidation
273 parameters, PV, tocopherols and volatile compounds were located in the same quadrant and
274 correlated with the following emulsions: 2_PC_com, 3_PC_C14_360, 6_PC_C16_360, and
275 9_PC_com_caf_360, which were most prone to lipid oxidation.

276 Emulsion 10_PC_com_caf_2160 was mostly explained by high zeta potential (less negative
277 zeta potential), high creaming, low viscosity and low non-adsorbed proteins. Emulsions
278 produced with middle and high concentrations of modified PCs were not described by any of
279 the specific physical parameters. However, they located far from the oxidative parameters,
280 which indicated that their oxidative stability were superior compared to the rest. In order to
281 obtain a better overall picture of the emulsions according to the physical parameters, see the
282 biplot (Supplementary Fig. 1) for the PCA model calculated only with physical parameters.

283 It was observed that the emulsions with PC_C14 were closer to viscosity and non-adsorbed
284 CAS compared to PC_C16, whereas emulsions with PC_C16 located closer to zeta potential
285 and creaming. This indicated differences in physical stability such as higher viscosity of the
286 emulsions with PC_C14 due to more non-adsorbed CAS content as well as higher protein
287 surface load, creaming and lower negative zeta potential for the emulsions with PC_C16.
288 However, the oxidation parameters showed that the emulsions with PC_C14 located closer to
289 oxidation parameters (volatiles, PV) compared to PC_C16 emulsions, which confirmed better
290 oxidative stability of the emulsions produced with PC_C16. In order to confirm this

291 interpretation of the PCA model, the original raw data of the physical and oxidative stability
292 parameters were studied in more detail.

293 **3.2. Characterization of emulsions**

294 **3.2.1. Creaming Index**

295 Emulsions produced with the combinations of CAS and soy PC were creamed 4-6%, whereas
296 emulsions produced with only CAS did not have any creaming during 12 days of storage
297 (Supplementary Fig. 2a,b). Modified emulsifiers also showed between 2-4% creaming during
298 12 days of storage. As these emulsions have lower creaming rate than 1 mm/day, they were
299 all considered as stable emulsions (McClements, 1999).

300 **3.2.2. Interfacial tension of the emulsifier combinations**

301 Oil-water interfacial tension of the aqueous phase of the emulsions was measured in order to
302 determine the effects of substituting 45% of the CAS with different PCs. The interfacial
303 tension values of all the samples at 15 min were significantly different from each other
304 ($p < 0.05$). It was observed that 2_PC_com had an oil-water interfacial tension of 11.47 ± 0.35
305 mN/m, whereas aqueous phase of 1_CAS had an interfacial tension of 8.34 ± 0.02 mN/m
306 (Fig. 2). Thus, including soy PC increased the interfacial tension. However, when 60% of the
307 soy PC in 2_PC_com was substituted with PC_C14 or PC_16, interfacial tension dropped
308 significantly from 11.47 ± 0.35 mN/m to 4.57 ± 0.01 or 3.94 ± 0.04 mN/m, respectively.
309 Aqueous phases with PC_C16 showed significantly lower interfacial tension compared to
310 PC_C14, which could be due to the interaction of PC_C16 with soy PC and CAS at the
311 interface as well as higher affinity to the oil phase. Low interfacial tension provided by

312 modified PCs implies a superior surface activity, which provided minimized contact area
313 between hydrophobic and hydrophilic regions.

314 **3.2.3. Droplet size**

315 The mean particle diameter ($D[4,3]$) of the emulsions was determined in order to observe the
316 impact of emulsifier types and different concentrations of modified PCs (Table 2). Substituting
317 45% of CAS with soy PC resulted in a significant increase in droplet size from 8.8 ± 0.3 to 17
318 $\pm 0.2 \mu\text{m}$. However, increasing concentration of modified PCs in the emulsions led to a
319 significant decrease in droplet size (Table 2). This could be due to the fact that modified PCs
320 had one alkyl chain instead of 2 alkyl chains compared to soy PC as well as a larger head
321 part as a result of covalently attached caffeic acid in the glycerol backbone, which might have
322 enhanced the surface activity. This was valid for both PC_C14 and PC_C16, which could be
323 attributed to their fast adsorption rates compared to proteins and soy PC, thereby producing
324 smaller oil droplets by inhibiting droplet coalescence during homogenization. Improved
325 performance of lysolecithins compared to conventional PC with 2 fatty acid chains was
326 reported previously by other researchers (Choi et al., 2011; Casado, Martin, Torres, &
327 Reglero, 2012). This could be due to higher HLB values of modified PCs compared to
328 conventional PC, which is around seven. It has been emphasized that lysolecithins usually
329 disperse better in the aqueous phase which influence their effectiveness (McClements et al,
330 2017).

331 When the droplet size of the emulsions with the equivalent concentrations of modified PCs
332 was compared, it was observed that PC_C14 provided smaller droplets compared to

333 PC_C16, which indicated faster adsorption of PC_C14 at the oil-water interface compared to
334 PC_C16 at their middle and higher concentrations, despite the fact the PC_C16 provided
335 lower interfacial tension than PC_C14. At the low concentration of modified PCs, there was
336 no significant effect of chain length observed for droplet sizes.

337 These results are generally consistent with the interfacial tension results, for which it was
338 found that the aqueous phase of 2_PC_com had higher oil-water interfacial tension compared
339 to 1_CAS, whereas addition of modified PCs decreased the interfacial tension significantly.
340 Higher surface activity of modified PCs improved emulsification of smaller droplets before
341 their coalescence. Moreover, it was obvious that the increasing concentration of modified PCs
342 provided significant decrease in droplet size (Table 2). The change in the droplet size with
343 increased concentration of modified PCs can also be seen in the optical microscope images
344 (Supplementary Fig. 3a,b). There was no significant increase in D[4,3] droplet sizes observed
345 during storage except for 5_PC_C14_2160 (Table 2, Supplementary Fig. 4a).

346 **3.2.4. Protein content in the aqueous phase and the surface load of proteins**

347 Protein content in the aqueous phase has several effects on physical and oxidative stability of
348 the emulsions. A previous study showed that viscosity of the emulsions was directly increased
349 by the concentration of sodium caseinate in the continuous phase when the concentration
350 was between 5-10% in 30% oil-in-water emulsion (Liang et al., 2014). Viscosity of the final
351 emulsion increases with the increasing viscosity of the continuous phase (Tesch & Schubert,
352 2002; Yesiltas et al., 2018a). On the other hand, proteins (e.g., CAS) inhibit oxidation due to
353 their radical scavenging and metal chelating activities, which allow proteins to deactivate

354 prooxidants either at the oil-water interface or in the aqueous phase (Faraji, McClements, &
355 Decker, 2004; Berton, Ropers, Viau, & Genot, 2011). For these reasons, we have determined
356 the protein content in the aqueous phases of the emulsions. Additionally, we have calculated
357 the protein surface load in order to indirectly infer the adsorption of modified PCs at the
358 interface. Forty to 66% of the protein was non-adsorbed in the aqueous phase, which
359 indicated that the proteins were available in the aqueous phase in concentrations ranging
360 between 2.25 – 6.23 wt%. Non-adsorbed protein of 8_PC_C16_2160 was significantly lower
361 compared to the rest of the emulsions except for 6_PC_C16_1080 and 7_PC_C16_2160
362 (Table 2). Changing modified PC concentration did not affect the protein content in the
363 aqueous phase significantly neither for PC_C14 nor PC_C16. Given that 5_PC_C14_2160
364 had significantly more protein content in the water phase compared to 8_PC_C16_2160,
365 PC_C14 showed better surface activity at the oil-water interface, thereby replacing more
366 protein at the oil-water interface. Protein surface load results revealed more information about
367 the adsorbed protein due to the impact of droplet size on the protein surface load.
368 Interestingly, involvement of soy PC led to 4 folds higher protein load at the oil-water interface
369 compared to 1_CAS (see 1_CAS versus 2_PC_com, 9_PC_com_caf_360, and
370 10_PC_com_caf_2160, Table 2). This could be due to the interaction between CAS and soy
371 PC, which presumably resulted in the adsorption of larger CAS aggregates and PC
372 multilayers at the interface. This hypothesis was supported by results from another study from
373 our lab, which indicated that emulsions produced with CAS and PC provided thicker interface
374 layer compared to only CAS emulsions and which provided a model for the complex structure
375 of 70 % oil-in-water emulsions with CAS and PC as emulsifiers (data submitted for
376 publication). Protein surface load decreased with increasing concentration of modified PCs

377 both for PC_C14 and PC_C16 (Table 2). Emulsions with PC_C14 had lower protein surface
378 load compared to emulsions with PC_C16, which would normally support that PC_C14 was
379 more surface active and replaced more proteins at the interface compared to PC_C16.
380 However, interfacial tension results (Section 3.1.2) indicated that aqueous phase with
381 PC_C16 had lower interfacial tension (4 mN/m) compared to PC_C14 (5 mN/m). This was
382 presumably due to PC_C16's more balanced molecular interactions with soy PC and CAS at
383 the interface, such as forming a densely packed interfacial layer with less permeability, which
384 are not merely related to the surface activity of the molecule. Therefore, these results could
385 be attributed to thicker oil-water interfacial layer formed in the presence of PC_C16, which
386 thereby led to a higher protein surface load compared to PC_C14.

387 **3.2.5. Zeta potential**

388 All the samples had negative zeta potential, which was mainly attributed to the sodium
389 caseinate being above its isoelectric point (pH 4.6) as the pH of the emulsion was 7
390 (O'Kennedy, 2011) (Table 2). Soy PC is a zwitterionic molecule, but it generally gives
391 negative surface charge at neutral conditions (Anankanbil et al., 2018). Modified PCs were
392 also negatively charged due to the phosphate groups at pH 7. Thus, when these emulsifiers
393 were used alone to emulsify 20% fish oil-in-water emulsions at pH 7, they resulted in a
394 surface charge of -37.20, -39.45 and -50.20 mV for PC_C14, PC_C16 and soy PC,
395 respectively (Anankanbil et al., 2018). In the current study, it was observed that the
396 substitution of some of the CAS with soy PC or soy PC + modified PCs resulted in less
397 negatively charged lipid droplets.

398 Free caffeic acid addition did not result in any significant change in surface charge of the
399 emulsions produced with CAS and soy PC (Table 2). Increasing the concentration of modified
400 PCs did not have a clear effect on surface charge. Comparison of the surface charge of the
401 emulsions produced with equivalent concentrations (360 and 1080 ppm caffeic acid) of
402 modified PCs showed that the emulsions with PC_C14 provided more negatively charged
403 particles compared to PC_C16. The determination of the protein content in the aqueous
404 phase and protein load at the interface showed that PC_C14 replaced protein at the oil-water
405 interface, whereas PC_16 interacted with CAS and soy PC at the oil-water interface. Since
406 CAS is more negatively charged than modified PCs, one would expect that the PC_16
407 emulsions would have a more negative charge than the emulsion with PC_14. However,
408 adsorption of these emulsifiers does not necessarily result in a monolayer. Protein surface
409 load results indicated that the interaction between CAS, soy PC and PC_C16 resulted in a
410 thicker interfacial layer, which gave less negative zeta potential due to the higher amount of
411 PC_C16 compared to the amount of PC_C14 when involved in the emulsions.

412 The reason for less negatively charged droplet surfaces for 2_PC_com compared to
413 3_PC_C14_360 and more negatively charged than 7_PC_C16_1080 could also be attributed
414 to more CAS, soy PC and PC_C16 adsorbed at the oil-water interface forming a thicker
415 interface, which results in less negatively charged interface compared to the emulsions with
416 PC_C14. Moreover, as there was no significant difference in protein surface load of
417 emulsions produced with PC_C14 and PC_C16 at their equivalent concentrations, different
418 surface charge could be attributed to the content of soy PC and modified PCs. As emulsions
419 with PC_C16 had less negative zeta potential, this indicates the presence of higher amount of

420 soy PC and PC_C16 at the oil-water interface compared to emulsions produced with
421 PC_C14.

422 **3.2.6. Apparent viscosity**

423 Emulsions were non-Newtonian and showed shear thinning behavior. Substitution of 45% of
424 the CAS with soy PC decreased viscosity significantly as expected due to the increase in
425 droplet size (D[4,3], Table 2). Viscosity of the emulsions decreased significantly during
426 storage except for 1_CAS and 9_PC_com_caf_360 (Table 2, Supplementary Fig. 4b).
427 Addition of 2160 ppm free caffeic acid into the CAS + soy PC emulsions resulted in significant
428 decrease in the viscosity as well, which correlated well with the significant increase in the
429 droplet size (D[4,3], Table 2). This negative correlation between droplet size and viscosity
430 could be due to smaller droplets leading to more friction between oil droplets at an expanded
431 surface-to-volume ratio of the dispersed phase. This results in less mobility of the droplets in
432 the emulsion and therefore provides higher viscosity compared to emulsions having larger
433 droplets (Yesiltas et al., 2019). Similar observations regarding decrease in viscosity with the
434 addition of caffeic acid were made for 70% fish oil-in-water emulsions produced with CAS and
435 diacetyl tartaric acid esters of mono- and diglycerides (DATEM) (Yesiltas et al., 2018b).

436 On the other hand, this argument was not valid when looking at the effects of increased
437 modified PCs concentration on viscosity. It was observed that the viscosity did not necessarily
438 increase with decreasing droplet size. This could be due to the effect of protein content in the
439 aqueous phase. As discussed in section 3.1.4, protein content in the aqueous phase might
440 increase the viscosity of the final emulsion when proteins are present in higher

441 concentrations. The concentration of CAS in the aqueous phase (2.25 – 6.23 wt%, Table 2)
442 might have affected the viscosity of the emulsions with modified PCs at their highest and
443 middle concentrations; 4_PC_C14_1080 and 5_PC_C14_2160 had significantly higher
444 viscosity compared to 7_PC_C16_1080 and 8_PC_C16_2160, respectively, whereas this was
445 not the case for the lower concentrations of modified PCs (3_PC_C14_360 and
446 6_PC_C16_360).

447 **3.3. Oxidative stability of emulsions**

448 **3.3.1. Primary oxidation products - peroxide value**

449 Lipid hydroperoxides were analyzed to compare the oxidation rate of the emulsions during 12
450 days of storage (Fig. 3). It was expected that modified PCs` involvement had an impact on the
451 oxidative stability of high fat fish oil-in-water emulsions. Peroxide value (PV) of 1_CAS was
452 lower compared to 2_PC_com, which showed that substitution of 45% of the CAS with soy
453 PC did not improve the oxidative stability of the 70% fish oil-in-water emulsion in terms of
454 primary oxidation products. When modified PCs were added in low concentrations (see
455 3_PC_C14_360 and 6_PC_C16_360, in Fig. 3), prooxidant effect was observed compared to
456 2_PC_com. PV of the emulsions with modified PCs were higher compared to CAS + soy PC
457 emulsions, which had the equivalent amount of caffeic acid but in free form. Thus, it was not
458 advantageous to have caffeic acid attached to PC molecules at both lower and higher
459 concentrations in terms of limiting the formation of hydroperoxides. The beneficial effect of
460 having free caffeic acid in the aqueous phase compared to having caffeic acid covalently
461 attached to the PC could be due to the enhanced ability of free caffeic acid in binding iron in

462 the aqueous phase, which limits metal catalyzed initiation and free radical formation (Frankel,
463 2012a).

464 Emulsions with PC_C16 had higher amount of primary oxidation products compared to
465 emulsions with equivalent amount of PC_C14. This could be due to the emulsions with
466 PC_C14 were forming and degrading hydroperoxides into secondary oxidation products
467 faster than the emulsions including PC_C16.

468 **3.3.2. Changes in tocopherol content**

469 Alpha-, gamma-, and delta-tocopherol levels in emulsions were in the range of 105.7 ± 0.7 –
470 133.6 ± 3.7 , 72.1 ± 0.1 – 74.2 ± 0.7 , 29.1 ± 0.7 – 30.5 ± 0.3 $\mu\text{g toc /g emulsion}$ at day 0
471 (Supplementary Fig. 5a-c). As the fish oil content was the same in all emulsions, significant
472 differences between tocopherol contents in emulsions were attributed to the consumption of
473 tocopherol during emulsion production at day 0. Emulsion 1_CAS had significantly higher
474 consumption of all types of tocopherols compared to 2_PC_com. When the soy PC was
475 substituted with increasing concentration of modified PCs, alpha tocopherol content
476 decreased significantly, which indicated that the alpha-tocopherols were acting as
477 antioxidants in the presence of modified PCs or there was an interaction between modified
478 PCs and alpha-tocopherols during emulsification. Free caffeic acid addition did not have any
479 significant effect on any tocopherols in the emulsions produced with CAS and soy PC, which
480 indicated that the alpha-tocopherol content was not affected by the presence of free caffeic
481 acid at day 0.

482 The changes in the tocopherol content were followed during 12 days of storage and it was
483 found that the alpha-tocopherol content decreased significantly only for 5_PC_C14_2160 and
484 8_PC_C16_2160, whereas gamma-tocopherol decreased significantly for 5_PC_C14_2160,
485 8_PC_C16_2160, and 10_PC_com_caf_2160 (Supplementary Fig. 5a,c). There was no
486 significant decrease in delta-tocopherol content of the emulsions during 12 days of storage.
487 These results indicated that the total tocopherol content of the emulsions with the highest
488 concentration of caffeic acid decreased significantly, which showed that part of the antioxidant
489 activity was due to tocopherols in these emulsions. However, the decrease in the amount of
490 tocopherol from day 0 to 12 was only around 5µg toc/g sample. It should also be considered
491 that alpha-tocopherols can be regenerated from oxidized tocopherol (e.g., tocopherol
492 quinone) with the proton-donating capacity of the amino group of phospholipids such as
493 phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (García-Moreno et
494 al., 2014; Samdani, McClements, & Decker, 2018). Therefore, the antioxidant activity of
495 alpha-tocopherol could be even higher than what was measured due to its possible
496 consumption and regeneration.

497 **3.3.3. Secondary volatile oxidation products – DHS GC-MS**

498 Volatile oxidation products formed in the emulsions showed similar trend in terms of their
499 content during 12 days of storage; therefore, sum of the volatiles was presented in Fig. 4.
500 Development of 1-penten-3-ol, (*E,E*)-2,4-heptadienal, 2-pentenal, and 2-ethylfuran, which
501 were in high concentration compared to rest of the volatile compounds, are also shown in
502 Supplementary Fig. 6a-d. It was observed that 360 ppm caffeic acid provided with modified

503 PCs resulted in a prooxidant effect. On the contrary, physical mixture of caffeic acid and PC
504 (9_PC_com_caf_360) did not cause a prooxidant effect, when compared to 2_PC_com.

505 For both modified PCs, oxidative stability was improved with the increasing amount of
506 modified PCs. All emulsions with modified PC added in concentrations above 360 mg/kg had
507 lower formation of volatile oxidation products than the emulsion with commercial PC as also
508 observed in the PCA plot. Improved oxidative stability of emulsions with modified PCs could
509 be due to the fact that caffeic acid was located at the interface showing antioxidant activity. It
510 could also be due to the larger surfactant head group of modified PCs compared to soy PC,
511 which led to thicker oil-water interfacial layer (Berton-Carabin et al., 2014).

512 Emulsion 8_PC_C16_2160 had better oxidative stability compared to 10_PC_com_caf_2160,
513 which showed that caffeic acid in high concentrations was more efficient when attached to
514 PC_16 than when present in its free form. This could be attributed to different interface
515 structure and composition of the emulsions due to the molecular structure differences
516 between PC and PC_16, which affects the adsorption performance of the molecules and the
517 location of caffeic acid. It is worth paying attention to the significant differences in physical
518 properties of these two emulsions such as droplet size. There has been contradicting results
519 on the impact of droplet size on oxidative stability (Berton-Carabin et al., 2014). Nevertheless,
520 studies which have shown an impact of droplet size have found that small droplets increase
521 lipid oxidation. Hence, the large droplets in the soy PC emulsions would favor decreased
522 oxidation and not increased oxidation as observed here.

523 Results also showed that on the last day of storage, CAS emulsion had higher concentrations
524 of volatile compound than emulsions with PC_C14_2160, PC_C16_1080 and PC_C16_2160
525 ppm.

526 On the other hand, PC_C16 had a lower amount of volatile compounds formed compared to
527 PC_C14 in all concentrations of added modified PCs (Fig. 3), which could be attributed to the
528 interface structure of the emulsions. This could be explained by the interfacial tension results
529 discussed under the section 3.2.2, where PC_C16 was indicated to have a better interaction
530 with CAS and soy PC, thereby forming a thicker interface, higher packing density and less
531 permeability at the oil-water interface compared to PC_C14 (McClements and Decker, 2018).
532 Moreover, as it was discussed in the section 3.2.4, PC_C16 had lower amount of non-
533 adsorbed protein in the aqueous phase compared to PC_C14 at their highest concentration,
534 thereby higher protein surface load, which supported the formation of a thicker interfacial
535 layer for PC_C16 and a better coverage of the oil droplets compared to PC_C14. Having
536 higher amount of emulsifiers at the interface resulted in a more protected interface against
537 prooxidant and increased oxidative stability due to the antioxidative activities of emulsifiers.

538 Another reason for higher oxidative stability could be attributed to the diffusion of some of the
539 PC_C16 into the oil phase in low concentrations (e.g., below its critical micelle concentration),
540 due to its high hydrophobicity and thereby acting as a chain-breaking antioxidant in the oil
541 phase. Individual PC_C16 molecules might act as an antioxidant owing to the covalently
542 attached caffeic acid on the head group of the molecule and thereby contribute to the overall
543 increased oxidative stability of the emulsions with PC_C16. Besides, both modified PCs could

544 also have potential hydrophobic interactions with emulsifiers or aggregates in the aqueous
545 phase, which might have improved their antioxidative effects (Shahidi and Zhong, 2011).

546 **4. Conclusion**

547 Soy and modified PCs in combination with CAS, as emulsifiers, decreased the viscosity of
548 70% omega-3 delivery emulsions compared to emulsions stabilized with only CAS. This
549 suggests that the emulsions can potentially be used in wider range of applications in food
550 systems when higher amount of omega-3 polyunsaturated fatty acids are needed. The use of
551 soy PC in combination with CAS decreased physical stability. However, the physical stability
552 was significantly improved when soy PC was partly replaced by modified PCs with C14 or
553 C16 alkyl chain as well as covalently attached caffeic acid due to their high surface activity.
554 The highest oxidative stability was observed for the emulsion with the highest concentration of
555 modified PC with C16 alkyl chain length and covalently attached caffeic acid. This was
556 attributed to its low interfacial tension and ability to interact with CAS and soy PC, which led to
557 thicker interfacial layer and less permeability for prooxidants, thereby resulting in better
558 oxidative stability.

559 **Abbreviations used**

560 **CAS** – sodium caseinate

561 **DHA** – docosohexaenoic acid

562 **DHS** – dynamic head space

563 **EPA** – eicosapentaenoic acid

564 **LC n-3 PUFAs** – Long chain omega-3 polyunsaturated fatty acid

565 **PC** – phosphatidylcholine

566 **PC_C14** – modified PC with covalently attached caffeic acid and C14 alkyl chain

567 **PC_C16** – modified PC with covalently attached caffeic acid and C16 alkyl chain

568 **PCA** – principle component analysis

569 **PV** – peroxide value

570 **Acknowledgements**

571 We would like to thank Lipoid GmbH, Arla Foods Ingredients amba and Maritex A/S Norway
572 for donating phosphatidylcholine, sodium caseinate and cod liver fish oil, respectively.
573 Furthermore, we are grateful to Lis Berner for her skillful work in the laboratory. We are
574 thankful to Terje Svingen for giving the instructions for taking microscopy images. Finally, we
575 would like to thank the Danish Council for Independent Research Technology and Production
576 Sciences for financing the project Mapping and Characterizing of Lipid Oxidation in Emulsified
577 Systems (MAPOX, DFF-4184-0123A).

578

579 **References**

580 Alemán, M., Bou, R., Guardiola, F., Durand, E., Villeneuve, P., Jacobsen, C., & Sørensen, A.-
581 D. M. (2015). Antioxidative effect of lipophilized caffeic acid in fish oil enriched mayonnaise
582 and milk. *Food Chemistry*, 167, 236-244.

583 Anankanbil, S., Pérez, B., Banerjee, C., & Guo, Z. (2018). New phenophospholipids equipped
584 with multi-functionalities: Regiospecific synthesis and characterization. *Journal of Colloid and*
585 *Interface Science*, *523*, 169-178.

586 AOCS Official Method Ce 8-89 (1998). Determination of tocopherols and tocotrienols in
587 vegetable oils and fats by HPLC. Champaign, IL, USA: AOCS.

588 Berton, C., Ropers, M. H., Viau, M., & Genot, C. (2011). Contribution of the interfacial layer to
589 the protection of emulsified lipids against oxidation. *Journal of Agricultural and Food*
590 *Chemistry*, *59*, 5052–5061.

591 Berton-Carabin, C. C., Ropers, M., & Genot, C. (2014). Lipid oxidation in oil-in-water
592 emulsions: Involvement of the interfacial layer. *Comprehensive Reviews in Food Science and*
593 *Food Safety*, *13*, 945–977.

594 Berton-Carabin, C. C., Sagis, L., & Schroën, K.. (2018). Formation, Structure, and
595 Functionality of Interfacial Layers in Food Emulsions. *Annual Review of Food Science and*
596 *Technology*, *9*, 24.1–24.37.

597 Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification.
598 *Canadian Journal of Biochemistry Physiol.*, *37*, 911–917.

599 Calder, P.C. & Yaqoob, P. (2012). Marine omega-3 fatty acids and coronary heart disease.
600 *Curr. Opin.Cardiol.*, *27*, 412-419.

601 Casado, V., Martin, D., Torres, C., & Reglero, G. (2012). Phospholipases in food industry: a
602 review. *Lipases Phospholipases Methods Protoc*, *861*, 495–523.

603 Cheatham, C. L., Colombo, J., & Carlson, S. E. (2006). n-3 Fatty acids and cognitive and
604 visual acuity development: methodologic and conceptual considerations. *Am. J. Clin. Nutr.*,
605 *83*, 1458S-1466S.

606 Choi, S. J., Decker, E. A., Henson, L., Popplewell, L. M., Xiao, H., & McClements, D. J.
607 (2011). Formulation and properties of model beverage emulsions stabilized by sucrose
608 monopalmitate: influence of pH and lyso-lecithin addition. *Food Res. Int.*, *44*, 3006–12.

609 Costa, M., Losada-Barreiro, S., Paiva-Martinsa, F., & Bravo-Diaz, C. (2017). Physical
610 evidence that the variations in the efficiency of homologous series of antioxidants in
611 emulsions are a result of differences in their distribution. *J Sci Food Agric*, *97*, 564–571.

612 EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) (2010). Scientific opinion on
613 dietary reference values for fats, including saturated fatty acids, polyunsaturated fatty acids,
614 monounsaturated fatty acids, trans fatty acids, and cholesterol. *EFSA Journal*, *8*, 1461–1568.

615 Fang, Y., & Dalgleish, D. G. (1996). Comparison of the effects of three different
616 phosphatidylcholines on casein-stabilized oil-in-water emulsions. *Journal of the American Oil*
617 *Chemists' Society*, *73*(4), 437–442.

618 Faraji, H., McClements, D. J., & Decker, E. A. (2004). Role of continuous phase protein on
619 the oxidative stability of fish oil-in-water emulsions. *Journal of Agricultural and Food*
620 *Chemistry*, *52*, 4558–4564.

621 Frankel, E. N. (2012a). Chapter 1: Free radical oxidation. *Lipid Oxidation* (pp 22-23). (2nd
622 ed.). Philadelphia, USA: Woodhead Publishing.

623 Frankel, E. N. (2012b). Chapter 2: Hydroperoxide formation. *Lipid Oxidation* (pp 43). (2nd
624 ed.). Philadelphia, USA: Woodhead Publishing.

625 García-Moreno, P. J., Horn, A. F., & Jacobsen, C. (2014). Influence of casein-phospholipid
626 combinations as emulsifier on the physical and oxidative stability of fish oil-in-water
627 emulsions. *Journal of Agricultural and Food Chemistry*, *62*(5), 1142–1152.

628 Gülcin, I. (2006). Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). *Toxicology*,
629 217, 213–220.

630 Horn, A. F., Nielsen, N. S., Andersen, U., Sogaard, L. H., Horsewell, A., & Jacobsen, C.
631 (2011). Oxidative stability of 70% fish oil-in-water emulsions: Impact of emulsifiers and pH.
632 *European Journal of Lipid Science and Technology*, 113, 1243–1257.

633 Jacobsen, C., Meyer, A. S., & Adler-Nissen, J. (1998). Oxidation mechanisms in real food
634 emulsions: Method for separation of mayonnaise by ultracentrifugation. *Journal of Food*
635 *Lipids*, 5, 87–101.

636 Laguerre, M., Lopez Giraldo, L. J., Lecomte, J., Figueroa-Espinoza, M. C., Barea, B., Weiss,
637 J., Decker, E. A., & Villeneuve, P. (2009). Chain length affects antioxidant properties of
638 chlorogenate esters in emulsion: The cutoff theory behind the polar paradox. *J. Agric. Food*
639 *Chem.*, 57, 11335–11342.

640 Laguerre, M., Bayrasy, C., Lecomte, J., Chabi, B., Decker, E. A., Wrutniak-Cabello, C.,
641 Cabello, G., & Villeneuve, P. (2013). How to boost antioxidants by lipophilization? *Biochimie*,
642 95, 20-26.

643 Liang, Y., Gillies, G., Patel, H., Matia-Merino, L., Ye, A., & Golding, M. (2014). Physical
644 stability, microstructure and rheology of sodium-caseinate stabilized emulsions as influenced
645 by protein concentration and nonadsorbing polysaccharides. *Food Hydrocolloids*, 36, 245-
646 255.

647 McClements, J. D. (1999). Chapter 7: Emulsion stability. *Food emulsions: Principles, practice*
648 *and techniques* (pp. 196). (1st ed.). Boca Raton, Florida: CRC Press LCC.

649 McClements, D. J., Bai, L., & Chung, C. (2017). Recent Advances in the Utilization of Natural
650 Emulsifiers to Form and Stabilize Emulsions. *Annu. Rev. Food Sci. Technol.*, *8*, 205–236.

651 McClements, D. J. & Decker, E. (2018). Interfacial Antioxidants: A Review of Natural and
652 Synthetic Emulsifiers and Coemulsifiers That Can Inhibit Lipid Oxidation. *J. Agric. Food*
653 *Chem.*, *66*, 20–35.

654 O’Kennedy, B.T. (2011). Chapter 2: Caseins. In G. O. Phillips & P. A. Williams (Eds.),
655 *Handbook of Food Proteins* (pp. 13-29). Cambridge, UK: Woodhead Publishing.

656 Samdani, G. K., McClements, D. J., & Decker, E. A. (2018). Impact of Phospholipids and
657 Tocopherols on the Oxidative Stability of Soybean Oil-in-Water Emulsions. *Journal of*
658 *Agricultural and Food Chemistry*, DOI: 10.1021/acs.jafc.8b00677

659 Shadidi, F. & Zhong, Y. (2011). Revisiting the Polar Paradox Theory: A Critical Overview. *J.*
660 *Agric. Food Chem.*, *59*, 3499–3504.

661 Shantha, N. C., & Decker, E. A. (1994). Rapid, sensitive, iron based spectrophotometric
662 methods for determination of peroxide values of food lipids. *Journal of AOAC International*,
663 *77*, 421–424.

664 Song, C., Shieh, C., Wu, Y., Kalueff, A., Gaikwad, S., & Su, K. (2016). The role of omega-3
665 polyunsaturated fatty acids eicosapentaenoic and docosahexaenoic acids in the treatment of
666 major depression and Alzheimer's disease: Acting separately or synergistically? *Progress in*
667 *Lipid Research*, *62*, 41–54.

668 Sørensen, A.-D. M., Haahr, A.-M., Becker, E. M., Skibsted, L. H., Bergenståhl, B., Nilsson, L.,
669 & Jacobsen, C. (2008). Interactions between Iron, Phenolic Compounds, Emulsifiers, and pH
670 in Omega-3-Enriched Oil-in-Water Emulsions. *J. Agric. Food Chem.*, *56*, 1740–1750.

671 Sørensen, A.-D. M., Durand, E., Laguerre, M., Bayrasy, C., Lecomte, J., Villeneuve, P., &
672 Jacobsen, C. (2014). Antioxidant properties and efficacies of synthesized alkyl caffeates,
673 ferulates and coumarates. *Journal of Agricultural and Food Chemistry*, *62*, 12553–12562.

674 Sørensen, A.-D. M., Villeneuve, P., & Jacobsen, C. (2017). Alkyl caffeates as antioxidants in
675 O/W emulsions: Impact of emulsifier type and endogenous tocopherols. *Eur. J. Lipid Sci.*
676 *Technol.*, *119*, 1600276.

677 Tesch, S. & Schubert, H. (2002). Influence of increasing viscosity of the aqueous phase on
678 the short-term stability of protein stabilized emulsions. *Journal of Food Engineering*, *52*, 305–
679 312.

680 Yesiltas, B., Sørensen, A.-D. M., García-Moreno, P. J., Anankanbil, S., Guo, Z., & Jacobsen,
681 C. (2018a). Combination of sodium caseinate and succinylated alginate improved stability of
682 high fat fish oil-in-water emulsions. *Food Chemistry*, *255*, 290–299.

683 Yesiltas, B., García-Moreno, P. J., Sørensen, A.-D. M., Anankanbil, S., Guo, Z., & Jacobsen,
684 C. (2018b). Effects of Modified DATEMs with Different Alkyl Chain Lengths on Improving
685 Oxidative and Physical Stability of 70% Fish Oil-in-Water Emulsions. *Journal of Agricultural*
686 *and Food Chemistry*, DOI: 10.1021/acs.jafc.8b04091.

687 Yesiltas, B., García-Moreno, P. J., Sørensen, A.-D. M., Akoh, C. C., & Jacobsen, C. (2019).
688 Physical and oxidative stability of high fat fish oil-in-water emulsions stabilized with sodium
689 caseinate and phosphatidylcholine as emulsifiers. *Food Chemistry*, *276*, 110–118.

690 Zhu, Z., Zhao, C., Yi, J., Liu, N., Cao, Y., Decker, E. A., & McClements, D. C. (2018). Impact
691 of Interfacial Composition on Lipid and Protein Co-Oxidation in Oil-in-Water Emulsions
692 Containing Mixed Emulsifiers. *J. Agric. Food Chem.*, *66*, 4458–4468

693 **Table 1.** Emulsion codes, descriptions and experimental design with the content of emulsions

Emulsion code	Description	Soy PC (%)	Modified PC (%)	caffeic acid (ppm)
1 CAS	CAS only	-	-	-
2 PC_com	CAS + comPC	1.27	-	-
3 PC_C14_360	CAS + com PC + mod PC C14	1.15	0.12	360*
4 PC_C14_1080	CAS + com PC + mod PC C14	0.90	0.37	1080*
5 PC_C14_2160	CAS + com PC + mod PC C14	0.52	0.75	2160*
6 PC_C16_360	CAS + com PC + mod PC C16	1.14	0.13	360*
7 PC_C16_1080	CAS + com PC + mod PC C16	0.88	0.39	1080*
8 PC_C16_2160	CAS + com PC + mod PC C16	0.48	0.79	2160*
9 PC_com_caf_360	CAS + com PC + caffeic acid	1.27	-	360
10 PC_com_caf_2160	CAS + com PC + caffeic acid	1.27	-	2160

694 All the emulsions have 70% fish oil, 2.8% total emulsifier content and the ratio between CAS
695 to PC is 1.2, which results in 1.53% CAS, except for 1CAS as it includes only CAS (2.8%).
696 Soy PC was substituted with modified PCs in different ratios in order to obtain different
697 concentrations of caffeic acid in the final emulsion (caffeic acid (ppm)).

698 *Free caffeic acid was not added in emulsion 3 to 8. Concentration was calculated according
699 to the added modified PC, which had caffeic acid attached to the compound itself.

700

701

702

703

704

705

706 **Table 2.** Droplet size, viscosity, zeta potential, protein in the aqueous phase, and protein
 707 surface load results of emulsions

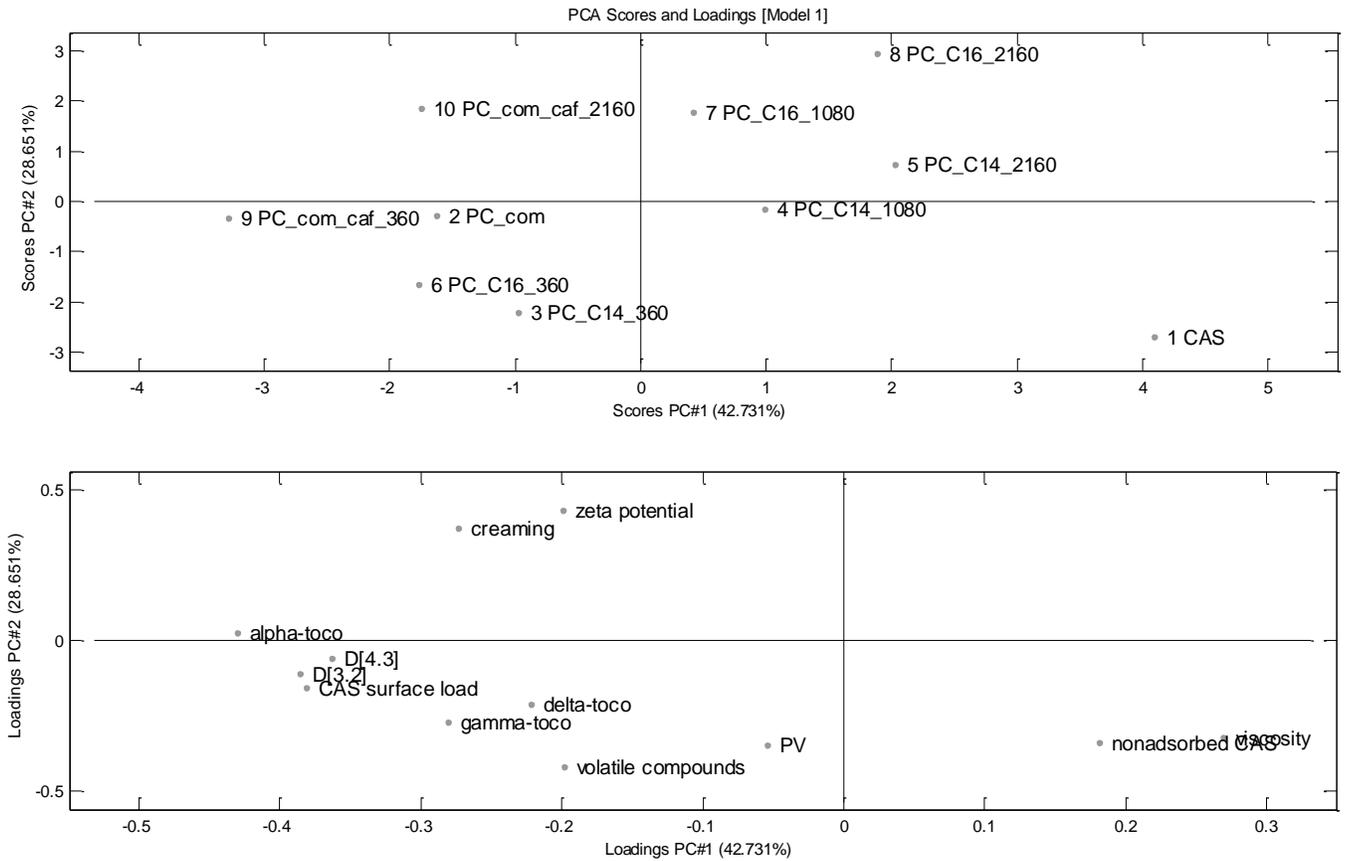
Emulsion code	D[3,2] (μm) (Day 1)	D[4,3] (μm) (Day 1)	Apparent viscosity (mPa·s) at 20 s^{-1} (Day 1)	Zeta potential (mV) (Day 2)	Protein in the aqueous phase (g) (Day 4)	Protein surface load (mg/m^2) (Day 4)
1 CAS	1.5 ± 0.7^b	8.8 ± 0.3^d	2457 ± 11^d	(-) 61.9 ± 1.4^a	1.70 ± 0.01^c	4.35 ± 0.05^a
2 PC_com	10.8 ± 0.2^d	17.0 ± 0.2^g	$923 \pm 14^{c*}$	(-) 52.8 ± 4.4^{cd}	0.95 ± 0.34^b	16.47 ± 9.67^b
3 PC_C14_360	8.3 ± 0.1^c	11.7 ± 0.0^f	$939 \pm 7^{c*}$	(-) 59.3 ± 5.2^{ab}	0.94 ± 0.05^b	12.81 ± 1.13^b
4 PC_C14_1080	1.0 ± 0.0^{ab}	8.0 ± 0.1^c	$841 \pm 9^{b*}$	(-) 56.3 ± 1.4^{bc}	0.91 ± 0.01^b	1.63 ± 0.01^a
5 PC_C14_2160	$0.7 \pm 0.1^{a*}$	$5.0 \pm 0.1^{a*}$	$971 \pm 3^{c*}$	(-) 52.2 ± 1.1^{cd}	0.91 ± 0.01^b	1.13 ± 0.02^a
6 PC_C16_360	$8.0 \pm 0.1^{c*}$	11.9 ± 0.0^f	$943 \pm 36^{c*}$	(-) 52.1 ± 1.2^{cd}	0.82 ± 0.10^{ab}	14.80 ± 2.05^b
7 PC_C16_1080	$1.8 \pm 0.3^{\S}$	$9.5 \pm 0.2^{e*}$	$740 \pm 32^{a*}$	(-) 47.0 ± 3.9^e	0.81 ± 0.06^{ab}	3.40 ± 0.30^a
8 PC_C16_2160	$1.1 \pm 0.3^{\S}$	6.7 ± 0.3^b	$777 \pm 83^{ab*}$	(-) 48.8 ± 1.3^{de}	0.61 ± 0.19^a	2.64 ± 0.54^a
9 PC_com_caf_360	11.1 ± 0.2^d	17.8 ± 0.1^h	$844 \pm 44^{b*}$	(-) 48.8 ± 1.9^{de}	0.96 ± 0.01^b	16.59 ± 0.17^b
10 PC_com_caf_2160	10.9 ± 0.6^d	18.4 ± 0.0^j	$831 \pm 93^{b*}$	(-) 49.4 ± 3.0^{de}	1.00 ± 0.08^b	15.00 ± 2.29^b

708 *Significant changes happened in droplet size and viscosity during 12 days of storage at
 709 $p < 0.05$.

710 ^{a-f}Letters indicate the significant differences between samples for the same physical
 711 parameter.

712 ^{\S}There was only one replicate for these 2 samples.

713



714

715 Figure 1. PCA scores and loading were plotted using the results of oxidation parameters (PV,
 716 tocopherols, volatiles) and physical parameters (D[3,2], D[4,3], viscosity, zeta potential,
 717 creaming, non-adsorbed CAS and CAS surface load).

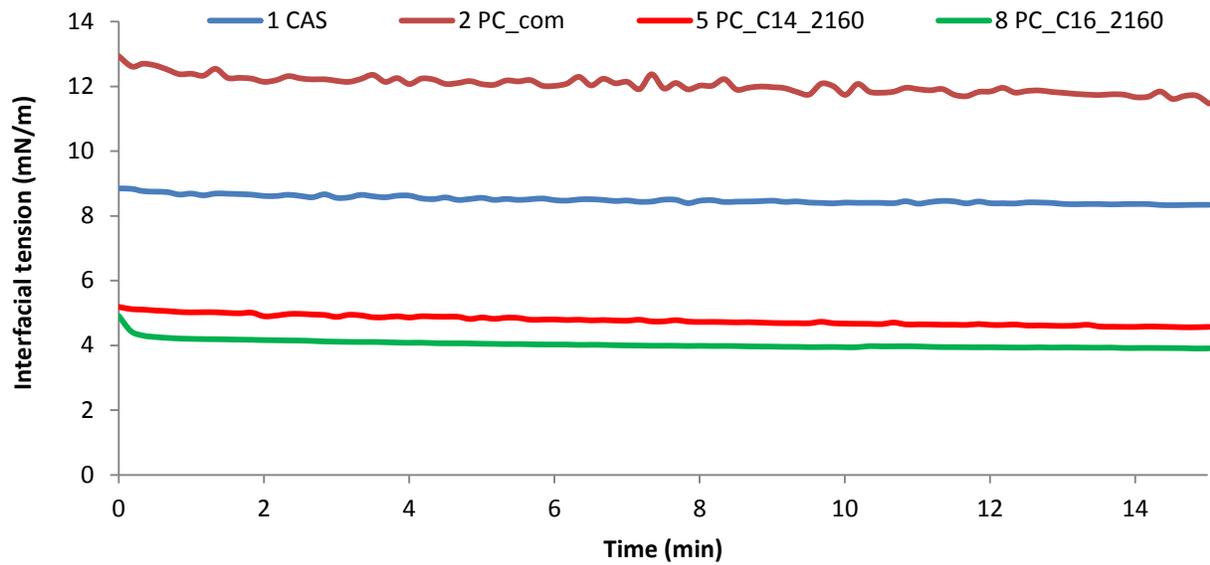
718

719

720

721

722

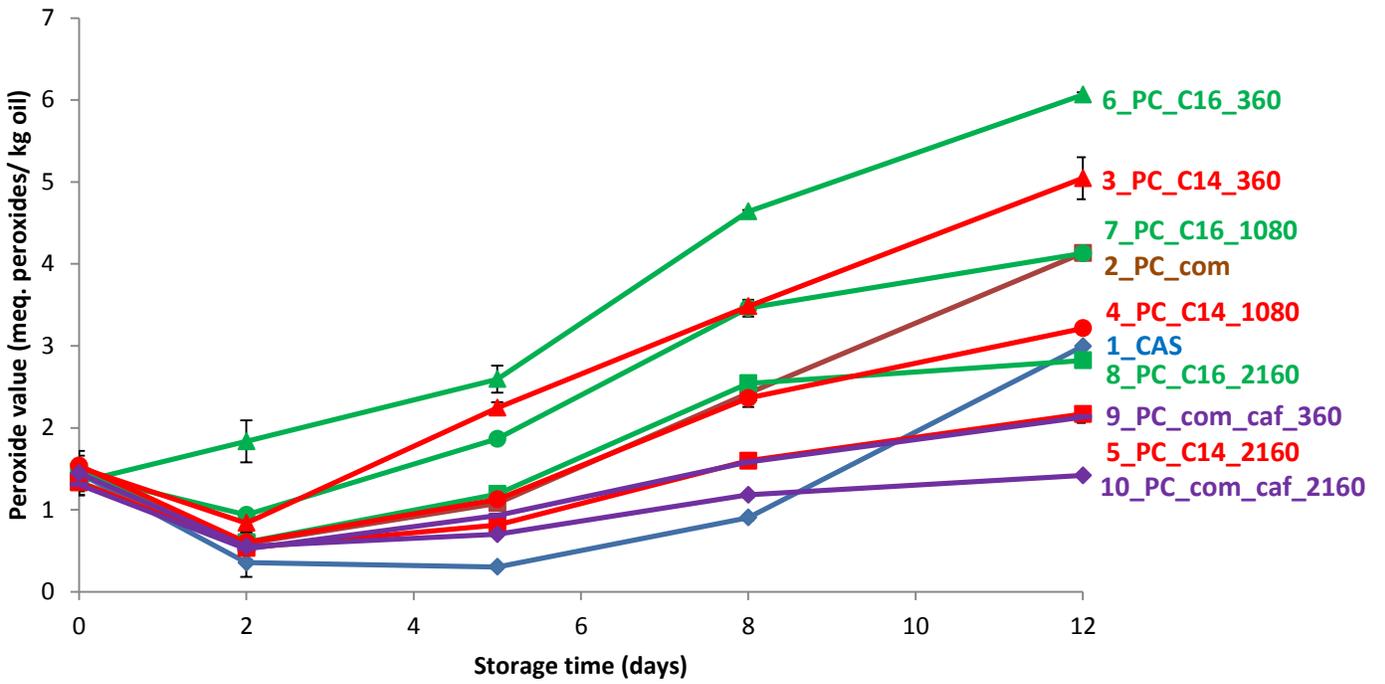


723

724 Relative standard deviation was lower than 6% in all samples.

725 Figure 2. Interfacial tension of emulsifier combinations

726

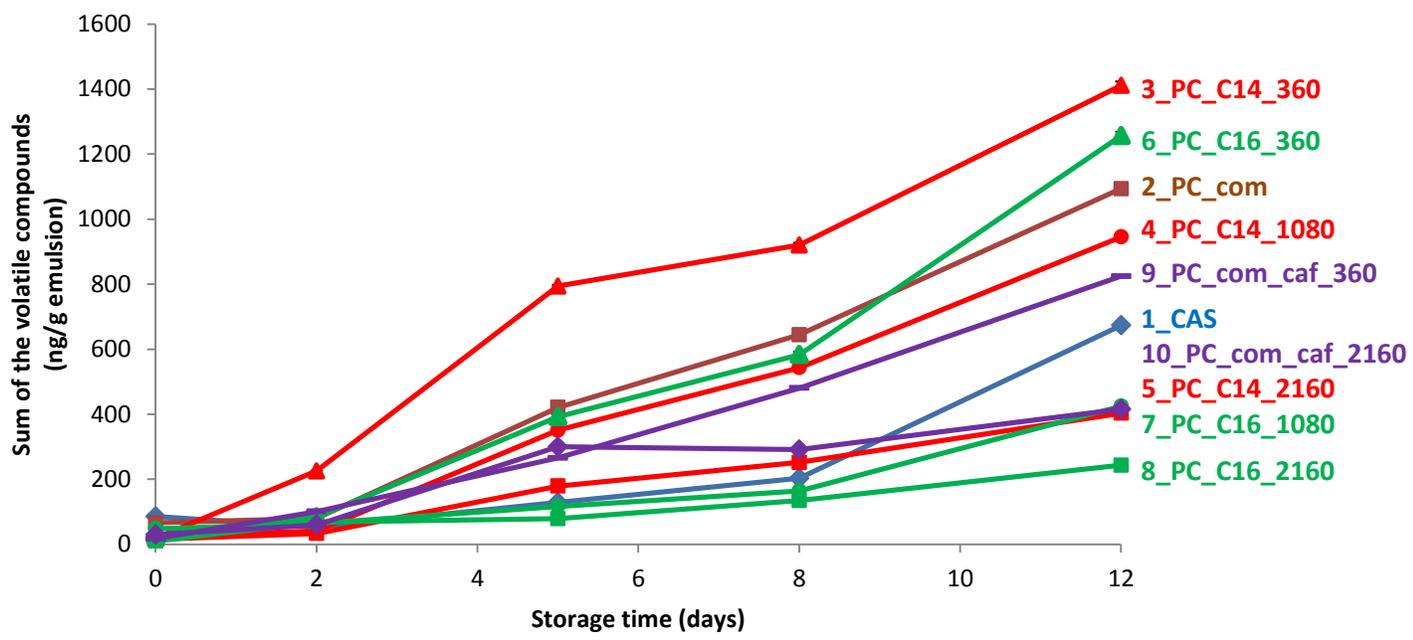


727
728

729 Figure 3. Formation of primary oxidation product in the emulsions during 12 days of storage.

730

731



732

733 Figure 4. Sum of the volatile secondary oxidation products formed in emulsion samples during

734 12 days of storage

735

736

737

738

739

740

741

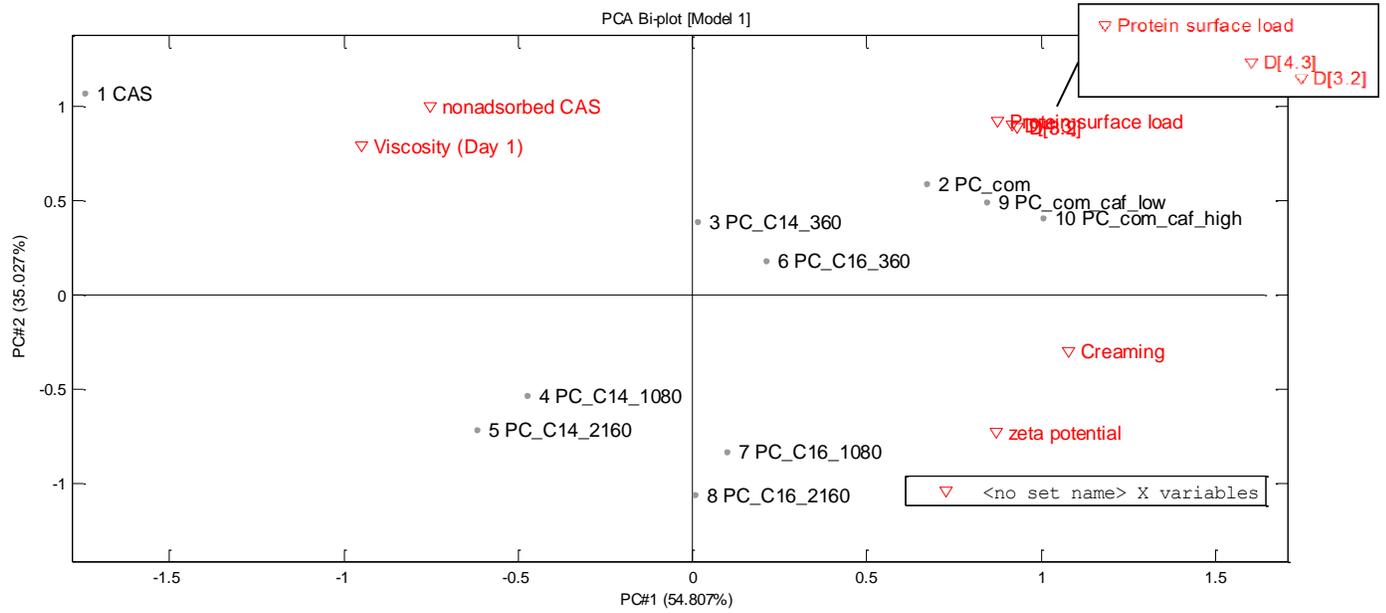
742

743

744

745

746 **Supplementary material**



747

748 Figure 1. PCA biplot plotted using physical parameters results from Table 2.

749

750

751

752

753

754

755

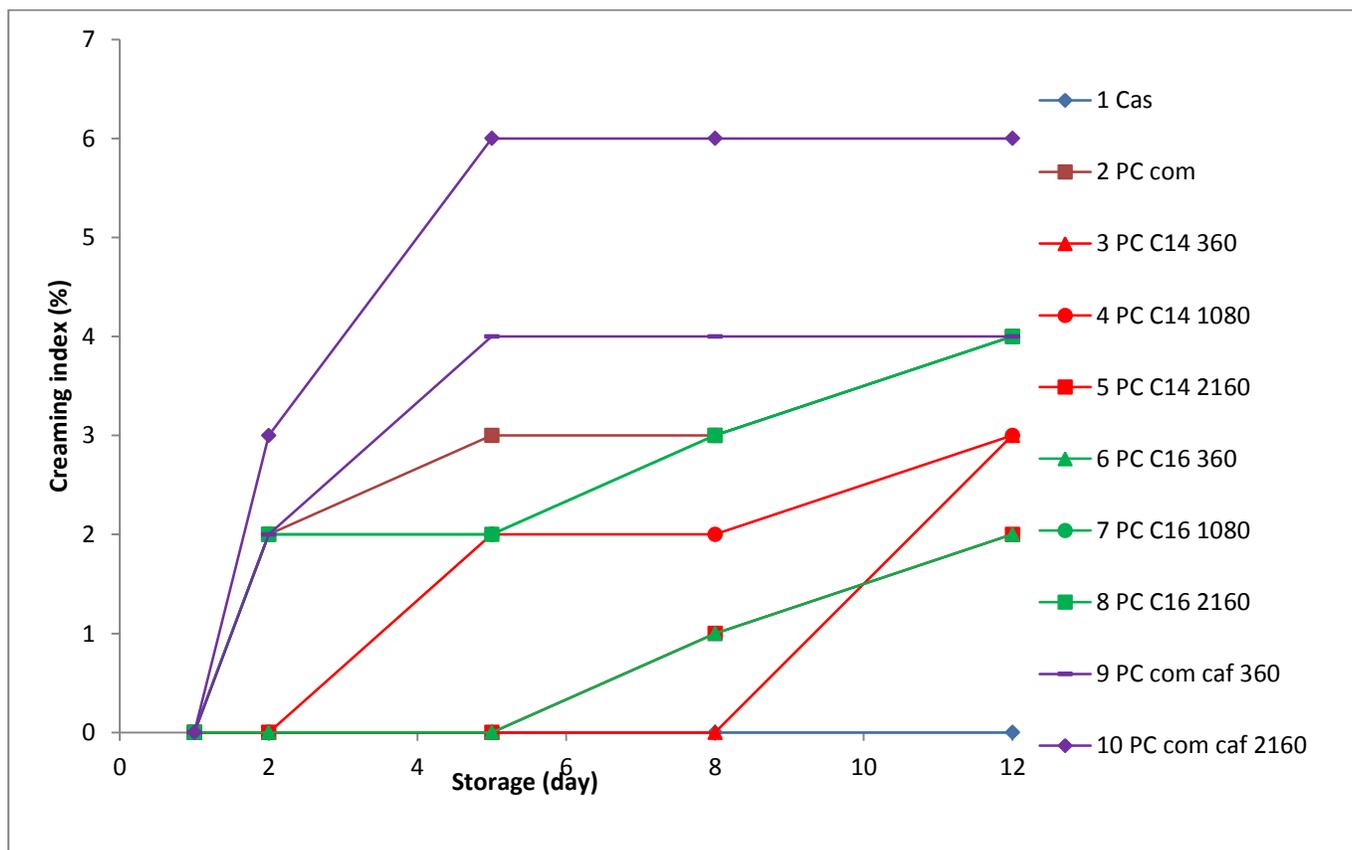
756

757



758 Figure 2a. Creaming of the emulsions samples on day 12, last day of the storage

759



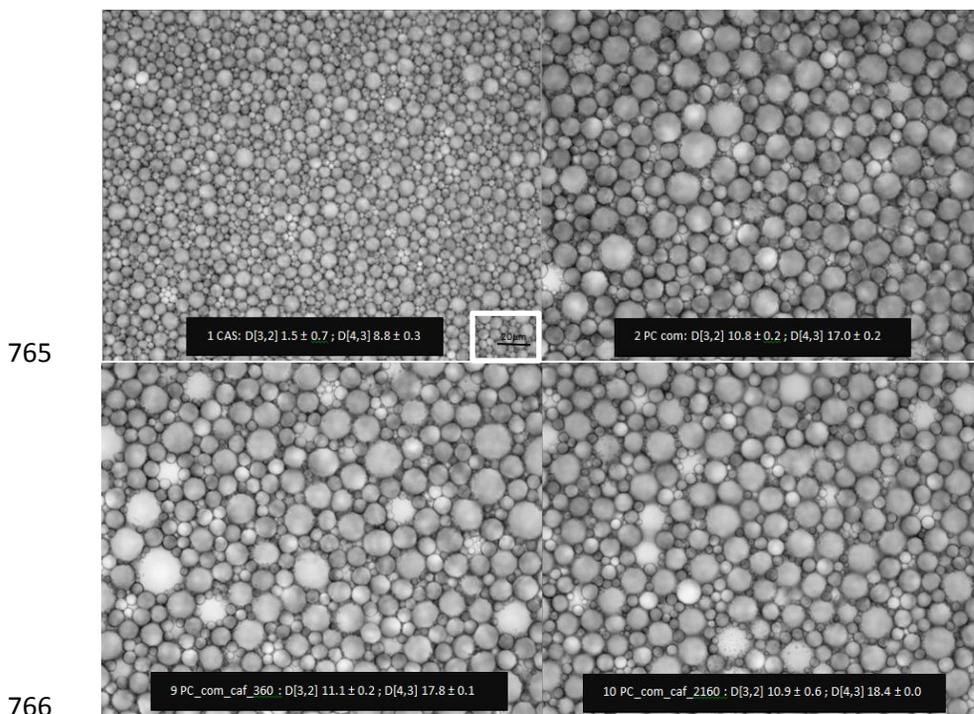
760

761 Figure 2b. Creaming index of the emulsions samples during 12 days of storage

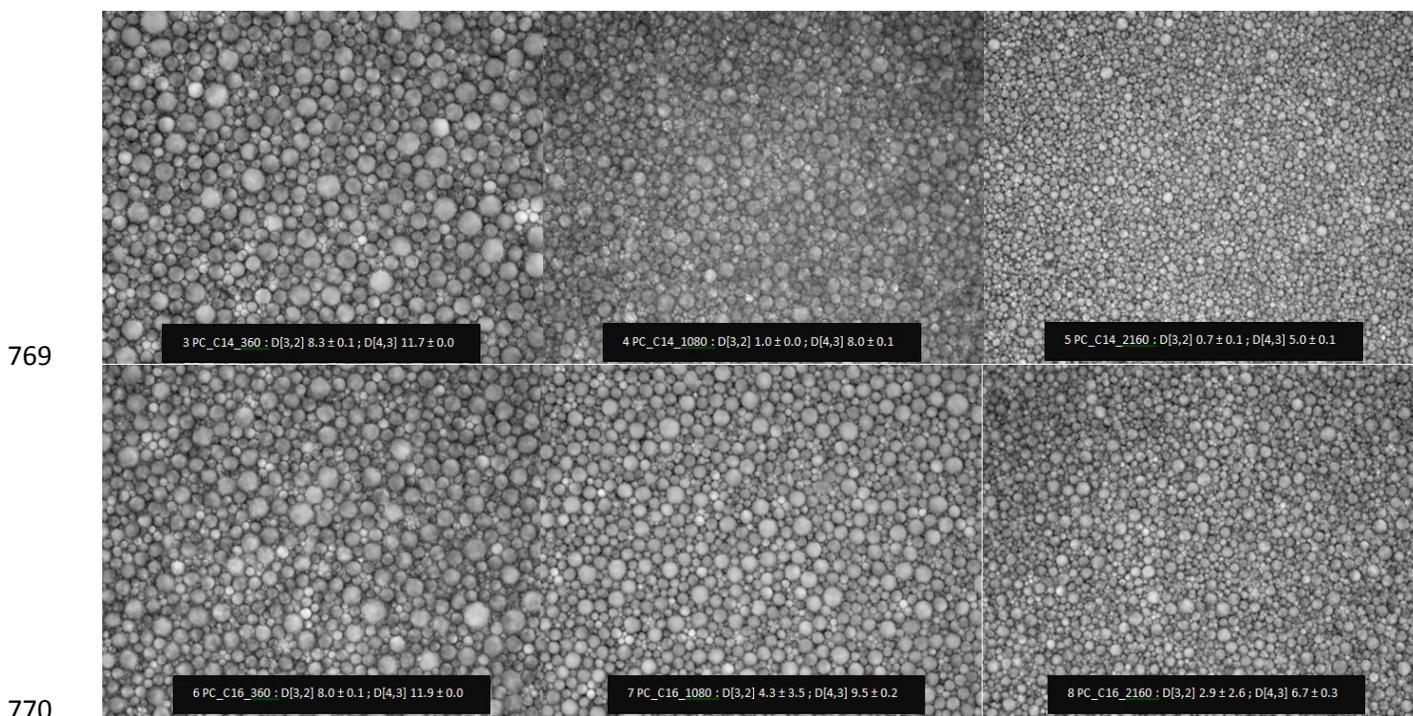
762

763

764



767 Figure 3a. Microscope images of emulsions produced with CAS and soy PC. Scale bar shown
768 inside the white square is 20 μm .



771 Figure 3b. Microscope images of emulsions produced with CAS, soy PC and modified PCs.

772

773

774

775

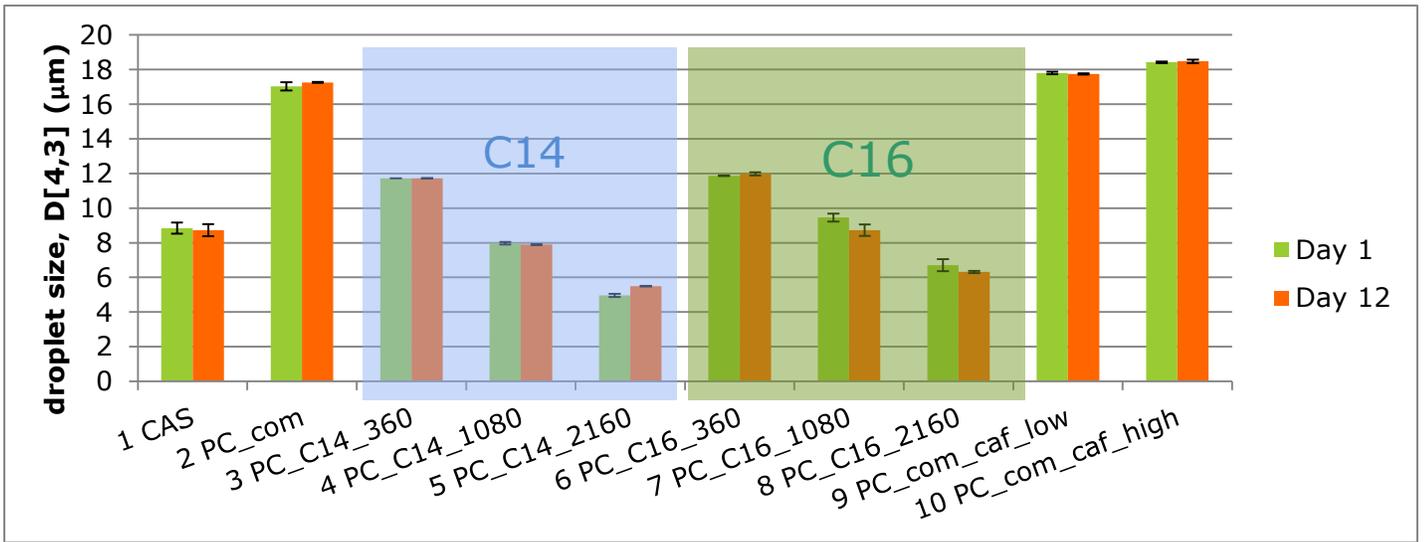
776

777

778

779

780



781 Figure 4a. Changes in D[4,3] mean droplet size during 12 days of storage

782

783

784

785

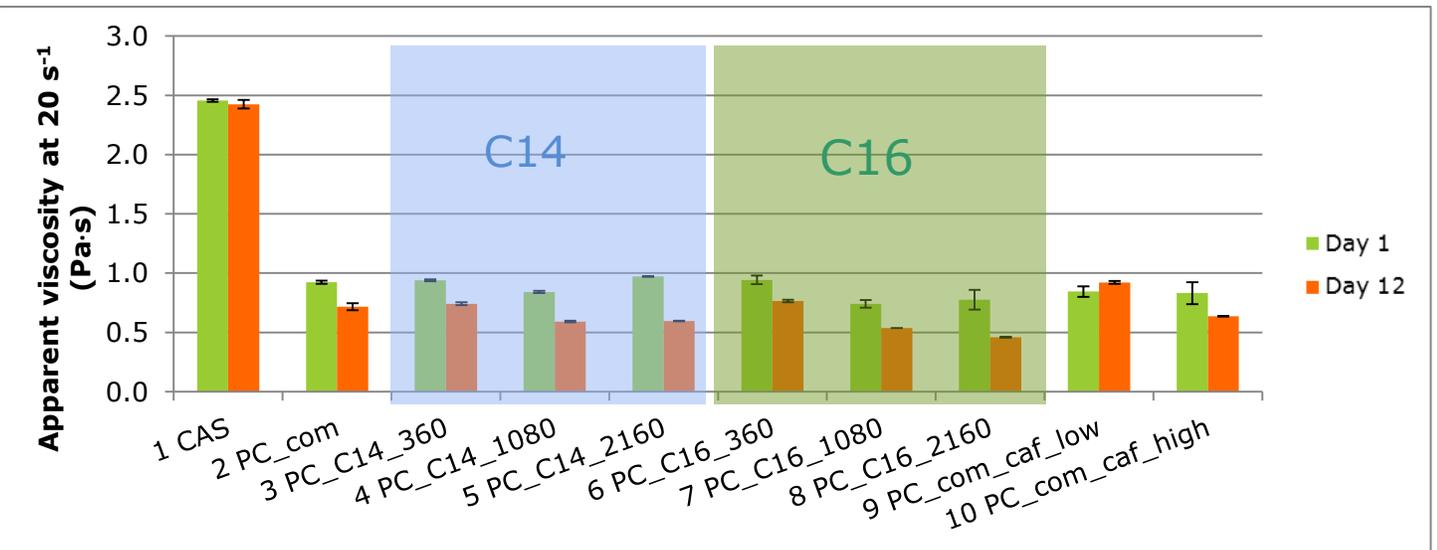
786

787

788

789

790

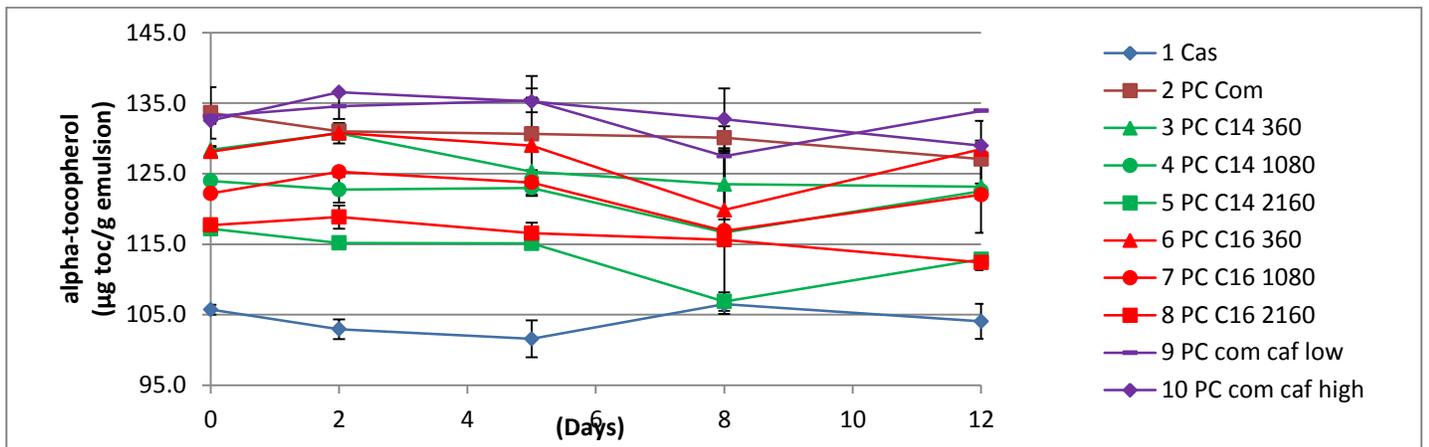


791

792 Figure 4b. Changes in D[4,3] mean droplet size during 12 days of storage

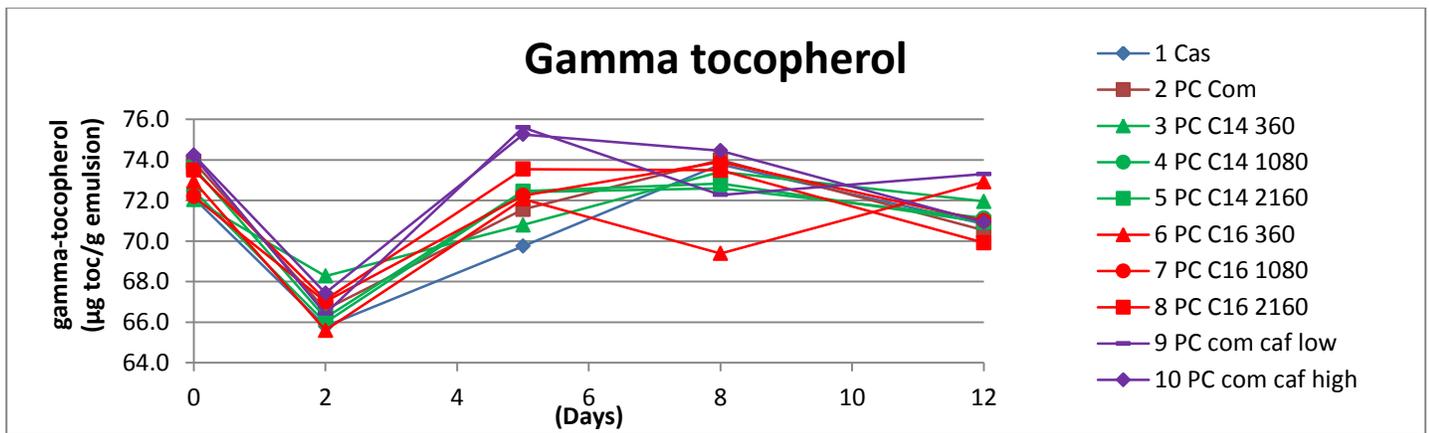
793

794



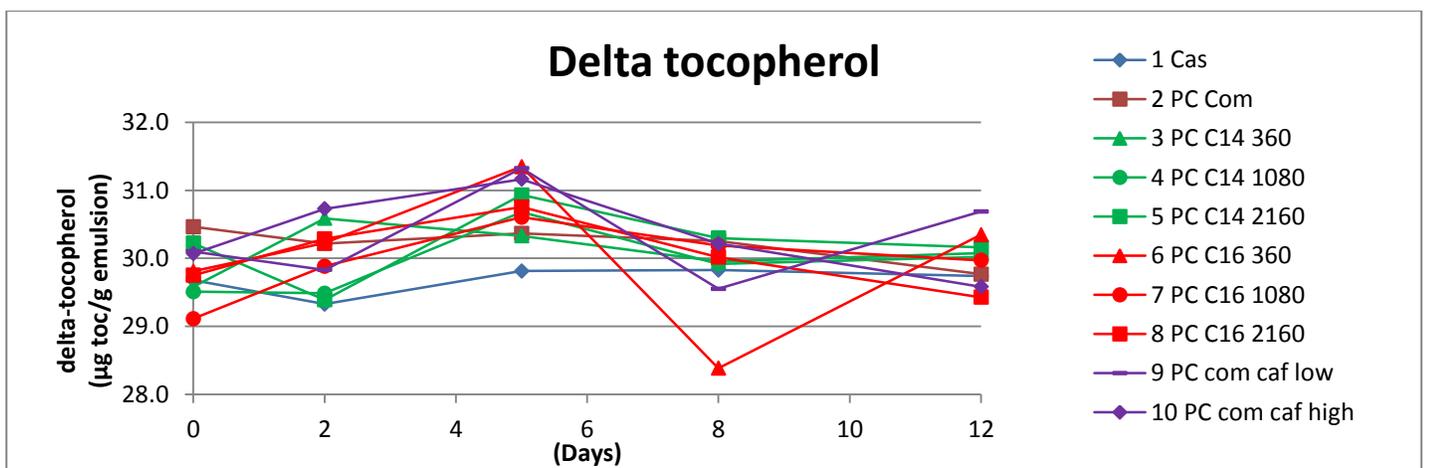
795 Figure 5a. Alpha-tocopherol content during 12 days of storage.

796

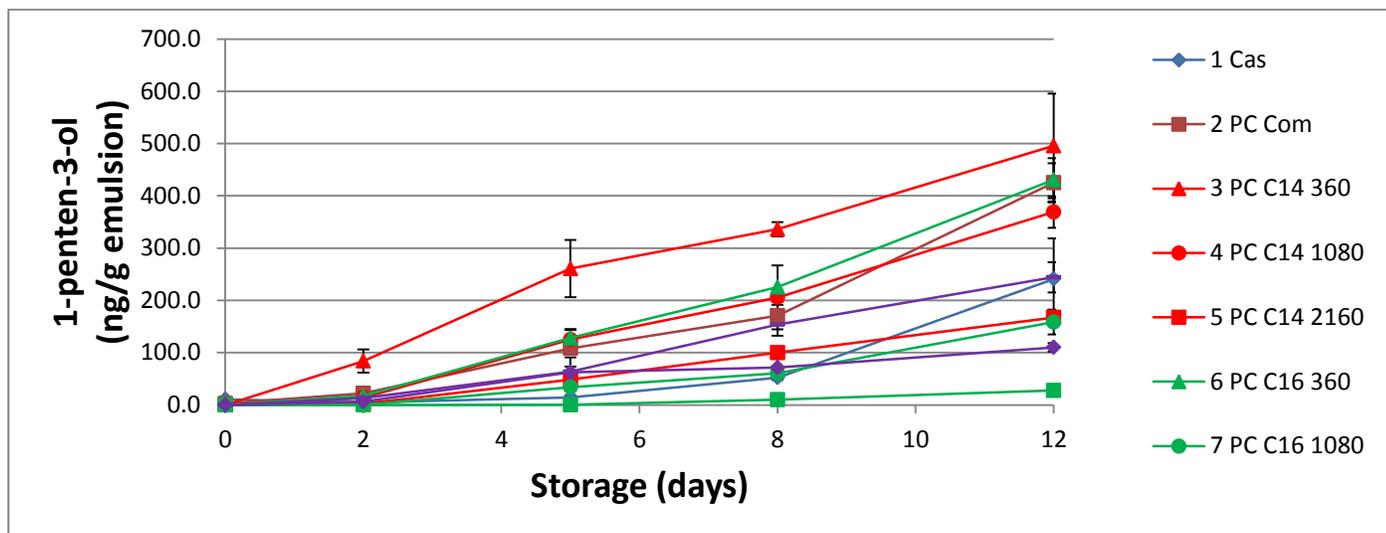


797 Figure 5b. Gamma-tocopherol content during 12 days of storage.

798

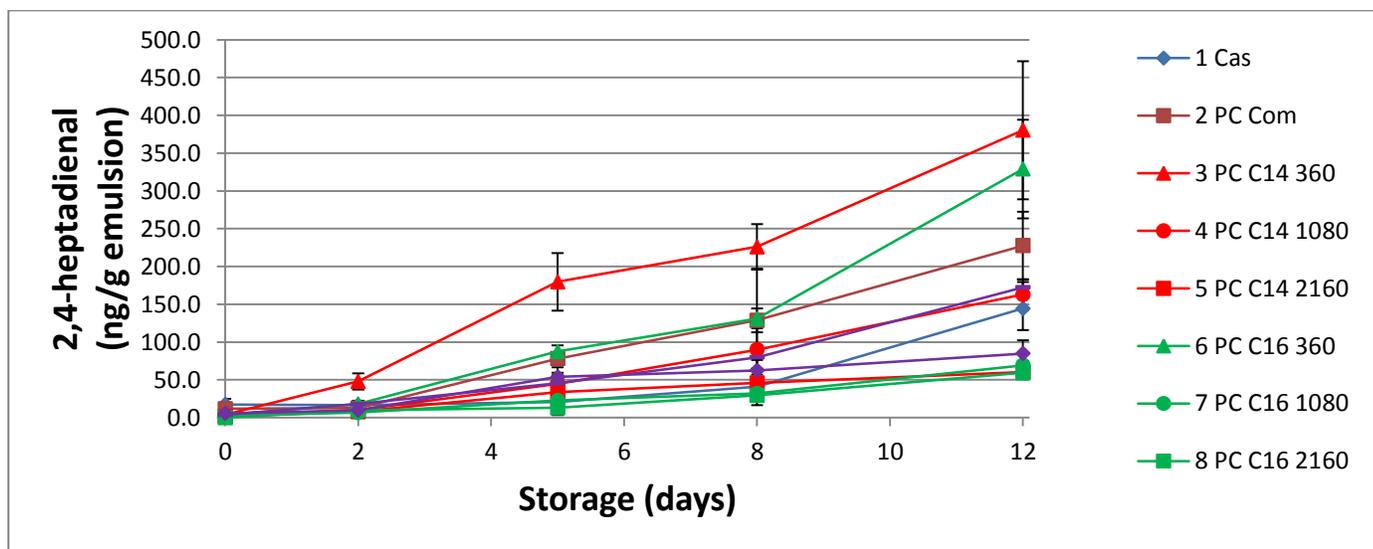


799 Figure 5c. Delta-tocopherol content during 12 days of storage.



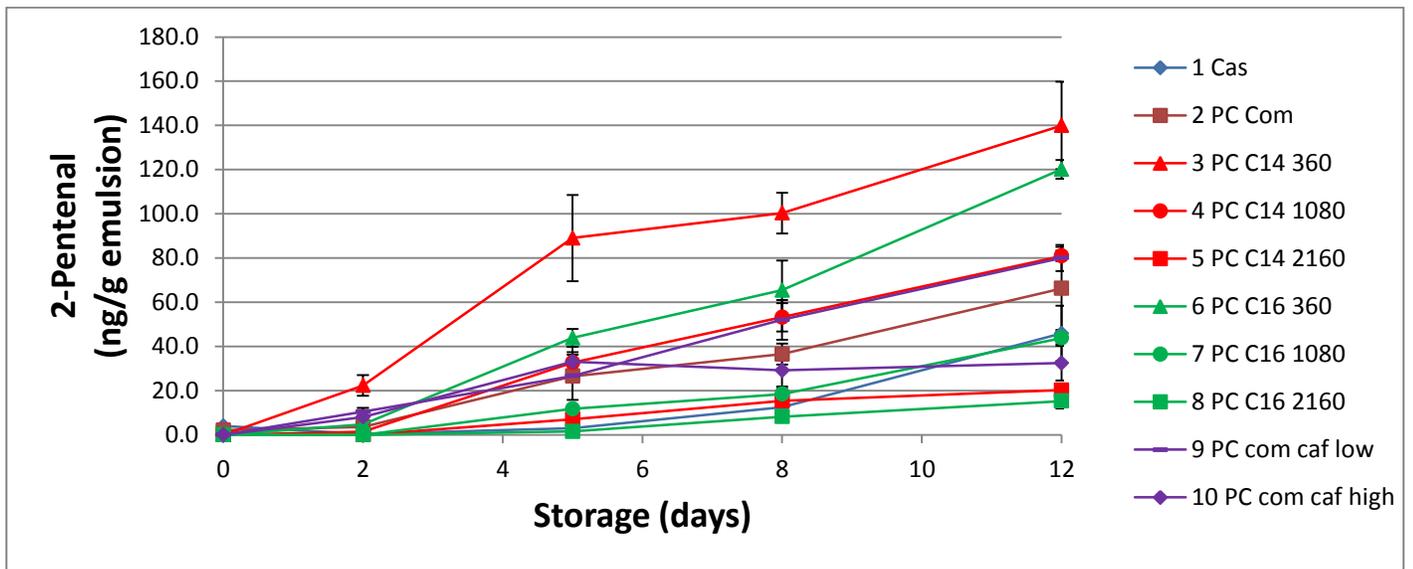
800

801 Figure 6a. 1-penten-3-ol formation during 12 days of storage. Concentration: 3 PC C14 360, 4
 802 PC C14 1080, 2 PC com, 6 PC C16 360 > 9 PC com caf low, 1 CAS > 5 PC C14 2160, 7 PC
 803 C16 1080 > 10 PC com caf high > 8 PC C16 2160



804

805 Figure 6b. (E,E)-2,4-heptadienal formation during 12 days of storage. Concentration: 3 PC
 806 C14 360, 6 PC C16 360, 2 PC com, 9 PC com caf low, 4 PC C14 1080, 1 CAS > 10 PC com
 807 caf high > 5 PC C14 2160, 7 PC C16 1080, 8 PC C16 2160

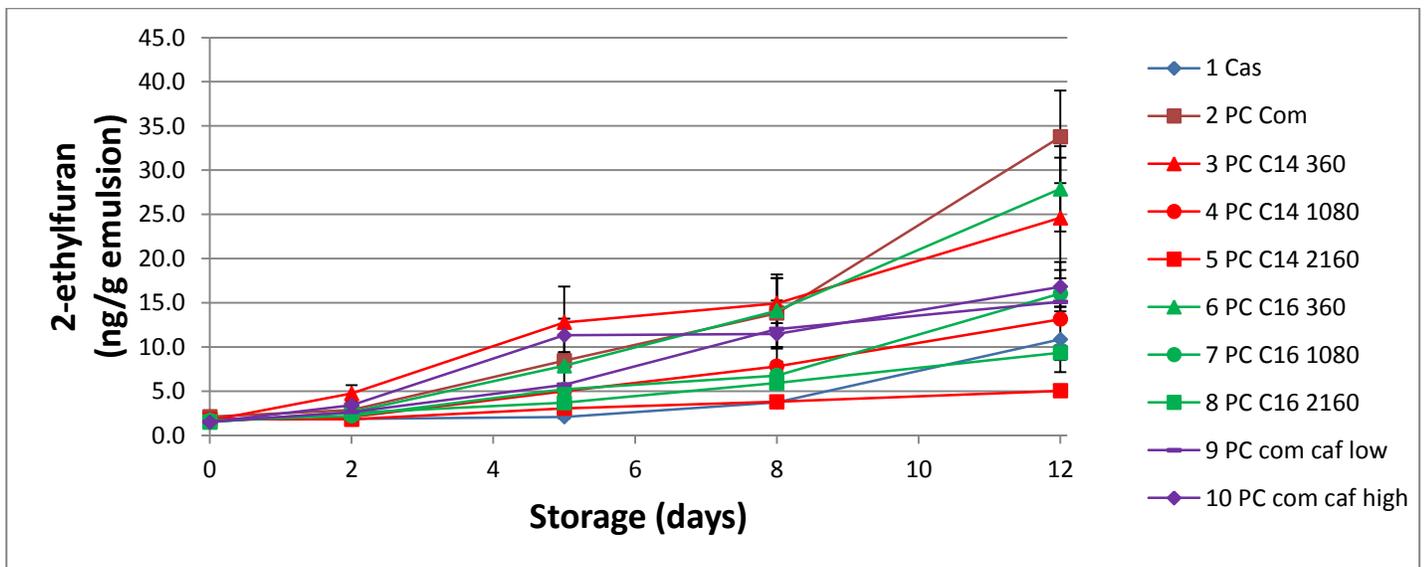


808

809 Figure 6c. 2-pentenal formation during 12 days of storage. Concentration: 3 PC C14 360, 6

810 PC C16 360 > 9 PC com caf low, 4 PC C14 1080, 2 PC com, 1 CAS, 7 PC C16 1080 > 10 PC

811 com caf high > 5 PC C14 2160, 8 PC C16 2160



812

813 Figure 6d. 2-ethylfuran formation during 12 days of storage. Concentration: 2 PC com, 6 PC

814 C16 360, 3 PC C14 360 > 10 PC com caf high, 7 PC C16 1080, 9 PC com caf low, 4 PC C14

815 1080, 1 CAS, 8 PC C16 2160 > 5 PC C14 2160

National Food Institute
Technical University of Denmark
Kemitorvet
2800 Lyngby

Tel: +45 35 88 70 00
www.food.dtu.dk

ISBN: 978-87-93565-40-1